Susceptibility of Purified Acetylcholinesterases from *Rhynchophorus Ferrugineus* towards Insecticides and Botanical Extracts

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ABSTRACT

The susceptibility of two purified acetylcholinesterases (AChEs), AChEIIb and AChEIIIb, from red palm weevil (RPW) *Rhynchophorus ferrugineus*, to inhibition by different synthetic insecticides and botanical leaves extracts *in vitro* has been investigated. In addition, the mechanism of inhibition has also been estimated. *R. ferrugineus* AChEs showed similar trends to inhibition by synthetic insecticides and the inhibition potency can be arranged in a descending order; deltamethrin > carbofuran > oxamyl > emamectin benzoate > chloropyrifos > malathion. All the examined insecticides competitively inhibited *R. ferrugineus* AChEs with *Ki* values ranging from 0.14 to 0.7 mM and *IC*₅₀ values from 0.15 to 0.75 mM, while malathion and emamectin benzoate showed noncompetitive inhibition manner. The susceptibility of *R. ferrugineus* AChEs to inhibition by botanical extracts can be arranged in a descending order: olives *Olea europaea* > neem *Azadirachta indica* > basil *Ocimum basilicum* with *Ki* values ranging from 3.5 to 14 mg and *IC*₅₀ values from 5 to 20 mg. *O. europaea* competitively inhibited *R. ferrugineus* AChEs, while the others noncompetitively. By HPLC, oleuropein is the major active compound present in the *O. europaea* (96.8%). Malathion and chloropyrifos, as organophosphate (OP) insecticides, have the least potency to inhibit *R. ferrugineus* AChEs. The susceptibility of *R. ferrugineus* AChEs to insecticides and botanical extracts seems to be a helpful approach for selecting the most efficient insecticide(s) for RPW management. These results may justify the complaint by the farmers regarding the low efficiency of OP insecticides for controlling RPW. *O. europaea* extract can be examined *in vivo* for introducing it as integral part of an integrated pest management programs against RPW.

**Keywords:** Acetylcholinesterase, botanical extracts, insecticides, inhibition, red palm weevil.

1. Introduction

Red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) is the major pest serious tissue-boring pest of more than 40 palm species in the Middle East, South and South East Asia, North Africa and Southern Europe (Sharaby and El-Dosary, 2016; Mohamed et al., 2020). The larval stage (grubs) is the most destructive stage and the longest period in the life-cycle of the RPW. The larva remains active for about 1-3 months (Salem, 2015; Salem and Ahmed, 2015). The larvae chew the tender, soft tissues of the palms and moves toward the interior part of the tree (Vatanparast et al., 2014; Sharaby and El-Dosary, 2016; Alzahrani, 2019). The damage caused by the larvae can be seen only long time after infection and finally larval damage results in the death of the infected tree (Sharaby and El-Dosary, 2016; Mahmoud et al., 2017; Alzahrani, 2019).

The management of RPW represents a tremendous challenge because of its cryptic life cycle. Current methods recommended for management of *Rhynchophorus* species have focused on integrated pest management (IPM) involving surveillance, pheromone lures, cultural control and chemical
insecticide treatments (Vidyasagar et al., 2000; Salama et al., 2004; Sharaby and El-Dosary, 2016; Salem and Abdel Salam, 2018). The application of synthetic insecticides remains the main strategy for control. However, the development of insect resistance, the high operational cost, the adverse effects of these synthetic chemical insecticides both on human and environmental health and the undesirable side effects have limited the usage of insecticides.

Along the late decades all over the world, so many plant species have been examined for their insecticidal activities as antifeedant, growth retarding, morphogenic, impairing, reproductive disturbing and oviposition deterrenting effects on various insect pests (Senthil-Nathan, 2013; Kolawole et al., 2014; Salem et al., 2016; Salem and Abdel Salam, 2018; Abdel-Aziz, 2019; Oni et al., 2019). The insecticidal effects of various plant extracts against RPW have been proven (Bream et al., 2001; Sharaby and Al-Dosary, 2014; 2016; Abdel Kareim et al., 2017; Salem and Abdel Salam, 2018; Ali et al., 2019). The toxic effects of basil, Osmium basilicum (Abdel Kareim et al., 2017) and neem, Azadirachta indica (Bream et al., 2001; Ali et al., 2019) on different stages of R. ferrugineus have been investigated. Although the toxic effects of the botanical insecticides on different insect species have been extensively published, the information about their mode of action is still so scanty. Oni et al (2019) suggested that once the insecticidal potential of a botanical extract has been discovered, its effects on various enzymes of insects should be addressed.

Insects can metabolize and degrade the toxic chemicals for surviving in a chemically unfriendly environment. The ineffectiveness of viable insecticides for management of R. phoenicis is due to the defense system inherent to the insect (Bamidele et al., 2013; 2017). The high activity level of acetylcholinesterase enzyme (AChE, EC 3.1.1.7) is one of the main resistance mechanisms in various insecticide-resistant pests (Yu et al., 2006; Pethuan et al., 2007; Yang et al., 2008; Kim et al., 2012; Mohamed et al., 2017). Acetylcholinesterase is a key enzyme catalyzing the hydrolysis of the neurotransmitter, acetylcholine, in the nervous system in various organisms (Zibae, 2011; Senthil-Nathan, 2013; Rana et al., 2015; Mohamed et al., 2017). AChE is primarily responsible for termination of cholinergic neurotransmission at synapses in the central nervous system of insects. Its inhibition produces a generalized synaptic collapse that lead to paralysis and insect death (Kim et al., 2010; Rajashekar et al., 2014; Oni et al., 2019). Most insects have two AChE isoenzymes but the mode of action is not well established. It has been reported that AChE2 of Bombyx mori and Apis mellifera is the main catalytic enzyme in synaptic transmission rather than AChE1 (Chen et al., 2009; Kim et al., 2012; Santos et al., 2019).

In a previous report, high AChE level has been recorded in the cuticles of RPW larvae, as the most important organ for protecting the larvae from the detrimental chemicals found in their environment (Mohamed et al., 2020). In addition, two predominant AChE isoenzymes have been purified and characterized. The inhibition of AChE activities in different insect species by a variety of plant extracts has been documented (Breuer et al., 2003; Begum et al., 2010; Ghoneim et al., 2012; Olmedo et al., 2015; Prakash, 2015; Rana et al., 2015; Oni et al., 2019). The study of R. ferrugineus AChE enzymes is motivated by the fact that those enzymes are the target site for inhibition by insecticides.

The understanding of the molecular basis of the inhibitory effects of different insecticides and botanical extracts on R. ferrugineus AChEs could provide an opportunity for developing RPW management strategy to ensure successful implication of such strategy. To achieve this objective, the present work is designed for evaluating the inhibitory effects and the mechanisms of inhibition of different synthetic chemical insecticides belonging to different classes of insecticides and the ethanolic leaves extracts of three different plant species on two purified R. ferrugineus AChEs.

2. Material and Methods

2.1. Chemicals:

Acetylthiocholine (AcSCh) and 5,5’-dithioibis (2-nitrobenoic acid) (DTNB) were purchased from Sigma Aldrich Chemical Co. Sephacryl S-200 and DEAE-Sepharose for chromatography were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All reagents and other general chemicals were of analytical grade. Insecticides, organophosphates (OPs) (chloropyrifos and malathion), carbamates
(oxamyl, carbosulfan), pyrethroid (deltamethrin) and avermectin (emamectin benzoate) were obtained from Agricultural Ministry, Dokki, Giza, Egypt.

2.2. Collection and preparation of botanical extracts

The leaves from trees of olive, *Olea europaea*; neem, *Azadirachta indica* and basil, *Ocimum basilicum* were obtained from the garden of Ministry of Agriculture, Dokki, Giza, Egypt. The leaves were rinsed with distilled water, dried in the shade and crushed to generate a fine powder. The plant powders (1g) for each plant were soaked in 10 ml of 70% ethanol and maintained for 48 h at room temperature. The suspension ethanol solution was centrifuged at 10,000 Xg for 10 min. The filtrates were evaporated to dryness by rotary evaporator, designated as botanical extract, kept inside air-tight container and stored at -4°C for subsequent use.

2.3. Insect

The 11th instar larvae of *R. ferrugineus* were obtained from Central Laboratory for Date Palm Research and Development (CLDPRD), Agricultural Research Centre, Dokki, Giza, Egypt.

2.4. Preparation of Purified AChEs

The two AChE isoenzymes; AChEIIb and AChEIIIb, have been previously purified from the crude extract of cuticles of the 11th instar larvae of *R. ferrugineus* that exhibited the highest AChEs level according to Mohamed *et al.* (2020).

2.5. Enzyme assay

The activity of AChE was estimated using AcSChI as a substrate according to Ellman *et al.* (1961). The reaction mixture contained in 1 ml: 60 mM Tris-HCl buffer, pH 8.5, 1mM AcSChI, 1 mM DTNB. The reaction mixtures were incubated at 37°C for 1h and the absorbance was measured at 412 nm. One unit of AChE activity was defined as the amount of enzyme that catalyzes the hydrolysis 1µmol of substrate per hour under standard assay conditions.

2.6. Susceptibility of AChEs to insecticides in vitro

The susceptibility of purified AChEIIb and AChEIIIb to inhibition by different classes of insecticides was performed. The classes of insecticides included organophosphates (OPs) (chloropyrifos and malathion), carbamates (oxamyl, carbosulfan), pyrethroid (deltamethrin) and avermectin (emamectin benzoate). The enzymes were pre-incubated with 6 different concentrations of each insecticide individually for 15 min at 25°C before substrate, AcSChI, addition for estimating the residual enzyme activities as described previously. Malathion, deltamethrin and oxamyl were used in the concentration ranges 0.25-2.0, 0.1-0.4 and 0.1-0.1 mM, respectively. Deltamethrin, carbosulfan and emamectin benzoate were used in the concentration ranges 0.1-0.4, 0.1-0.8 and 0.1-0.6 mM, respectively. The median inhibition concentration (IC$_{50}$) for each botanical extract were used in the concentration ranges 0.1-0.4, 0.1-0.8 and 0.1-0.6 mM, respectively. The median inhibition concentration (IC$_{50}$), the concentration of insecticide that inhibited 50% of *R. ferrugineus* AChEs activities was determined based on the log-concentration versus log (% residual activity) according to Devonshire and Moores (1982). The bimolecular rate constant ($K_i$) for each insecticide was estimated by the double reciprocal plots of initial velocities versus reciprocal concentrations of AcSChI in the absence and presence of 3 different insecticide concentrations according to Dixon and Webb (1964). The $K_i$ values were calculated from the replot of S/V against insecticide concentrations. The mechanism of AChEs inhibition by an insecticide, competitive or non-competitive was determined according to Dixon and Webb (1964).

2.7. Susceptibility of AChEs to botanical extracts in vitro

The susceptibility of purified AChEIIb and AChEIIIb to inhibition by different botanical extracts; *O. europaea*, *A. indica* and *O. basilicum* were examined. The enzymes were pre-incubated with 6 different concentrations of each botanical extract individually for 15 min at 25°C before substrate, AcSChI, addition for estimating the residual enzyme activities as described previously. *O. europaea*, *A. indica* and *O. basilicum* were used in the concentration ranges 1-10, 2-16 and 2-30 mg, respectively. IC$_{50}$ and $K_i$ for each botanical extract were estimated as mentioned before.
2.8. Evaluation of the active compounds present in *O. europaea*

The high performance liquid chromatography (HPLC) analysis was carried out for 70% ethanol leaves extract of *O. europaea* using an Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector. The analytical column was eclipse XDB-C18 (150 x 4.6 µm; 5 µm) fitted with 4.0 x 3.0 mm i.d. guard column. The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was 1.0 ml/min for a total run time of 70 min and the gradient program was as follows: 100-85% B in 30 min, 85-50% B in 20 min, 50-0% B in 5 min and 0-100% B in 5 min. There was 10 min of post-run for reconditioning. Peaks were monitored simultaneously at 280, 320 and 360 nm (Kim et al., 2006). All samples were filtered through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectra and compared with those of the standards.

3. Results

3.1. Susceptibility of AChEs to insecticides

The susceptibility of two purified *R. ferrugineus* AChEs, AChEIIb and AChEIIIb, to inhibition by six different insecticides were investigated using AcSChI as a substrate. The inhibition kinetic parameters, IC$_{50}$ and Ki, and the mechanisms of inhibition are presented in Table (1).

<table>
<thead>
<tr>
<th>Insect species</th>
<th>Insecticides</th>
<th>Chloropyrifos IC$_{50}$ a</th>
<th>Chloropyrifos Ki b</th>
<th>Malathion IC$_{50}$ a</th>
<th>Malathion Ki b</th>
<th>Oxamyl IC$_{50}$ a</th>
<th>Oxamyl Ki b</th>
<th>Carbosulfan IC$_{50}$ a</th>
<th>Carbosulfan Ki b</th>
<th>Deltamethrin IC$_{50}$ a</th>
<th>Deltamethrin Ki b</th>
<th>Avermectin IC$_{50}$ a</th>
<th>Avermectin Ki b</th>
<th>Emamectin benzoate IC$_{50}$ a</th>
<th>Emamectin benzoate Ki b</th>
<th>References</th>
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<tr>
<td><em>R. ferrugineus</em></td>
<td>AChEIIb</td>
<td>0.75</td>
<td>0.57</td>
<td>1.5</td>
<td>1.21</td>
<td>0.23</td>
<td>0.19</td>
<td>0.6</td>
<td>0.5</td>
<td>0.2</td>
<td>0.16</td>
<td>0.35</td>
<td>0.35</td>
<td>Present study</td>
<td>Wu et al., (2011)</td>
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<td>AChEIIIb</td>
<td>1.0</td>
<td>0.8</td>
<td>1.0</td>
<td>0.85</td>
<td>0.25</td>
<td>0.21</td>
<td>0.5</td>
<td>0.7</td>
<td>0.15</td>
<td>0.14</td>
<td>0.3</td>
<td>0.23</td>
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<td><em>O. chinensis</em></td>
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<td>0.8</td>
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<td>Wu et al., (2011)</td>
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<td><em>S. littoralis</em> (Field strain)</td>
<td>OP</td>
<td>10$^{4}$</td>
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<td>Gaaboub et al., (2005)</td>
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<td><em>S. littoralis</em> (laboratory strain)</td>
<td>Carbamate</td>
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<td><em>A. millera</em></td>
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<td>Villatte et al., (1998)</td>
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<td><em>A. ipsilon</em></td>
<td>5x10$^{-5}$</td>
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<td>Loewenstein et al., (1993)</td>
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<td><em>C. elegans</em></td>
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<td>11.2x10$^{-3}$</td>
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<td><em>T. California</em></td>
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<td>49x10$^{-3}$</td>
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<td><em>D.melanogaster</em></td>
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<tr>
<td><em>Drosophila</em></td>
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<td>38x10$^{-3}$</td>
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3.1.1. Susceptibility towards chloropyrifos

The effect of different chloropyrifos concentrations (Fig. 1) on the activities of *R. ferrugineus* AChEs revealed that a gradual decrease in AChEIIb and AChEIIIb activities with increasing chloropyrifos concentrations and incubation time was observed. Upon incubation of each with 2 mM for 15 min, 94% and 87.3% of the enzyme activities were suppressed, respectively with IC$_{50}$ values 0.75 and 1.0 mM for AChEIIb and AChEIIIb, respectively. Chloropyrifos competitively inhibited *R. ferrugineus* AChEIIb and AChEIIIb with Ki values 0.57 and 0.8 mM respectively (Fig. 2 a, b).
Fig. 1: Effect of different chloropyrifos concentrations on the activities of *R. ferrugineus* AChEs. Purified AChEIIb and AChEIIIb were incubated for 15 min with different concentrations ranging from 0.1-2.0 mM at room temperature followed by estimating the residual activities.

Fig. 2: Reciprocal of initial velocities of (a) AChEIIb and (b) AChEIIIb versus reciprocal concentrations of AcSChI in presence of different concentrations of chloropyrifos. Inhibition constant (*Ki*) of chloropyrifos was shown in the inset.
3.1.2. Susceptibility towards malathion

A decrease in *R. ferrugineus* AChEIIb and AChEIIIb activities was recorded with increasing malathion concentrations (Fig. 3), where 67% and 84% of the activities, respectively were inhibited upon incubation of each with 2.0 mM for 15 min. The IC_{50} values were 1.5 and 1.0 mM for AChEIIb and AChEIIIb, respectively. Malathion non-competitively inhibited *R. ferrugineus* AChEIIb and AChEIIIb with *K*/* values 1.2 and 0.85 mM, respectively (Figs. 4 a, b).

![Fig. 3: Effect of different malathion concentrations on the activities of *R. ferrugineus* AChEs. Purified AChEIIb and AChEIIIb were incubated for 15 min with different malathion concentrations ranging from 0.25 – 2.0 mM at room temperature followed by estimating the residual activities.](image)

3.1.3. Susceptibility towards oxamyl

The enzymatic activities of *R. ferrugineus* AChEIIb and AChEIIIb were reduced with increasing oxamyl concentrations (Fig. 5). Upon incubation of each with 0.8 mM for 15 min, 92.5% and 93% of the activities were inhibited, respectively. The IC_{50} values were 0.23 and 0.25 mM, respectively. Oxamyl competitively inhibited *R. ferrugineus* AChEIIb and AChEIIIb with *K*/* values 0.19 and 0.21 mM, respectively (Figs. 6 a, b).

3.1.4. Susceptibility towards carbosulfan

*R. ferrugineus* AChEIIb and AChEIIIb enzyme activities were inhibited with increasing carbosulfan concentrations (Fig. 7). Upon incubation of each with 0.8 mM for 15 min, 61% and 73% of the activities were lost. The IC_{50} values are 0.6 and 0.5 mM, respectively. Carbosulfan competitively inhibited *R. ferrugineus* AChEIIb and AChEIIIb with *K*/* values 0.5 and 0.7 mM, respectively (Figs. 8 a, b).

3.1.5. Susceptibility towards deltamethrin

The activities of *R. ferrugineus* AChEIIb and AChEIIIb were suppressed with increasing deltamethrin concentration (Fig. 9). Upon incubation of each with 0.4 mM for 15 min, 80% and 91.3% of activities were suppressed with IC_{50} values 0.2 and 0.15 mM, respectively. Deltamethrin competitively inhibited *R. ferrugineus* AChEIIb and AChEIIIb with *K*/* values 0.16 and 0.14 mM, respectively (Fig. 10 a, b).
Fig. 4: Reciprocal of initial velocities of (a) AChEIIb and (b) AChEIIIb versus reciprocal concentrations of AcSChI in presence of different concentrations of malathion. $K_i$ of malathion was shown in the inset.

Fig. 5: Effect of different oxamyl concentrations on the activities of *R. ferrugineus* AChEs. Purified AChEIIb and AChEIIIb were incubated for 15 min with different oxamyl concentrations ranging from 0.1 – 1.0 mM at room temperature followed by estimating the residual activities.
Fig. 6: Reciprocal of initial velocities of (a) AChEIIb and (b) AChEIIIb versus reciprocal concentrations of AcSChI in presence of different concentrations of oxamyl. Inhibition constant ($K_i$) of oxamyl was shown in the inset.
Fig. 7: Effect of different carbosulfan concentrations on the activities of *R. ferrugineus* AChEs. Purified AChEIIb and AChEIIIb were incubated for 15 min with different carbosulfan concentrations ranging from 0.1 – 0.8 mM at room temperature followed by estimating the residual activities.

Fig. 8: Reciprocal of initial velocities of (a) AChEIIb and (b) AChEIIIb versus reciprocal concentrations of AcSChI in presence of different concentrations of carbosulfan. *Ki* of carbosulfan was shown in the inset.
Fig. 9: Effect of different deltamethrin concentrations on the activities of *R. ferrugineus* AChEs. Purified AChEIIb and AChEIIIb were incubated for 15 min with different deltamethrin concentrations ranging from 0.1 – 0.4 mM at room temperature followed by estimating the residual activities.

Fig. 10: Reciprocal of initial velocities of (a) AChEIIb and (b) AChEIIIb versus reciprocal concentrations of AcSChI in presence of different concentrations of deltamethrin. *K*\textsubscript{i} of deltamethrin was shown in the inset.
3.1.6. Susceptibility towards emamectin benzoate

A reduction in *R. ferrugineus* AChEs, AChEIIb and AChEIIIb activities were recorded with increasing emamectin benzoate concentration (Fig. 11). Upon incubation of each with 0.6 mM, 80% and 85% of the activities were lost with IC$_{50}$ values 0.35 and 0.3 mM, respectively. Emamectin benzoate non-competitively inhibited *R. ferrugineus* AChEIIb and AChEIIIb with $K_i$ values 0.47 and 0.23 mM, respectively (Figs. 12 a, b).

3.2. Susceptibility of *R. ferrugineus* AChEs towards botanical extracts

3.2.1. Susceptibility towards *O. europaea* extract

The susceptibility of *R. ferrugineus* AChEs to inhibition *in vitro* by different concentration of the *O. europaea* extract revealed that, above concentration 3 mg, *O. europaea* exerted a strong inhibitory effect. Such effect increased by increasing the extract concentration (Fig. 13). Upon incubation *R. ferrugineus* AChEIIb and AChEIIIb with 10 mg of *O. europaea* for 15 min, 79% and 87% loss in enzyme activities were recorded and the IC$_{50}$ values are 7 and 5 mg, respectively. Competitive inhibition mechanisms were established for *R. ferrugineus* AChEIIb and AChEIIIb with $K_i$ values 5 and 3.5 mg, respectively (Fig. 14 a, b).

3.2.2. Susceptibility towards *A. indica* extract

*R. ferrugineus* AChEs are susceptible to inhibition by different concentrations of the *A. indica* extract where increasing in the inhibitory effect by increasing the botanical extract concentration (Fig. 15). *R. ferrugineus* AChEIIb showed higher susceptibility than AChEIIIb, where 69 and 58% of inhibition was recorded upon incubation with 16 mg of *A. indica*, and the IC$_{50}$ values are 14 and 12 mg, respectively. Noncompetitive inhibition mechanisms were estimated for *R. ferrugineus* AChEIIb and AChEIIIb with $K_i$ values 10 and 9 mg, respectively (Fig. 16 a, b).

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**Fig. 11:** Effect of different emamectin benzoate concentrations on the activities of *R. ferrugineus* AChEs. Purified AChEIIb and AChEIIIb were incubated for 15 min with different emamectin benzoate concentrations ranging from 0.1 – 0.6 mM at room temperature followed by estimating the residual activities.
Fig. 12: Reciprocal of initial velocities of (a) AChEIIb and (b) AChEIIIb versus reciprocal concentrations of AcSChI in presence of different concentrations of emamectin benzoate. $K_i$ of emamectin benzoate was shown in the inset.

Fig. 13: Effect of different *O. europaea* concentrations on the activities of *R. ferrugineus* AChEs. Purified AChEIIb and AChEIIIb were incubated for 15 min with different *O. europaea* concentrations ranging from 1 to 10 mg at room temperature followed by estimating the residual activities.
Fig. 14: Reciprocal of initial velocities of (a) AChEIIb and (b) AChEIIIb versus reciprocal concentrations of AcSChI in presence of different concentrations of *O. europaea*. Inhibition constant (*Ki*) of *O. europaea* was shown in the inset.

Fig. 15: Effect of different *A. indica* concentrations on the activities of *R. ferrugineus* AChEs. Purified AChEIIb and AChEIIIb were incubated for 15 min with different *A. indica* concentrations ranging from 2 to 16 mg at room temperature followed by estimating the residual activities.
Fig. 16: Reciprocal of initial velocities of (a) AChEIIb and (b) AChEIIIb versus reciprocal concentrations of AcSChI in presence of different concentrations of A. indica. Inhibition constant (Ki) of A. indica was shown in the inset.

3.2.3. Susceptibility towards O. basilicum

The susceptibility of R. ferrugineus AChEs to inhibition by O. basilicum revealed that the inhibitory effect increased with regard to the botanical extract concentration (Fig. 17). Upon incubation with 30 mg of O. basilicum, 77 and 71% of R. ferrugineus AChEIIb and AChEIIIb activities were suppressed with IC50 values 15 and 20 mg, respectively. Noncompetitive inhibition mechanisms (Figs 18 a, b) were deduced with Ki values 12 and 14 mg, respectively. The inhibition kinetic parameters, IC50 and Ki, and the mechanisms of inhibition of R. ferrugineus AChEs by different plant extracts are cumulative in Table (2).

3.3 The active compounds present in O. europaea

The active compounds present in the ethanolic leaves extract of O. europaea, as the promising botanical extract for inhibiting R. ferrugineus AChEs, by HPLC analysis are demonstrated. The fragmentation patterns of the peaks were compared with those of standards (Fig. 19). Thirteen peaks of active compounds are present in the extract with percent ranged from 0.05-96.8%. Only, oleuropein is
the major active compound and represented 96.8% Table (3).

**Fig. 17:** Effect of different *O. basilicum* concentrations on the activities of *R. ferrugineus* AChEs. Purified AChEIIb and AChEIIIb were incubated for 15 min with different *O. basilicum* concentrations ranging from 2 to 30 mg at room temperature followed by estimating the residual activities.

(a)

**Fig. 18:** Reciprocal of initial velocities of (a) AChEIIb and (b) AChEIIIb versus reciprocal concentrations of AcSChI in presence of different concentrations of *O. basilicum*. Inhibition constant (*Ki*) of *O. basilicum* was shown in the inset.
Fig. 19: HPLC- chromatogram of ethanol extract of *O. europaea*

Table 2: Kinetic parameters and mechanism of inhibition for inhibiting *R. ferrugineus* AChEs by different botanical extracts

<table>
<thead>
<tr>
<th>Plant species</th>
<th>IC₅₀ (mg)</th>
<th>Kᵢ (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AChEIIb</td>
<td>AChEIIIb</td>
</tr>
<tr>
<td><em>Olea europaea</em></td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td><em>Azerachita indica</em></td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td><em>Ocimum basilicum</em></td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>c</em>: Competitive</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>n</em>: Non-competitive</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Active compounds present in the leaves extract of *O. europaea* using HPLC analysis

<table>
<thead>
<tr>
<th>No</th>
<th>Compound name</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gallic</td>
<td>0.000</td>
</tr>
<tr>
<td>2</td>
<td>Protocatechuic</td>
<td>0.000</td>
</tr>
<tr>
<td>3</td>
<td>ρ-hydroxybenzoic</td>
<td>0.000</td>
</tr>
<tr>
<td>4</td>
<td>Gentisic</td>
<td>0.000</td>
</tr>
<tr>
<td>5</td>
<td>Catechin</td>
<td>0.70</td>
</tr>
<tr>
<td>6</td>
<td>Chlorogenic</td>
<td>0.134</td>
</tr>
<tr>
<td>7</td>
<td>Cafécic</td>
<td>0.054</td>
</tr>
<tr>
<td>8</td>
<td>Syringic</td>
<td>0.074</td>
</tr>
<tr>
<td>9</td>
<td>Vanillic</td>
<td>0.104</td>
</tr>
<tr>
<td>10</td>
<td>Ferulic</td>
<td>0.05</td>
</tr>
<tr>
<td>11</td>
<td>Sinapic</td>
<td>0.384</td>
</tr>
<tr>
<td>12</td>
<td>ρ-coumaric</td>
<td>0.348</td>
</tr>
<tr>
<td>13</td>
<td>Rutin</td>
<td>0.96</td>
</tr>
</tbody>
</table>
4. Discussion

Current tactics employed to manage R. ferrugineus are largely based on chemical insecticides application. The choice of the chemicals used in the field regularly was developed through laboratory experiments (Shawir et al., 2014). Although chemical insecticides have been used for controlling RPW for long time, there is scarcity of knowledge for estimating the inhibitory effects of these insecticides and the mechanisms of inhibition on R. ferrugineus AChEs as the target site for inhibition and is responsible for the intoxication resulting in the target pest death. In addition, the usage of botanical extracts from different plant species as toxic compounds alternative to chemical insecticides through an integrated pest management (IPM) programs for management RPW have been investigated. The toxic effects of the ethanolic extract of Juniperus communis (Sharaby and Al-Dosary, 2014; 2016), A. indica (Bream et al., 2001) and O. basilicum (Ali et al., 2019) on different stages of RPW in vivo have been investigated. However, the information about the mode of action of the plant base insecticides is still so scanty.

This is the first report for evaluating the susceptibility of purified R. ferrugineus AChEs to inhibition by different insecticides and botanical extracts in vitro, as the target site for inhibition, and estimating the inhibition parameters (IC\(_{50}\), Ki, and the mechanism of inhibition). Such study was carried out for understanding the mechanism of R. ferrugineus AChEs for scavenging different insecticides to provide opportunities to develop the control strategies for mitigating the resistance problem and for preventing the failure of an insecticide(s) for management RPW. The present report aimed to predict and nominate in vitro the insecticide(s) and the botanical extract(s) that have high inhibitory effect against AChEs of the target pest R. ferrugineus. These parameters could be reliable measures of RPW susceptibility to insecticides and botanical extracts, since no physiological variables are incorporated in the system.

The failure of the available insecticides for management RPW is complained by the farmers (Al-Ayedh et al., 2016). This could be due to the development of insecticide resistance. Target-site insensitivity of AChE is a physiological mechanism for conferring resistance in different insect species by metabolic detoxification of synthetic insecticides (Pethuan et al., 2007; Kim et al., 2012; Dang et al., 2017).

R. ferrugineus AChEs were susceptible to inhibition by all the insecticides examined, albeit weak with IC\(_{50}\) values ranged from 0.16 to 1.5 mM. Deltamethrin recorded the lowest IC\(_{50}\) values 0.2 and 0.15 mM for AChEIIb and AChEIIb, respectively. On the contrary, malathion recorded the highest IC\(_{50}\) of 1.5 and 1.2 mM, respectively. Based on IC\(_{50}\) and Ki, our data showed that R. ferrugineus AChEs have similar susceptibility to inhibition by the examined insecticides and the potency of inhibition could be arranged as follows; deltamethrin > carbosulfan > oxamyl > emamectin benzoate > chloropyrifos > malathion. A significant difference in susceptibility of R. ferrugineus AChEs could be observed among the examined insecticides where, the susceptibility of R. ferrugineus AChEs towards deltamethrin is in average 7.5- and 8.0-fold higher than that of malathion.

The inhibition parameters, IC\(_{50}\) and Ki, values for R. ferrugineus AChEs towards different insecticides examined demonstrate that the highest and the lowest susceptibility of R. ferrugineus AChEs towards insecticides was observed for deltamethrin and malathion, respectively. The inhibition kinetic parameters have been compared with those previously reported for different insect species (Table 1). Except for malathion and emamectin benzoate, all the insecticides examined competitively inhibited R. ferrugineus AChEs with Ki values ranged from 0.16 to 1.2 mM.

This type of inhibition, competitive, decrease the Km values of R. ferrugineus AChE. Since the Km has an inverse relationship with the substrate concentration required to saturate the active sites of the enzyme, this indicates that most of the insecticides, i.e., chloropyritos, oxamyl, carbosulfan and deltamethrin, decreased the affinity of R. ferrugineus AChEs towards substrate. In other words, Km is the measurement of the stability of the enzyme-substrate complex, a high Km indicate weak binding and
vice versa. While, the type of inhibition, non-competitive, diminished the \( V_{\text{max}} \) of \( R. \text{ferrugineus} \) AChEs which refer that the presence of malation and emamectin benzoate, interfere with the rate of breakdown of the enzyme-substrate complex as deduced by Zibaee, (2011).

Chemical structure of the insecticide is significantly important for determining the susceptibility of AChEs to inhibition by insecticides (Shi et al., 2002). \( R. \text{ferrugineus} \) AChEs have high IC\(_{50}\) and \( K_i \) values for malathion and chloropyrifos. It can be concluded that \( R. \text{ferrugineus} \) AChEs, have the lowest sensitivity to inhibition by malathion and chloropyrifos, OP insecticides, with several folds than those for various insect species (Loewenstein et al., 1993; Villatte et al., 1998; Gaaboub et al., 2005; Wu et al., 2011). While chloropyrifos competitively inhibited \( R. \text{ferrugineus} \) AChEs, it noncompetitively inhibited laboratory and field strains of \( A. \text{ipsilon} \) and \( A. \text{millefraz} \) AChEs (Gaaboub et al., 2005). Chloropyrifos do not directly inhibited AChE, but must first be metabolized (Chambers and Chambers, 1989; Timechalk et al., 2002; Gaaboub et al., 2005).

Therefore it can be interpreted that \( R. \text{ferrugineus} \) AChEs have the least susceptibility towards such insecticides that belong to OP insecticides, justifying the concern of farmers regarding the low efficiency of these insecticides for management of RPW and could explain the lower efficiency of such insecticides upon field trail treatments. The insensitivity of \( R. \text{ferrugineus} \) AChEs may play a critical role in the tolerance of RPW to these insecticides. These results are congruent to that reported by Shawir et al. (2014) where insecticides belonging to OP group, chloropyrifos and dimethoate, have the least relative toxicity LC\(_{50}\) and are the least toxic insecticides against RPW.

Based on IC\(_{50}\) values, \( R. \text{ferrugineus} \) AChEIIb and AChEIIib are 2.1- and 3.3- fold more susceptible to emamectin benzoate than chloropyrifos as an OP insecticide. These results confirmed the in vivo finding by Shawir et al. (2014), where emamectin benzoate had remarkable effect on the larvae of \( R. \text{ferrugineus} \) and the relative toxicity of emamectin benzoate at the level of LD\(_{50}\) was about 18.5-times of chloropyrifos. In addition, Al-Jabr et al. (2013) found that emamectin benzoate was also a highly toxic insecticide and resulted in 92% cell mortality of mid-gut cell line of \( R. \text{ferrugineus} \) and 74% growth inhibition. Emamectin benzoate is a novel semi-synthetic derivative of natural product a barneyct in Avermactin family. It blocks post-synaptic potentials of the neuromuscular junction, leading to paralysis and finally the death of the target pest (Putter et al., 1981; Abdel-aziz, 2019). The low inhibitory effect of chloropyrifos is in accordance to the interpretation recorded by Gaaboub and coworkers where, chloropyrifos exerted a weak inhibitory effect against AChEs of cotton leafworm, \( Spodoptera \text{littoralis} \), cutworm, \( Agrotis \text{ipsilon} \) and honey bee \( A. \text{millefraz} \) (Gaaboub et al., 2005).

Studies of the inhibition kinetic of \( R. \text{ferrugineus} \) AChEs by different insecticides in vitro appear to be a useful tool to make a rational selection of the promising and the most efficient insecticides for management of RPW. AChE is the target of many OP and carbamate insecticides. The accepted mode of their action is the promotion of the phosphorylation or carbamylation type modifications of the active site of the AChEs. These modifications inhibit AChE activity and block the hydrolysis of ACh (Hsu et al., 2008). This step results in an increase ACh level at the nerve fibers and the eventual fail of the neuromuscular junction, leading to paralysis and finally the death of the target pest. In the present study, the results of in vitro inhibition of RPW AChEs by insecticides as OPs, carbamates, pyrethroids and avermectins agreed quite well with the previously published in vivo bioassays data (Shawir et al., 2014).

The insecticidal activity of sweet basil, \( O. \text{basilicum} \) against \( R. \text{ferrugineus} \) has been evaluated and the major essential oil components are: methyl chavicol (estragole) (27.82%), linalool (25-35%), eugenol (8.81%), eucalyptol (4.92%) and terpinen (2.1%) (Abdel Kareim et al., 2017). Eugenol, the principle compound of the essential oils of basil, has a strong repellent activity against insect pests. Linalool is the second essential oil present also in basil has toxic effect to the Bruchid zabrotes subfasciatus and other storage pests (Weaver et al., 1991; Kim and Lee, 2014). Recently, Ali et al. (2019) documented the insecticidal potential of basil against different stages of RPW and concluded that \( O. \text{basilicum} \) could be used for management RPW in the egg and larva stages. The present study represents the first report to establishment the susceptibility of purified \( R. \text{ferrugineus} \) AChEs to inhibition by \( O. \text{basilicum} \) extract. \( O. \text{basilicum} \) extract inhibited \( R. \text{ferrugineus} \) AChEIIb and AChEIIib noncompetitively with \( K_i \) value 12 and 14 mg and with IC\(_{50}\) values 15 and 20 mg, respectively.

\( A. \text{indica} \) has long been recognized as a source of environment friendly biopesticide. The high insecticidal effect of neem leaves extract could be attributed to the presence of various compounds that are lethal to a wide range of insects and their complex mode of action (Schmutterer, 1990). Its physiological and insecticidal effects against Lepidopteran insects have been reviewed (Senthil-Nathan,
Leaves extract showed marked insect control potential and can be recommended for many IPM programs (Khan et al., 2007; Senthil-Nathan, 2013; Rana et al., 2015; Abd-Elsalam et al., 2016). The insecticidal effect of neem extract A. indica on the respiratory metabolism during the pupal stage of RPW R. ferrugineus (Bream et al., 2001) has been investigated.

The present study demonstrated a significant (P<0.01) reduction in R. ferrugineus AChEIIb and AChEIIIb activities, where 69 and 58 % of the enzyme activities were inhibited by 16 mg of A. indica, respectively. A. indica noncompetitively inhibited R. ferrugineus AChEIIb and AChEIIIb with Kᵢ values 10 and 9 mg, respectively. Similarly, A. indica leaves extract suppressed Musca domestica (Rana et al., 2015) and Drosophila melanogaster AChEs (Khan et al., 2007) with 20.35% and 45% inhibition in presence of 16.4 and 16.9 µg/ml, respectively. Senthil-Nathan et al. (2008) reported that the active ingredients from A. indica alter AChE activity and the LC₅₀ concentration of A. indica significantly inhibited AChE activity of the brown plant hopper, Nilaparvata lugens. However, certain essential oils from aromatic plants, monoterpenes, competitively inhibited AChE in vitro (Grundy and still, 1985; Miyazawa, et al., 1997).

R. ferrugineus AChEIIb and AChEIIIb have high susceptibility to inhibition by O. europaea leaves extract with IC₅₀ values 7 and 5 mg, respectively. According to the IC₅₀, O. europaea extract exerted the highest inhibitory effect where, the susceptibility of the enzymes ranged from 2.0-4.0-fold higher than that recorded for A. indica and O. basilicum. The most promising botanical extract for inhibition R. ferrugineus AChEs is O. europaea. O. europaea extract competitively inhibited R. ferrugineus AChEs. This revealed that O. europaea extract decreased the affinities of R. ferrugineus AChEs to the substrate and weak binding of the enzymes to the substrate.

The mode of action of the botanical extracts, as bioinsecticides is not obviously known. However, it is evident that botanical extracts affect insect physiology as; repellent, antifeedant and growth regulation effects, in diverse ways. Botanical extract constituents affect biochemical processes, which specifically disrupt the endocrinologic balance of insects (Rattan, 2010; Kumar et al., 2011), blocked insect AChEs synthesis by which play role in cholinergic synapses in insects and higher animals (Fournier and Mutero, 1994; Kumar et al., 2011) for nerve conduction and thus maintain a general coordination in the neuromuscular system. However, this action may not be correlated with toxicity to insects in vivo where a direct correlation between insect toxicity and AChE inhibition could not be recorded (Lee et al. 2001; Isman, 2000).

The HPLC analysis for O. europae, as the promising botanical extract with the highest inhibitory effect on R. ferrugineus AChEs, showed that oleuropein constitutes the highest percentage (96.8) of the total compounds present in O. europae. The inhibitory effect of O. europae may be attributed to the active compounds that are contained in the extract. Rajashekar et al. (2014) reported that the active compounds of the botanical extract have broad impact across the nervous system which is attenuated by modified acetyl choline and acetate function.

In conclusion, the susceptibility of R. ferrugineus AChEs toward different insecticides appear to be a useful tool in vitro approach for selecting the most promising insecticides for controlling RPW. R. ferrugineus AChEs have the least sensitivity towards malathion and chloropyrifos as OP insecticides, and this can justify the complaint by the farmers regarding the low efficiency of these insecticides for controlling RPW. The combination of the promising insecticide, deltamethrin, and O. europaea extract will be investigated in future, in vivo, for estimating the synergistic relation between them for management of R. ferrugineus, reducing the amounts of insecticides released in the environment and as an attempt to increase their efficacy against RPW to overcome the failure of synthetic insecticide(s) upon field application for controlling R. ferrugineus.

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