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A study on lethal doses of various pesticides on honeybees (*Apis mellifera* L.) – a laboratory trial

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Abstract. Exposure of honeybee (*Apis mellifera* L.) to pesticides disturbed normal physiological and behavioral functions required for normal foraging and colony maintenance. The present study was aimed towards establishing the mean lethal concentration (LC_{50}) of three pesticides viz. carbamate (carbaryl), organophosphate (chlorpyrifos) and neonicotinoid (imidacloprid) in honeybees through feeding bioassay laboratory trial. Deleteriousness was confirmed through mortality rate, number of survival bees, acetylcholinesterase (AChE), total protein (TP) levels, AChE gene transcription level, and gut tissues histological analysis of exposed honeybees. Mean mortality rate was calculated for 96 h interval at three different concentrations of tested pesticides (5, 2.5, and 1.25 mg L^{-1}), and LC_{50} values calculated for 48 and 96 h interval. AChE enzyme and TP level are determined by ELIZA and spectrophotometer, respectively. Results revealed that imidacloprid had the lowest LC_{50} (0.477 ng/bee) values as compared with carbaryl and chlorpyrifos. High mortality rate was observed at highest dose, being impidacloprid have more lethal effects as compared with other pesticides. Similarly, biochemical analysis revealed that imidiacloprid and chlorpyrifos significantly ($P \leq 0.05$) increased AChE and TP levels, whereas carbaryl significantly ($P \leq 0.05$) decreased them. Similarly, probe based RT qPCR revealed that imidacloprid and chlorpyrifos treatments significantly ($P \leq 0.05$) enhanced the AChE level whereas carbaryl decreased it. Histological analysis showed that the gut tissues of honeybees exposed to pesticide treatment had substantial morphological abnormalities. In a nutshell, imidacloprid, carbaryl and chlorpyrifos have substantial toxic effects on all the study attributes of honeybees with imidacloprid being most toxic.

Key words. Acetylcholinesterase, gene transcription, LC_{50}, pesticides.

Introduction

Honeybees (*Apis mellifera* L.) have always been a vital part of the economy and environment. Not only are they known for their hive products (royal jelly, pollen and honey), but are also considered to be one of the best pollinators, globally (Hayat *et al.*, 2018). An estimate has reported that they contribute to more than 80% of total agri-pollination (Tavares *et al.*, 2017), hence, deserving the title of "major pollinators". Since the dawn of this decade, an alarming rate of their decline is being witnessed throughout the world (Goulson *et al.*, 2015; Porrini *et al.*, 2016). Literature reveals that mass disappearances are being reported from USA (Garibaldi *et al.*, 2013), Europe (Stokstad, 2007), and Asia (Hayat *et al.*, 2018). Owing to this drastic phenomena, all the stakeholders such as apiarists, academicians, researchers, industrialists and governmental agencies have raised their concerns to contribute positively towards this alarming decline in honeybee population.

Climate change, industrialization, globalization, deforestation, lack of biodiversity and their habitat, and bee-killing pesticides are pivotal factors responsible for this decline (Becher *et al.*, 2013). Amongst these factors, use of pesticides has been determined as a major contributor, and detailed studies have revealed their disastrous effects on honeybee colony characteristics i.e. larvae and pupal development abnormalities, enhanced risk of pest attack, foraging bees die (Gill *et al.*, 2012), physiological functions (Fasasi, 2012), oxidant and antioxidant activities, learning (Decourtye *et al.*, 2004; Aliouane *et al.*, 2009), possing behavioral stresses (Hayat *et al.*, 2018), acetylcholinesterase (AChE) activity (Boily *et al.*, 2013),

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survival rate (Husain *et al.*, 2014) and other biological aspects of honeybees. It has been studies that AChE is one of the best indictaor of exposure to various pesticides in order to predict population decline in invertebrates (Hyne & Maher, 2003; Pohanka, 2011). Measurement of AChE inhibition has been increasingly used in the last two decades as a biomarker of effect on nervous system following exposure to organophosphate and carbamate pesticides (Lionetto *et al.*, 2013).

AChE is an enzyme that hydrolyses the neurotransmitter acetylcholine (ACh) in cholinergic synapses for the rapid and precise control of nerve transmission. Thus, an increase in AChE level might be a biological response to compensate the permanent activation of cholinergic neurons due to the strong binding of pesticides to ACh receptors (Tavares *et al.*, 2017). In vertebrates only one locus of gene encoding the AChE whereas in insects there are two gene loci encoding AChE i.e. ace1 encoding AChE1 and ace2 encoding AChE2 (Kim & Lee, 2013). In honeybees, cholinergic signaling is the basis of neurological and physiological functions. A key cholinergic protein is AChE the enzyme (Shapira *et al.*, 2001). Sub lethal doses of pesticides that target AChE enzyme can disturb bee foraging behaviour which ultimately leads to colony loss (Williamson & Geraldine, 2013).

Among all pesticides being used, neonicotinoid class shares 25% of global pesticide market (Jeschke *et al.*, 2011; Swenson & Casida, 2013). Other insecticides such as carbaryl and chlorpyrifos are also extensively used which are agonistic to insect nicotinic ACh receptors. Pakistan is one such country which, owing to its geo-location, is highly vulnerable to climate change. These changing weather patterns have turned it into a vulnerable state in terms of declining biodiversity. Lately, concerns are being expressed regarding the decline of honeybee population in Pakistan which is seriously affecting honey production and quality fruits (Hayat *et al.*, 2018). In Pakistan, imidacloprid and carbaryl is being used for the control of sucking and borer insects in different developmental stages of various crops e.g. wheat, cotton, barley, sorghum and corn. These contaminated crops pose threat to beneficial animals and insects (Naveed *et al.*, 2010; Khan *et al.*, 2015). The present work is carried out to study these pesticides as they affect the honeybees through laboratory trials. It was aimed towards assessment of lethal doses (LC_{50}) of three pesticides viz. carbamate (carbaryl), organophosphate (chlorpyrifos) and neonicotinoid (imidacloprid) as determined through mortality rate, AChE, total protein (TP) levels, relative abundance of AChE1 and AChE2 genes and histological analysis of honeybees.

Materials and methods

Pesticides

Technical grades of pesticides representing three chemical classes carbaryl (Carbamate), chlorpyrifos (Organophosphate) and imidacloprid (Neonicotinoid) with 99% purity were used (Bayer, Pakistan). Following correction factor (CF) equation was used (Heong *et al.*, 2013).

$$CF = 100\%/\%\text{active ingredient of the pesticides} \quad (1)$$

$$CF = 100\%/99.0\% = 1.01 \quad (2)$$

From the given CF, the weight of the technical pesticide needed can be computed and required volume and concentration can be prepared by using following formula.

Concentration of pesticide required × Volume × CF.

According to CF equation to prepare 10 $mg\,L^{-1}$ pesticide stock solution of 10 mL volume, the weight of pesticide needed i.e. $10\,mg\ L^{-1} \times 10\ mL \times 1.01 = 101\,mg = 0.101\,g$. First, 0.101 g of technical grade pesticide was weighed in a vial using an analytical balance and add 10 mL ethanol. From this stock solution, pesticide dilutions were prepared in 50% sucrose solution.

Bee hives

For the collection of honeybee samples, six beehives were purchased from local private apiarists. Each hive box contained 10 frames. New beeswax sheet foundation frames were used to avoid possible contamination of pesticide residues. These sheet frames were used as a template for the construction of comb hive. Within 25 days, workers bees built the frames completely. Each hive had a healthy queen as determined through visual inspection. Brood frames were checked for the presence of larvae and pupae in case of nonvisibility of queen due to large bee population. It was also assured that no chemical treatment was done on frame during and before study. A white paper sheet coated with a thin layer of petroleum jelly was placed at the bottom of the hive to observe mites. As hives were present under controlled environmental conditions (confirm by regular inspection that no mite infected or disease hive present in the apiary), so no mite infestation was observed. The bees were fed with 50% (w/v) sucrose solution ad libitum along with Sarsson (*Brassica compestris*), Berseem (*Trifolium alexdndrium*) and Sunflower (*Helianthus annus*) vegetation. Before placing bee hives it was also ensured no pesticide spray was done on plants.

Honeybee collection

For laboratory bioassays, a small plastic container was taken and a thin layer of honey was applied to attract foragers honey bees in hive towards the container (It was assured queen did not come along with worker bees by careful observing external morphology of capture bees). As bees came to the container, it was covered with a net and transported to the Entomology Research Laboratory, Lahore College for Women University, Lahore, Pakistan. Only adult worker bees present in the hive were used for the experimentation. The bees were acclimatized in net cage ($11 \times 10 \times 14$ cm) for 2 h (to acclimatized them) at 25 ± 2 °C, relative humidity of 45% providing 50% (w/v) sucrose solution and cages were put in an incubator under darkness to provide conditions similar to hive.

Determination of mean lethal concentration-feeding test

Feeding test was conducted to determine the lethal effects of tested pesticides as per Williamson & Geraldine (2013). Experiment was carried out in net cage (11 × 10 × 14 cm). All tested pesticide solutions were diluted in sugar syrup and fed ad libitum in the following concentrations (5, 2.5 and 1.25 mg L^{-1}). One control group received only sugar syrup as 50% (w/v) food source. Second control group received ethanol (70% purity) diluted in 50% (w/v) sucrose solution. 3 mL ethanol solution per cage was used in a single vial. Plastic feeding vials (5 mL) with cut feeding holes (1 × 0.5 cm) were inserted horizontally into the cages, provided fresh daily. Two feeding vials per cage were inserted. Amount of pesticide solution consumed each day was noted. Group of 50 bees/replicate were released in each cage. Three replicates were performed for each concentration of pesticide. Worker bees in each cage were exposed to one of these concentration. The net cage were incubated in an incubator at 25 ± 2 °C, with relative humidity of 45% in darkness to simulate conditions similar to the hives. Daily mean mortality was noted for about 4 days. Oral lethal concentration LC_{50} was determined after 48 and 96 h of treatments.

Honeybee mortality rate

Dead and remaining live bees in treated and control cages were counted at the end of trials. The mean mortality rate was assessed in all the treatment and control groups at 96 h for all concentrations of exposure groups. The corrected mortality was calculated as per following (Abbott, 1925):

$$\mathrm{Pr} = [(\mathrm{Po} - \mathrm{Pc})/(100 - \mathrm{Pc})] \times 100 \quad (3)$$

Pr = corrected mortality, Po = observed mortality, Pc = control mortality.

Dissection and homogenization

Bees exposed to 5 and 2.5 mg L^{-1} concentration of tested pesticides during feeding laboratory test were used for further tests i.e. AChE and TP levels after 48 h exposure. Honeybees (three per vial) were collected and transferred into plastic vials (5 mL) which were immediately frozen in dry ice and stored in subsequent analysis (Nikolic *et al.*, 2019). Wings and legs were removed. For a single reaction, gut and head of three bees was crushed. Head and gut tissues were homogenized by using tissue lyse buffer (1 µL) and phosphate buffer (5 µL) per reaction. Samples were vortexed for about 5 min and centrifuged at 15 000 rpm at 4 °C. Supernatant was removed and used to determine the AChE and TP levels (Chan & Foster, 2008).

Cholinesterase assay

Method of Williamson & Geraldine (2013) was used to perform cholinesterase assay. The activity of AChE was determined through Bio-Tek ELx800 ELISA reader using Acetylcholinesterase Activity Assay Kit Cat. No. MAK 119 by Sigma-Aldrich, USA as per manufacturer's instructions. Three honey bees head and thorax (bees collected from each replicate) were used per assay reaction. Assay in total volume of 200 µL per microplate well was used. The working reagents were prepared by mixing kit reagent (2 mg) with assay buffer (200 µL) per reaction. For blank assay, double distilled H_2O (200 µL) was used and read the absorbance at 412 nm. 10 µL of samples were added into separate wells of the 96-well plate. The freshly prepared working reagent (190 µL) was transferred to all sample wells and tap plate briefly to mix. Calibrator (200 µL) was used as standard. Initial absorbance measurement was taken at 412 nm $(A_{412})_{\mathrm{initial}}$. The plate was incubated at room temperature and after 10 min the final measurement $(A412)_{\mathrm{final}}$ was taken. Following formula used to determine AChE activity.

$$\mathrm{AChE\ activity\ (unit)} = (A412)_{\mathrm{final}} - (A412)_{\mathrm{initial}} / (A412)_{\mathrm{calibrator}} - (A412)_{\mathrm{blank}} \times 20 \quad (4)$$

Total protein (TP) assay

Method of Williamson & Geraldine (2013) was used for TP detection. The TP test was performed by using kit of Crescent Diagnostics Cat.No.CS.610 based on Biuret method. Colour development was directly proportional to protein concentration. Wells were labelled for blank, standard and samples. Blank assay contained only distilled water. Supernatant of three honeybee head and thorax were used per assay reaction. Standard assay contained standard reagent provided with kit. Fresh supernatant (0.02 mL) was added to appropriate well. Biuret reagent (0.08 mL) and supernatant (0.02 mL) was added in each plate well. Incubation was done at room temperature for about 5 min. Absorbance was read at 546 nm. Following formula was used to determine protein concentration.

$$\mathrm{Total\ protein}\left(\frac{\mathrm{g}}{\mathrm{dL}}\right) = \mathrm{Absorbance\ of}\frac{\mathrm{sample}}{\mathrm{Absorbance\ of\ standard}} \times \mathrm{conc.of\ standard} \quad (5)$$

Histological assessment

For histological study, laboratory bioassay similar to feeding bioassay was performed. Surviving bees in the feeding test which were exposed to 5 and 2.5 mg L^{-1} pesticide concentrations were used in histological study to assess toxic effects of these pesticides on gut tissues. Surviving bees after 72 h were used as in dead bees other morphological changes occur in dead tissues due to necrosis. Method of Szymas *et al.* (2012) was used for histological assessment. Bees were chilled at 4 °C. Wings and legs were cut off and gut was removed by pulling the abdomen. After removing the gut, it was washed with distilled water and dried on filter paper. Gut tissues were fixed in

10% buffered formalin solution to prevent autolysis of cellular morphology. After fixation dehydration of tissues done in following grades of ethanol: 80% (overnight at room temperature), 90% (2–4 h at room temperature) and 100% ethanol (2–4 h at room temperature). Samples were transferred to clove oil to clear and transparent the tissues at room temperature. Samples were then paraffinized in paraffin wax and block form. About 5 µm thin sections were cut on rotatory microtome ERM – 2301 equipped with a sharpened blade. The ribbons with tissues were stretched and fixed to clean albumenized glass slide and warm at 60 °C. These glass slides were then placed in an incubator overnight for stretching and removal of bubbles. The slides were de-paraffinized in xylene (75%; histological grade) overnight. Gut tissues were hydrated in descending order of alcohol grade i.e. 100%, 90%, 70%, 50% and 30% (3–5 min in each grade). Then washed in distilled water for 5–10 min and stain them with hematoxylin–eosin stains. Thereafter, slides were dehydrated in graded ethyl alcohol (30%, 50%, 70%, 90% and 100%) cleared through xylene (75%) and slides were mounted with Canada balsam. Slides were photographed and examined under microscope (Trinocular E – 200, digital microscope Camera – Nikon Japan Eil –12).

Total RNA extraction, cDNA synthesis and amplification

Total RNA was extracted by using TRIzol reagent (Evans *et al.*, 2013) protocol. For each sample, five bees were taken (number of bees per reaction = 3 bees) from each replicate units and crushed. TRIzoI reagent (0.5 mL) was added and tissue was gently mixed and homogenized the tissue for 2–3 min. About 133.3 µL chloroform (analytical grade 100% pure) was added and incubated for 15 min at room temperature followed by centrifugation at 12 000 rpm (5415 CMEB 1039) for 10 min at 4 °C. Supernatant (3 µL) was transferred to new centrifuge tube. Afterward, 100% isopropanol (500 µL) was added, invert 3–5 times gently and incubated at room temperature for 10 min and centrifuged it at 12 000 rpm for 15 min at 4 °C, RNA pallet was formed. All liquids were carefully siphoned off from centrifuge tube. Pallet was subsequently washed with 70% ethanol and centrifuged. Finally, RNA pellet was air dried and dissolved in 50 µL nuclease free water. RNA was quantified using spectrophotometer (OPTIZEN Nano Q) at an absorbance ratio 260/280 nm. The extracted RNA was reverse transcribed into cDNA by using Fermentase revert Aid TM First stand cDNA synthesis kit no. K1622 (Thermo Fisher Scientific). The resultant cDNA was amplified by adding the following chemical reagents; double distilled water (10 µL), dNTPs (2 µL), forward primer (0.5 µL), reverse primer (0.5 µL), $MgCl_2$ (2.5 µL), buffer (2.5 µL), Taq polymerase (1 µL) and cDNA (2 µL). The thermocycler (Biorad I Cycle, 009193) was programmed to run for 40 cycles of denaturation at 94 °C (45 s), annealing 60 °C (15 s) and extension at 72 °C (45 s). The resulting PCR product was run on 4% agarose gel. A 0.5 µL of PCR product was loaded in each gel well and 50 bp DNA ladder was used.

To quantify the genetic expression of acetylcholinesterase (AChE1 and AChE2) probe based q Real time PCR reaction was performed by using Taq Man Universal Master Mix (Thermo Fisher Scientific) Kat no. 4304437, in triplicates, on step one real time PCR (Applied biosystem qRT PCR Step One™ 4369074). Thermocycler was programmed to run 40 cycles of denaturation 94 °C (40 s), annealing 60 °C (15 s) and extension at 72 °C (45 s).

Primers and probe design

Primers and probes were designed by using Primer 3 software. Primer sequence for AChE1 gene was 189 bp (forward primer 5′-CCCGAGGAGGTGAATCTGTC-3′), (reverse primer 5′-CCTCACTCTAGGCCCATTCC-3′), AChE2 220 bp (forward primer 5′-ATCTACGGGGGTGGGTTC-3′), (reverse primer 5′-CGTTGTCCCTCAG CCATC-3′). The studied genes were normalized by using housekeeping gene GAPDH (reference gene). GAPDH was 570 bp (forward primer: 5′-GCCGTATTGGCCGTC TTG-3′) (reverse primer: 5′-AAGCACCACGAC CGTCTC-3′). The probe sequence was 5-FAM 3-TEMRA dye based. Probe sequence for AChE1 was 5′-AGTACATGACGCTGGACACG-3′, AChE 2 was 5′-ACG TGTACAACGCGGACATA-3′ and GAPDH was 5′-GGTGCTCAGGTTGTTGCC-3′. Fold changes in targeted gene expression determined through ΔCT values (Livak & Schmittgen, 2001).

$$\Delta CT(\text{control}) = CT(\text{target control gene}) - CT(\text{reference gene}) \quad (6)$$

$$\Delta CT(\text{test}) = CT(\text{target test gene}) - CT(\text{reference gene}) \quad (7)$$

Normalize ΔCT of the test samples to ΔCT of the control and calculate fold changes.

$$\Delta\Delta CT = \Delta CT(\text{test}) - \Delta CT(\text{control}) \quad (8)$$

$$2^{-\Delta\Delta CT} = \text{fold changes in targeted gene} \quad (9)$$

Statistical analyses

The lethal concentration viz. LC_{50} along with their respective 95% CI intervals, and mortality rates were assessed by EPA computer Probit Analysis Program (Version 1.5). For the assessment of mortality, corrected mortality was determined by using Abbott's formula and mean (±SE) was calculated. Significance between treatment and control groups was determined through repeated measure multiple ANOVA followed by Tukey's multiple comparison test. Difference in honeybee survival in treated and control group was determined by using cox regression model log-ranke along with Kaplan–Meier survival curves at different time intervals. The $P \leq 0.05$ value from cox regression model summary were considered significantly different from control. Mean (±SE) values of AChE, TP levels and AChE1 and AChE2 gene transcription level were calculated and significant difference was determined by using ANOVA test between treated and control groups, followed by Tukey's post-hoc test. Results with $P \leq 0.05$ were considered statistically significant. All the statistical analyses were carried out using GraphPad-Prism (Version 4.03; La Jolla, CA).

Table 1. Values for acute lethal concentrations (LC_{50}) of imidacloprid, carbaryl and chlorpyrifos in *A. mellifera* L. through feeding bioassay.

Chemicals	Time (h)	LC_{50} ng/bee	$CI_{95\%}$	X^2
Imidacloprid	48	2.359	0.988–1.727	0.191
	96	0.477	0.076–0.885	1.397
Carbaryl	48	4.331	0.570–1.312	0.440
	96	0.715	0.076–0.885	2.823
Chlorpyrifos	48	8.670	0.076–0.885	0.324
	96	1.957	0.076–0.885	0.740

Results

Mean lethal concentration-feeding test

The results regarding LC_{50} of imidacloprid, carbaryl and chlorpyrifos feeding test to honeybees is shown in Table 1. Imidacloprid had more toxic effects with LC_{50} values of 2.359 ng/bee ($X^2 = 0.191$, df = 45, $P = 0.001$) and 0.477 ng/bee ($X^2 = 1.397$, df = 45, $P = 0.001$) at 48 and 96 h interval, respectively, in comparison to other compounds.

Bee mortality rates

The honeybee mean (±SE) mortality rates in all the exposure concentrations (5, 2.5 and 1.25 mg L^{-1}) as determined at 96 h in feeding bioassay are given in Fig. 1. Higher pesticide concentrations had pronounced effects as compared with lower concentrations (dose – dependent). Imidacloprid showed significantly ($F = 2365$; df = 14; $P = 0.0001$) higher mean (±SE) mortality rates of 50.0 ± 0.0, 43.6 ± 0.6 and 39.3 ± 0.3 at 5, 2.5 and 1.25 mg L^{-1} exposure respectively, in comparison to other compounds and control groups.

Survival proportions (cox regression model)

The cox regression model indicated a high significant effects of different concentrations of all pesticide treatment on honeybee survival compared with control units ($P < 0.05$). Pesticide treatment significantly affected the bee survival proportion compared with control; Imidacloprid ($X^2 = 83.84$, df = 47, $P = 0.0011$), chlorpyrifos ($X^2 = 76.33$, df = 47, $P = 0.0014$) and carbaryl ($X^2 = 72.32$, df = 45, $P = 0.0012$). Kaplan–Meier curves indicated that hazards for each treatment group on bee survival varied over time. The mean survival rate in imidacloprid was 0.00 ± 0.0, 6.4 ± 0.6 and 10.7 ± 0.55 at 5, 2.5 and 1.25 mg L^{-1} exposure, respectively. Whereas in chlorpyrifos 3.0 ± 0.57, 8.0 ± 0.6 and 15.5 ± 0.57 at 5, 2.5 and 1.25 mg L^{-1} exposure respectively. Similarly, in carbaryl treatment numbers of survive honey bees were 5.7 ± 0.57, 10.5 ± 0.57 and 19.5 ± 0.57. In all treated group bee survival rate significantly decreased compared with control (Fig. 2).

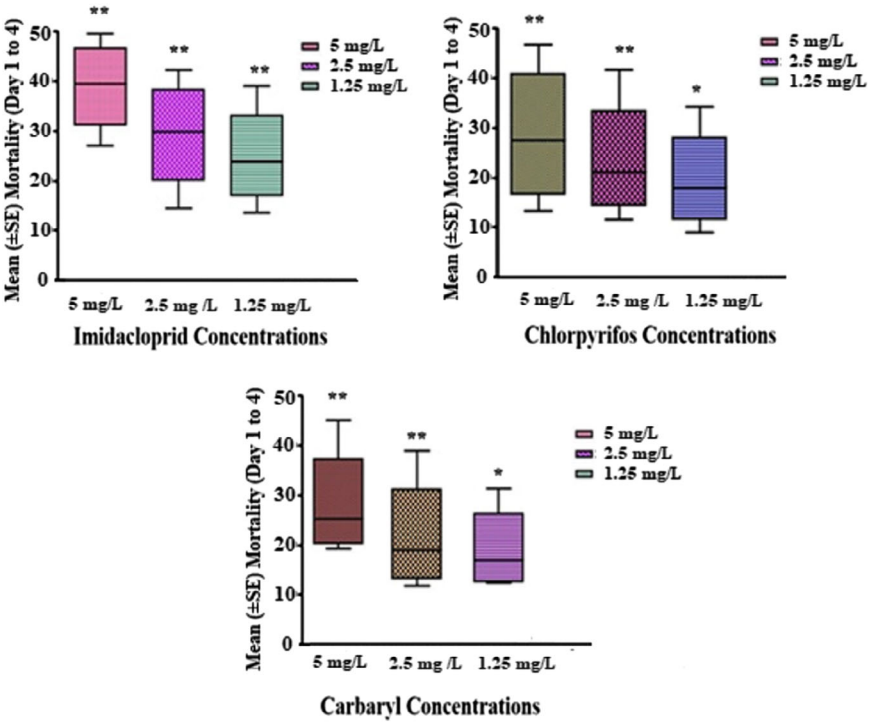

Fig. 1. Box – plot graphs showing mean (±SEM) mortality in feeding bioassay of *A. mellifera* at different concentrations of imidacloprid, carbaryl and chlorpyrifos (96 h). Upper vertical lines represent highest observation values and lower vertical represent lowest observation values, Each box represent mean number of bees died at each concentration, $n = 50$. Significant at $^{**}P \leq 0.001$; $^{*}P \leq 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com].

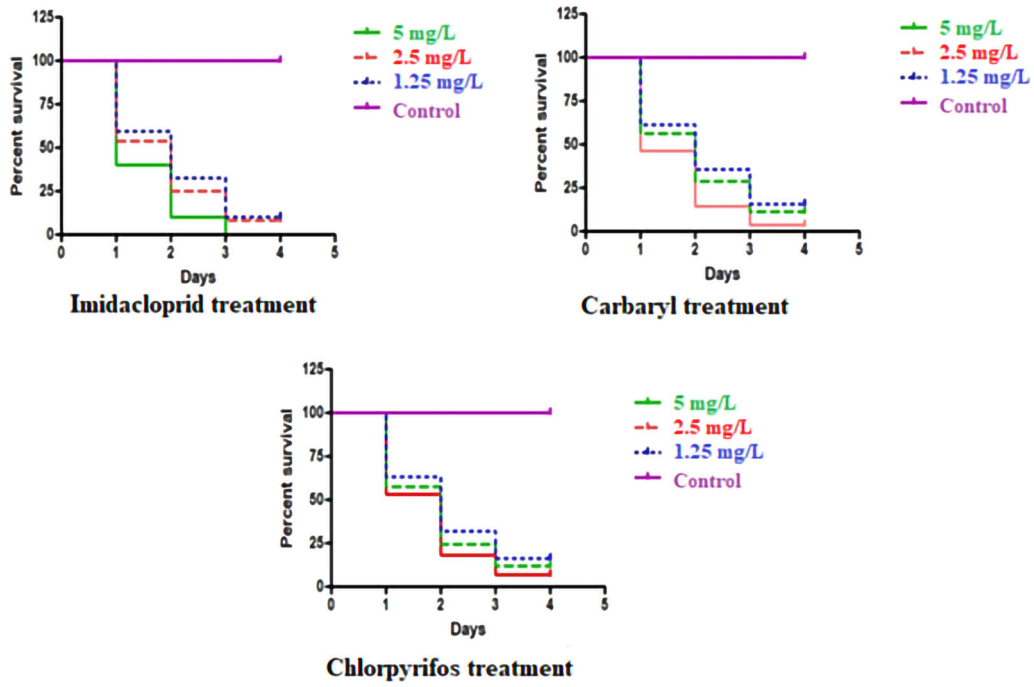

Fig. 2. Kaplan Meier survival curves between different concentrations of treated groups and control at different time intervals (1–4 days) in feeding bioassay. [Colour figure can be viewed at wileyonlinelibrary.com].

Table 2. Mean (±SE) values for acetylcholinesterase level for head and thorax tissues of *A. mellifera* in treatment and control groups.

Pesticides	Treated groups Mean unit value (U/L)	Control (ethanol) Mean unit value (U/L)	Control (sucrose) Mean unit value (U/L)	*P* values
Head tissue				
IMD (5 mg L^{-1})	86.6 ± 5.1	59.2 ± 2.2	57.5 ± 7.2	$P = 0.0031$, df = 47, $F = 1.750$
IMD(2.5 mg L^{-1})	74.4 ± 6.5	58.3 ± 9.3	54.2 ± 2.3	$P = 0.004$, df = 47, $F = 1.732$
CAR (5 mg L^{-1})	39.4 ± 3.05	60.2 ± 9.7	56.2 ± 9.4	$P = 0.03$, df = 47, $F = 1.078$
CAR (2.5 mg L^{-1})	40.4 ± 2.05	56.3 ± 2.4	57.7 ± 2.2	$P = 0.04$, df = 47, $F = 2.106$
CHP (5 mg L^{-1})	77.3 ± 3.45	63.2 ± 2.3	59.5 ± 9.2	$P = 0.0071$, df = 47, $F = 2.196$
CHP (2.5 mg L^{-1})	65.5 ± 4.51	60.6 ± 9.5	56.4 ± 9.3	$P = 0.0084$, df = 47, $F = 3.210$
Thorax tissue				
IMD (5 mg L^{-1})	55.3 ± 4.2	43.1 ± 2.2	52.1 ± 9.3	$P = 0.031$, df = 47, $F = 1.621$
IMD(2.5 mg L^{-1})	53.2 ± 5.1	41.3 ± 7.3	48.7 ± 12.4	$P = 0.032$ df = 47, $F = 1.021$
CAR (5 mg L^{-1})	34.4 ± 2.06	39.3 ± 5.7	42.4 ± 13.0	$P = 0.024$, df = 47, $F = 1.231$
CAR (2.5 mg L^{-1})	32.7 ± 3.15	41.2 ± 2.4	44 .3 ± 9.0	$P = 0.040$, df = 47, $F = 1.421$
CHP (5 mg L^{-1})	57.3 ± 4.52	43.2 ± 11.3	40.2 ± 12.3	$P = 0.041$, df = 47, $F = 1.561$
CHP (2.5 mg L^{-1})	55.4 ± 7.41	45.3 ± 13.0	43.2 ± 11.2	$P = 0.022$, df = 47, $F = 1.321$

IMD, imidacloprid; CAR, carbaryl; CHP, chlorpyrifos.

Acetylcholinesterase and total protein levels

The mean (±SE) values for AChE and TP level in head and thorax tissues of honeybees in treatment (5 and 2.5 mg L^{-1}) and control groups is presented in Tables 2 and 3, respectively. Results revealed that all the treatment groups were significantly affected by the pesticides as compared with the control group. Imidacloprid and chlorpyrifos significantly increased AChE level and TP level compared with control ($P < 0.05$). Whereas carbaryl treatment significantly decreased AChE and TP level in comparison to control group.

Qualitative and quantitative measure of alteration in AChE gene transcription

Regarding AChE gene transcription, in all control samples both AChE1 and AChE2 duplicate genes were presented.

Table 3. Mean (±SE) values for total protein level for head and thorax tissues of *A. mellifera* in treatment and control groups.

Pesticides	Treated groups Mean unit value (U/L)	Control (ethanol) Mean unit value (U/L)	Control (sucrose) Mean unit value (U/L)	*P* values
Head tissue				
IMD (5 mg L^{-1})	3.51 ± 0.22	2.0 ± 0.24	2.54 ± 0.24	$P = 0.002$, df = 47, $F = 2.31$
IMD(2.5 mg L^{-1})	3.10 ± 0.08	2.0 ± 0.22	2.76 ± 0.15	$P = 0.001$, df = 47, $F = 2.54$
CAR (5 mg L^{-1})	0.92 ± 0.133	2.3 ± 0.56	1.27 ± 0.57	$P = 0.004$, df = 47, $F = 3.21$
CAR (2.5 mg L^{-1})	1.27 ± 0.05	2.0 ± 0.67	2.94 ± 0.23	$P = 0.001$, df = 47, $F = 3.45$
CHP (5 mg L^{-1})	3.21 ± 0.089	2.8 ± 0.20	2.14 ± 0.20	$P = 0.003$, df = 47, $F = 2.78$
CHP (2.5 mg L^{-1})	2.98 ± 0.067	2.1 ± 0.57	2.20 ± 0.15	$P = 0.004$, df = 47, $F = 2.31$
Thorax tissue				
IMD (5 mg L^{-1})	3.48 ± 0.377	2.12 ± 0.42	2.57 ± 0.35	$P = 0.002$, df = 47, $F = 3.01$
IMD (2.5 mg L^{-1})	3.15 ± 0.10	2.01 ± 0.24	2.65 ± 0.17	$P = 0.002$, df = 47, $F = 2.21$
CAR (5 mg L^{-1})	0.95 ± 0.120	2.22 ± 0.57	2.60 ± 0.23	$P = 0.001$, df = 47, $F = 4.02$
CAR (2.5 mg L^{-1})	1.33 ± 0.03	2.31 ± 0.67	2.66 ± 0.41	$P = 0.001$, df = 47, $F = 4.21$
CHP (5 mg L^{-1})	3.21 ± 0.089	2.16 ± 0.24	2.54 ± 0.25	$P = 0.004$, df = 47, $F = 1.03$
CHP (2.5 mg L^{-1})	2.88 ± 0.066	2.57 ± 0.24	2.56 ± 0.67	$P = 0.003$, df = 47, $F = 1.23$

IMD, imidacloprid; CAR, carbaryl; CHP, chlorpyrifos.

DNA ladder of 50 bp was run. In imidacloprid and chlorpyrifos samples, bright bands showed that AChE1 (189 bp) and AChE2 (220 bp) gene transcription level up-regulated whereas; in carbaryl treated samples, the brightness of bands were dimmed indicating down-regulation of targeted genes. It was also revealed that in all samples AChE1 and AChE2 bands were presented but brightness of AChE1 bands was lower as compared with AChE2 bands. GAPDH gene (570 bp) was also presented among all samples.

*Quantitative relative abundance of AChE1 and AChE2 gene transcription level in pesticides treated honeybee (*A. mellifera*) samples*

Quantitative transcription level of AChE1 and AChE2 genes was measured by RT qPCR. Results showed that higher fold changes were appeared in imidacloprid treated samples being AChE1 4.359 and 3.74 folds upregulated at 5 and 2.5 mg L^{-1}, respectively compared with control. Analysis of variance showed that there was significant difference in upregulation of AChE1 gene level ($F = 3.577$; df = 7; $P = 0.0016$). Similarly AChE2 was 8.6 and 6.9 folds upregulated at 5 and 2.5 mg L^{-1} respectively compared with control showing significant difference ($F = 2.76$; df = 5; $P = 0.0012$).

In carbaryl treated samples being AChE1 3.22 and 2.78 folds down regulated at 5 and 2.5 mg L^{-1}, respectively compared with control. Analysis of variance showed that there was significant difference in downregulation of AChE1 gene level ($F = 6.34$; df = 5; $P = 0.003$,). Similarly AChE2 was 3.66 and 2.92 folds downregulated at 5 and 2.5 mg L^{-1} respectively, compared with control showing significant difference ($F = 7.510$; df = 5; $P = 0.008$).

In chlorpyrifos treated samples being AChE1 1.60 and 4.2 folds upregulated at 5 and 2.5 mg L^{-1}, respectively compared with control. Analysis of variance showed that there was significant difference in upregulation of AChE1 gene level ($F = 2.321$; df = 5; $P = 0.0243$). Similarly AChE2 was 3.86 and 3.55 folds upregulated at 5 and 2.5 mg L^{-1} respectively compared with control showing significant difference ($F = 2.244$; df = 5; $P = 0.02536$).

Histological observations

The results regarding morphological cell alterations, as affected by pesticide treatment, at 5 and 2.5 mg L^{-1} are given in Figs 3 and 4, respectively. Gut abnormalities were analysed by comparing gut tissues of control with treated groups. The shape of midgut epithelial cells was normal in tissues of control group honeybees. The epithelial cells were elongated and possessed single nuclei with a homogeneous smooth cytoplasm. Intact peritrophic membrane was present which allows direct absorption of nutrients into hemolymph. Large number of small regenerative cells was present. These cells help to regenerate the damaged epithelium but in treated groups, number of regenerative cells reduced which indicated decreasing cell repairing capacity (Fig. 3 A, B).

On the contrary, the tissues of honeybees exposed to pesticide treatment 5 mg L^{-1} (Fig. 3 C–E) and 2.5 mg L^{-1} (Fig. 4 F–H) had substantial morphological abnormalities compared with control. The epithelial cells of gut were large and irregular in shape with irregular shaped nuclei. Cytoplasm of these cells was heterogeneous showing cellular aggregates.

Discussion

The study of pesticides effects on honeybees is vital because of the need to control a wide variety of agricultural pests without hurting foraging bees. The present study investigated the possibility of using mortality rate, AChE enzyme, TP, AChE gene transcription level and gut tissue for assessing

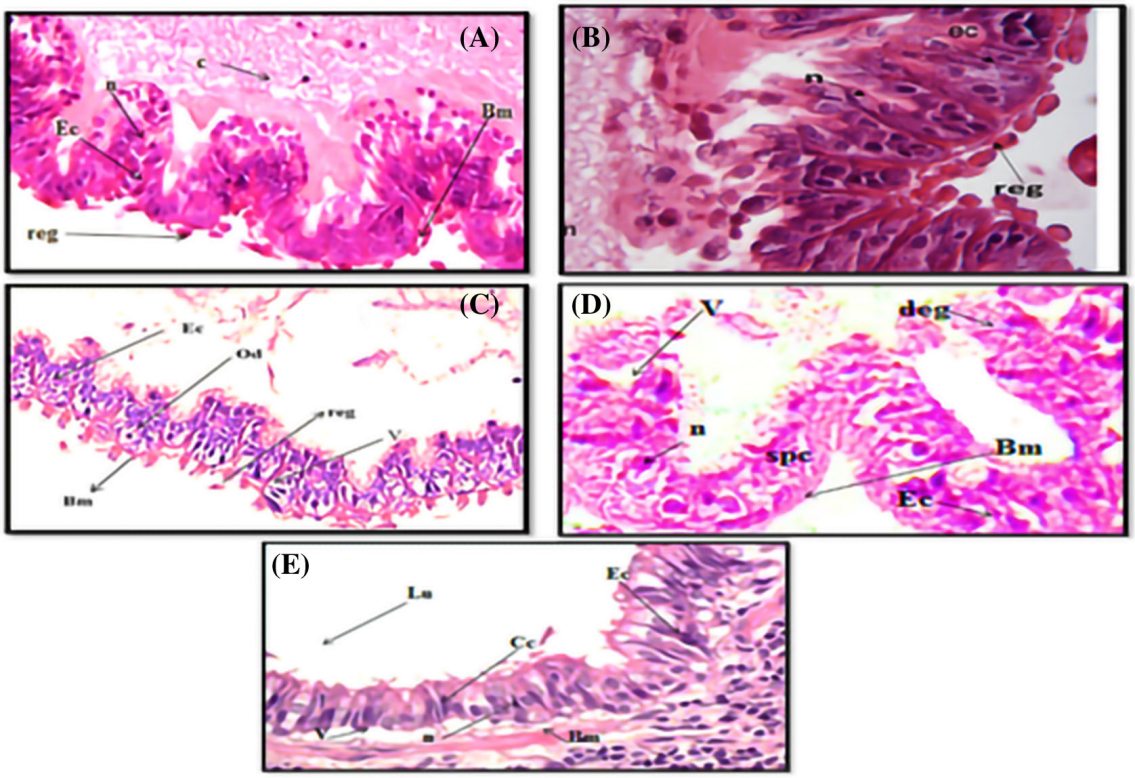

Fig. 3. Histological sections of *A. mellifera* midgut in control group (Sucrose) (A), Solvent control (Ethanol) (B), imidacloprid (C), carbaryl (D), chlorpyrifos (E) at 5 mg L^{-1} treatments. V, vacuole; Ec, epithelial cells; c, cytoplasm; Bm, basement membrane; n, nucleus; deg, degeneration; Lu, lumen; Cc, cell cytoplasm; Od, Odema; reg, regenerating cells. [Colour figure can be viewed at wileyonlinelibrary.com].

pesticide toxicity in honeybees. The usefulness of measuring such biochemical parameters is in determining probable cause of lethal and sublethal effects of pesticides. The present work is a novel one and is a part of our continued effort to study the deleterious aspects of different pesticides as they affect the honeybees.

In feeding bioassay laboratory trial, all tested pesticides proved toxic against honeybees. Results indicated that higher concentrations had pronounced effects as compared with lower concentrations (dose-dependent). It was revealed that LC_{50} values of imidacloprid at 48 and 96 h had more toxic effects (2.359 and 0.477 ng/bee) than other tested pesticides. Extensive work has earlier been reported regarding LD_{50} and LC_{50} values for different pesticides. Costa *et al.* (2014) whereas working on an acute lethal dose of imidacloprid on *Malipona scutellaris* has reported topical LD_{50} value of 2.41 ng/bee for 24 h and 1.29 ng/bee for 48 h; and oral LC_{50} value of 2.01 ng/bee and 0.81 ng/bee for 24 and 48 h, respectively which are in accordance to our results. Higher LC_{50} values for imidacloprid i.e. 17.9 ng/bee at 24 h (Iwasa *et al.*, 2004), 24 ng/bee at 24 h (Suchail *et al.*, 2001) and 49 ng/bee at 48 h (Nauen *et al.*, 2001) have been reported. The LC_{50} values of our study are also lower than those of 40 ng/bee and 81 ng/bee reported earlier by Schmuck *et al.* (2001) and Nauen *et al.* (2001), respectively. It may be due to differential honeybee pesticide sensitivity, experimental and laboratory conditions.

The present results for honeybee mortality showed that imidacloprid incurred higher mortality rates as compared with other tested pesticides. These results are in line with previous reports of Costa *et al.* (2014), Schmuck *et al.* (2001) and Nauen *et al.* (2001), they demonstrated pesticide mortality increased after exposure to pesticides. Laboratory study carried by Laurino *et al.* (2011) recorded toxic effects of neonicotinoid insecticides on honeybees. High mortality rate was noted in honeybees exposed orally to thiamethoxam, clothianidin, acetamiprid and thiacloprid compared with control group. In another laboratory study acute toxicity of chlorpyriphos, dichlorvos, malathion, profenofos and deltamethrin was determined on honey bees. It was evaluated that all tested pesticides caused 100% mortality of exposed bees (Stanley *et al.*, 2015). Martinello *et al.* (2020) stated that pesticides exposure is directly related to honeybee mortality in Italy. About 150 pesticides residues were found in dead honeybee samples.

The AChE is a reliable biomarker of neurotoxicity in insects, and various studies have affirmed its efficacy as a reliable diagnostic tool for pesticide exposure (Badiou *et al.*, 2008; Boily *et al.*, 2013; Tavares *et al.*, 2017). In the present study, imidacloprid and chlorpyrifos significantly increased AChE and TP levels, whereas carbaryl significantly decreased them. It has been confirmed that precise control of motor activity and nerve transmission are, in fact, maintained through hydrolyses of ACh at cholinergic synapses by the AChE. Hence, an elevated

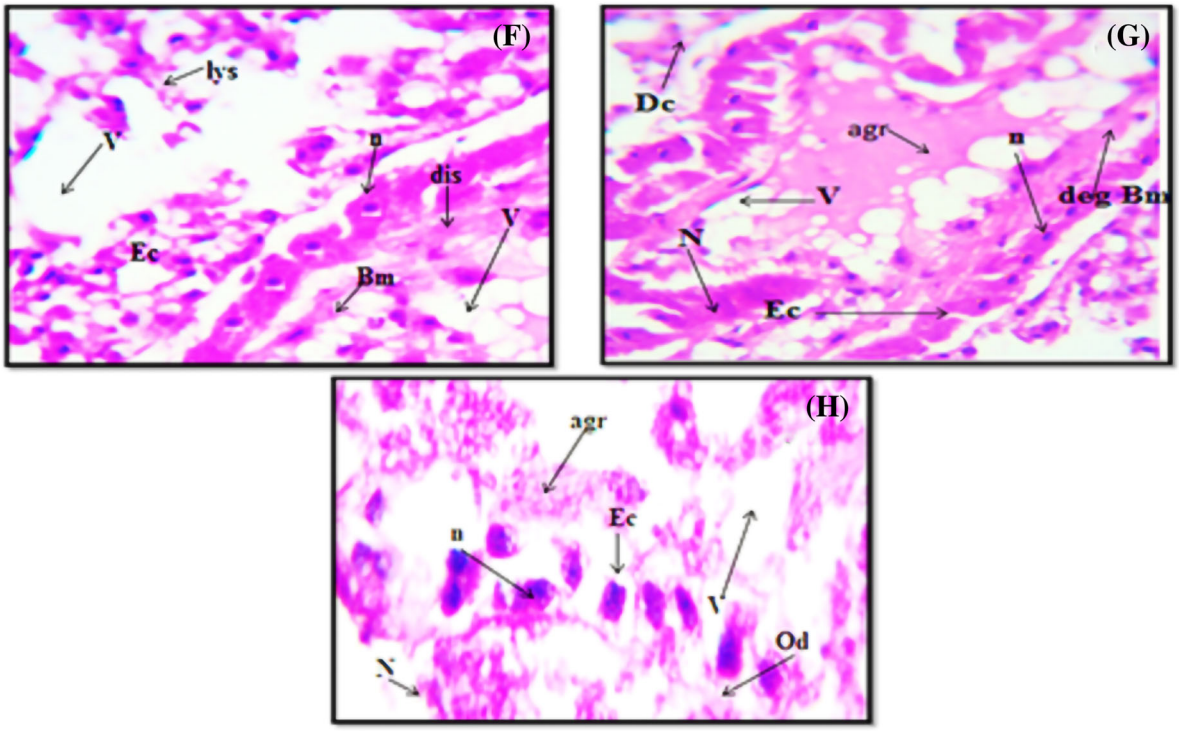

Fig. 4. Histological section of *A. mellifera* midgut after 2.5 mg L^{-1} imidacloprid (F), carbaryl (G) and chlorpyrifos (H) treatments. V, vacuole; Ec, epithelial cells; c, cytoplasam; Bm, basement membrane; n, nucleus; deg, degeneration; agr, aggregation; N, necrosis; lys, lysis; dis, disintegration; Od, odema; Dc, damage cells. [Colour figure can be viewed at wileyonlinelibrary.com].

AChE level may be a biological response to compensate the permanent activation of cholinergic neurons due to the strong binding of pesticides to ACh receptors (Tavares *et al.*, 2017). Direct alteration in AChE activity in the thorax of exposed honeybees provides an explanation for the abdominal spasms and disruption of normal gut functions. It may be suggested that presence of different levels of different AChE enzymes varies between tissues (Badiou *et al.*, 2008).

Alterations in AChE gene transcript level were also investigated. In honeybee, there are two isotype of AChE gene (AChE1 and AChE2). Both qualitative and quantitative approaches were used to measure relative transcript level of AChE1 and AChE2 in laboratory treated samples. It was noted that bands of AChE2 gene were more prominent in all treated samples as compared with AChE1. GAPDH expressing quite stable in all treated and control groups. In imidacloprid and chlorpyrifos treatments AChE level upregulates whereas in carbaryl treatment gene level downregulated. This could be explained by the fact that chlorpyrifos inhibit the AChE enzyme is irreversible. Once it bind to enzyme target site it permanently block enzyme active site and requiring more enzyme to control overstimulation of nerve impulses. Requirement of new enzyme upregulates the AChE gene transcription level. Whereas, carbaryl bind to target enzyme reversibly and after few hours enzyme target site freely available for ACh, but long term exposure caused noxious effects on insect normal physiological function and down-regulate the AChE gene transcript level (Pohanka, 2011). In present study relative abundance of AChE2 is more prominent than AChE1. Recent study revealed that in some social insects belonging to hymenoptera such as bees, wasps and ants AChE2 was predominantly expressed. In honeybees, AChE2 had more catalytic activities than AChE1 (Kim & Lee, 2013; Kim *et al.*, 2017).

Morphological analysis of gut epithelia of exposed honeybees conducted through histology in the present study revealed that these pesticides had harmful effects on honeybees gut tissues. In pesticide treated groups, the epithelial tissue cells were deformed, and had abnormal nuclei as compared with control. At certain places, cells were aggregated due to rupturing of their plasma-membrane, whereas at others vacuolation occurred due to autophagy and necrosis of cells. The cytoplasm in treated cells was coarse and heterogeneous. In present study, regenerative cells were absent or reduced in all treated groups which indicated decreasing capacity of epithelial cells to repair after injuries. Regenerative cells are important because they help to regenerate damaged epithelial cells and are found at the base of epithelium (Maiolino *et al.*, 2013). Similar results have been documented. Tapparo *et al.* (2012) demonstrated that bees orally exposed to neonicotinoid pesticides showed cytoplasmic vacuolization and reduction in number of their regenerative cells. Such changes may lead to short life span, decreased flight and foraging activities, impairment of coordination among colony members, disruption of learning which ultimately lead to decline and disappearance of honey bees (El-Hassani *et al.*, 2008; Neumann & Carreck, 2010).

Conclusion

In a nutshell, imidacloprid, carbaryl and chlorpyrifos have substantial effects on mortality rate, AChE level, TP level, and gut epithelia of honeybees. Imidacloprid and chlorpyrifos significantly increased AChE gene transcription, AChE and TP levels, whereas carbaryl significantly decreased them. Histological analysis showed that the gut tissues of honeybees exposed to insecticide treatment had substantial morphological abnormalities. AChE is an excellent enzyme to determine pesticide toxicity in honeybee. Imidacloprid revealed most toxic effects as compared with its counterpart pesticides. Lethal effects induced by various pesticides may be taken into account for risk assessment schemes whereas considering pollinators such as honeybees. We recommend a broader laboratory study allied with field trials to further confirm sensitivity of *A. mellifera* for various pesticides.

Declaration of interest statement

The authors present that there is no conflict of interest.

Data availability statement

All required data are given in research manuscript in the form of tables and figures.

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