



# Toxicity of Imidacloprid Administered Alone and in Combination with Heavy Metal Lead on Silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

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## ABSTRACT

In this study, we evaluated the single and joint toxicities of insecticide imidacloprid (commercial formulation and technical grade) and heavy metal lead (Pb) against 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> instars of silkworm. For treatment, leaf-dipping method was used and toxicity calculated after 48h exposure to the treatment. Both chemicals showed high toxicity against all instars of silkworm. However, results indicated that imidacloprid was more toxic for silkworm larvae compared to lead. The acute toxicity of imidacloprid formulation (IMF), imidacloprid technical grade (IMT) and Pb showed positive correlation with the concentration and negative with the age of larvae. From second to fifth instars, LC<sub>50</sub> values of IMF ranges from 0.72 to 2.96, IMT from 0.86 to 2.75 and Pb from 1.93 to 6.88 mg/L. The toxicity of binary mixture of IMF + Pb showed synergism against all instars of silkworm. While, binary mixture of IMT + Pb displayed synergism, additive, and antagonism and antagonism activity in silkworm larvae in silkworm larvae. The results of our study provide useful data for the assessment of ecological risk of the tested compounds (single or mixture) against different silkworm instars.

## Article Information

Received 23 June 2021

Revised 28 September 2021

Accepted 19 October 2021

Available online 11 February 2022

(early access)

## Authors' Contribution

AB conceived the idea and designed the experiment. SAK provided experimental basis for research. SA and SAK performed experiments. SA and AB analysed the data and wrote the article. AB revised the manuscript.

## Key words

Insecticides, Heavy metal, Combined toxicity, Silkworm larvae, Instars

## INTRODUCTION

Silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae) is an economically important insect that was domesticated for more than 5000 years ago for silk production. It utilizes less valuable resources (mulberry leaves) in most effective manner to produce high quality silk. Its silk and other products are used in textile, medical, cosmetic and food industries (Lee *et al.*, 2011; Longvah *et al.*, 2011; Zhang *et al.*, 2011; Dutta *et al.*, 2012; Gupta *et al.*, 2015). It is a source of income generation in many areas of the world and play crucial role to increase the employment of the people.

Although silkworm is domesticated and reared in isolated insectaries but still exposed to different xenobiotics through food contamination. The major food of *B. mori* is mulberry (*Morus alba*) leaves, that is attacked by many insect pests like mites, grasshoppers, mealy bug, beetles, cutworms etc. (Singh and Saratchandara, 2002). To control these pests, different types of insecticides are sprayed on mulberry plants. Some other pesticides used in surrounding area also reach to mulberry plants through

spray drift, runoff and leaching. Imidacloprid is widely used insecticide throughout the world to control chewing and sucking insect pests. It is a broad spectrum neonicotinoid insecticide with contact and systemic activity. In crop fields, it is used as foliar spray or seed treatment. Plants absorb it from the soil or leaves and translocate it to their different parts. In insects, imidacloprid bind with nicotinic acetylcholine receptors of central nervous system that cause paralysis and ultimately its death (Matsuda *et al.*, 2001, 2005). Liu *et al.* (2018) reported low toxicity of imidacloprid against silkworm larvae as compared to pyrethroids. Yu *et al.* (2016) studied acute toxicity of different insecticides against silkworm larvae and reported high toxicity of imidacloprid based on LC<sub>50</sub> values after 96 h exposure.

In few decades, heavy metals pollution in the environment has increased many folds due to industrialization and agriculture practices. Lead (Pb) is non-essential heavy metal. Its concentration in the air and soil is increasing due to deposition of pollutants from automobile, smelters, mining and paint industry (Zia *et al.*, 2011). Long term persistence and accumulation of Pb in environment produce high risk for organisms that exposed to Pb directly or indirectly (Peterson *et al.*, 2017). Mulberry plants absorb different heavy metals including Pb from the soil and accumulate them in their different parts (Zhou *et*

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0030-9923/2022/0001-0001 \$ 9.00/0  
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*al.*, 2015; Jiang *et al.*, 2020). Silk worm larvae exposed to Pb through the consumption of contaminated mulberry leaves. The large quantity of consumed Pb passed through the gut of silkworm larvae and excreted in feces. While, some quantity is absorbed by the gut and later excreted from the body through spinning or pupal case (Jiang *et al.*, 2012). Different detoxification processes also help to reduce the effects of Pb accumulation in silkworm larvae (Zhou *et al.*, 2015).

Many studies have reported toxicity of many pesticides administered alone and in combination with heavy metals against silkworm larvae (Zhang *et al.*, 2008; Zhou *et al.*, 2015; Yu *et al.*, 2016; Meng *et al.*, 2017; Liu *et al.*, 2018). However, effect of insecticide and heavy metal mixture on the survival, biology and physiology on silkworm larvae is not reported according to our knowledge. The objective of this study was to assess single and joint toxicity of imidacloprid and Pb against different instars of silkworm. The results of this study will provide useful guidelines to assess the harmful effects of xenobiotics and their mixtures against silkworms and other nontarget organisms.

## MATERIALS AND METHODS

### Insects

The eggs of silkworm (Bulgarian hybrid H1\*KK\*G2\*V2) were obtained from Sericulture Department, 108 Ravi Road, Lahore, Pakistan. These eggs were reared in the laboratory at 25±2 °C, 70±5% relative humidity and photoperiod of 12:12 h (L: D). Fresh mulberry leaves were harvested from untreated mulberry plants present in the University of the Punjab, Lahore, Pakistan and fed to the silkworm larvae *ad libitum*.

### Chemicals

In present study, both technical grade and commercial formulations of the imidacloprid were used. Commercial formulation of imidacloprid (Confidor 200 SC) was purchased from local office of Bayer. Technical grade imidacloprid (CAS No. 138261-41-3) and lead chloride (PbCl<sub>2</sub>, CAS No. 7758-95-4) were purchased from Sigma Aldrich, USA. Stock solution of Imidacloprid formulation (IMF) and imidacloprid technical grade (IMT) was prepared at field application rate in acetone and lead chloride at rate of 20 mg/L in distilled water. All stock solutions were stored at 4 °C till further used. Each stock solution was diluted with distilled water to the required concentrations of the bioassays.

The binary mixtures of imidacloprid commercial formulation + lead chloride (IMF+PbCl<sub>2</sub>), and imidacloprid technical grade + lead chloride (IMT+PbCl<sub>2</sub>) were prepared by mixing the two solutions at their LC<sub>50</sub>: LC<sub>50</sub> ratio.

### Bioassay method

To assess the toxicity of above mentioned chemicals, 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars of silkworm were exposed to a series of IMF, IMT and Pb concentrations and to their binary combinations at LC<sub>50</sub>: LC<sub>50</sub> ratio. Before experiment, preliminary bioassays were performed to determine the concentrations of each chemical that cause 0-100% mortality in silkworm larvae after 48h of exposure. On the basis of this study, six concentrations were designed for bioassays to calculate LC<sub>50</sub> values for each chemical.

For assays, leaf dip method described by Yu *et al.* (2011) was used. Fresh and healthy mulberry leaves were plucked from mulberry trees present in the vicinity of University of the Punjab, Lahore, Pakistan and washed with water and air dried. These clean mulberry leaves were immersed in required concentration of each chemical for 30s, drained and spread on filter paper to dry under fan. The treated mulberry leaves were placed in wooden rearing trays (dimension 25cm x 15cm). Thirty same size and same age silkworm larvae were placed in each tray and fed with treated mulberry leaves for 48h. Assay for each concentration of a chemical was replicated four times. Mortality of the silkworm larvae was recorded after 24 and 48h of exposure. Control group silkworms were fed with water treated mulberry leaves that contain acetone at a concentration present in the highest dose of insecticide used in bioassay. The bioassay conditions were same as described above for the rearing of silkworm.

### Data analysis

The silkworm larvae that did not show any movement on touching with camel hair brush were considered dead. The mortality of silkworm larvae after 48 h of exposure to treatments was used to calculate LC<sub>50</sub> and median effect using Compusyn software (Chou and Martin, 2005). The following formula was used:

$$\frac{fa}{fu} = \left(\frac{D}{Dm}\right)^m$$

Where *fa* is the fraction of the total organism affected by the dose *D*; *fu* is the fraction of the total organism remains unaffected by the dose *D*; *Dm* is concentration of chemicals at which 50% mortality occurs (LC<sub>50</sub>); *m* is coefficient that define the shape of dose-mortality curve. The value of *m* may be < 1, = 1, > 1 that represent flat sigmoidal, hyperbolic, and sigmoidal dose-mortality curves, respectively.

To identify and quantify the interaction between insecticide and heavy metal, data was analyzed using formula of combination index (Chou, 2006). The combination index (*CI*) values for binary mixtures were also calculated using compusyn software. The *CI* value may be >1, <1 or =1 that indicate antagonism, synergism

and additive effect of binary mixture, respectively. The formula used to calculate n-chemicals combinations for  $x\%$  mortality is given below:

$$(CI)_x = \sum_{j=1}^n \frac{(D)_j}{(Dm)_j}$$

$$= \sum_{j=1}^n \frac{(Dx)_{1-n} \{(D)_j / \sum_1^n (D)\}}{(Dm)_j \{(fa_x)_j / [1(fa_x)_j]\}^{1/m_j}}$$

Where  $(D)_{1-n}$  is the sum of the concentrations of  $n$  chemicals that cause  $x\%$  mortality due to mixture,  $\{(D)_j / \sum_1^n (D)\}$  is the fraction of individual concentration of  $n$  chemicals that cause  $x\%$  mortality and  $\{(Dm)_j \{(fa_x)_j / [1(fa_x)_j]\}^{1/m_j}\}$  is the concentration of single chemical cause  $x\%$  mortality.

General linear model was used to compare the acute toxicity of IMF, IMT and Pb individually and their mixtures. ANOVA followed by Tukey's test was used to compare the  $LC_{50}$  values of a chemical in different instars. Pearson correlation was used to find relationship between mortality and concentration of chemical, and instar of silkworm.

On the basis of  $LC_{50}$  values, four toxicity classes of chemicals were devised following Yu *et al.* (2016). These were (i) extreme ( $LC_{50} < 0.5$  mg/L), (ii) high ( $LC_{50} > 0.5-20$  mg/L), (iii) moderate ( $LC_{50} > 20-200$  mg/L), and low ( $LC_{50} > 200$  mg/L) toxicity producing chemicals.

## RESULTS

In the study, mortality rate of silkworm larvae in control groups ranged from 0–4% which depicted the credibility of the experiments. The concentration-mortality curve parameters ( $Dm$ ,  $m$  and  $r$ ) of the single and binary mixture of Pb, IMF, and IMT against 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> instars of silkworm are shown in Tables I and II. For all single and combination assays, the values of  $m$  were  $>1$  which represented that the sigmoidal curves of median-effect equation fit best on our data (Fig. 1). For all assays,  $r$  values  $> 0.90$  also confirmed that sigmoid curve fit best on our data. According to  $LC_{50}$  values ( $Dm$ ), the toxicity of all chemicals showed positive correlation with the concentration (Table 1) and negative with the age of silkworm larvae (IMF  $r^2 = 96.6\%$ ; IMT  $r^2 = 90.7\%$ ; Pb  $r^2 = 98.4\%$ ). In single chemicals decreasing order of toxicity was IMF = IMT > Pb in all instars after 48 h of exposure (2<sup>nd</sup> instar,  $F_{2,9} = 214.65$ ; 3<sup>rd</sup> instar,  $F_{2,9} = 196.40$ ; 4<sup>th</sup> instar,  $F_{2,9} = 78.72$  and 5<sup>th</sup> instar,  $F_{2,9} = 137.61$ ,  $P < 0.001$  for all). The  $LC_{50}$  values of IMF and IMT did not differ significantly in all instars.

The susceptibility of binary mixtures also showed negative correlation with the age of silkworm larvae ( $r^2 = 92.3\%$  for IMF+Pb and  $r^2 = 95.7\%$  for IMT+Pb;  $P < 0.001$ ; Table II). For all instars, binary mixture of IMF+Pb

was more toxic than IMT+Pb ( $F_{1,24} = 72.80$ ,  $P < 0.001$ ).  $CI$  values were calculated at  $LC_{10}$ ,  $LC_{50}$  and  $LC_{90}$  to assess the behavior of each mixture (Table II). The binary mixture of IMF+Pb showed strong synergistic effect in 2<sup>nd</sup> and 3<sup>rd</sup> instars larvae of silkworm at all affective levels ( $fa$ ). However, 4<sup>th</sup> and 5<sup>th</sup> instars showed moderate to low level synergism with the increase of  $fa$  (Fig. 2). The binary mixture of IMT +Pb showed strong synergistic effect at all  $fa$  values in 2<sup>nd</sup> instar of silkworm. In 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> instars, binary mixture of IMT +Pb showed strong to moderate synergism at low  $fa$  values and additive to low antagonism at high  $fa$  values (Fig. 2).

**Table I.  $LC_{50}$  values (Dm) with dose-mortality curve coefficient (m) against different instars of silkworm *B. mori* after 48h of exposure.**

Chemi- calcs	Instar	Dm (mg/L)	m	r	Toxicity grade
Pb	2 <sup>nd</sup>	1.93 <sup>d</sup> ± 0.121	2.03 ± 0.15	0.97	Highly toxic
	3 <sup>rd</sup>	2.28 <sup>c</sup> ± 0.163	2.72 ± 0.14	0.94	Highly toxic
	4 <sup>th</sup>	4.45 <sup>b</sup> ± 0.392	3.30 ± 0.25	0.97	Highly toxic
	5 <sup>th</sup>	6.88 <sup>a</sup> ± 0.576	3.85 ± 0.49	0.98	Highly toxic
	IMT	2 <sup>nd</sup>	0.86 <sup>c</sup> ± 0.082	2.06 ± 0.11	0.93
IMF	3 <sup>rd</sup>	1.09 <sup>c</sup> ± 0.071	1.70 ± 0.14	0.98	Highly toxic
	4 <sup>th</sup>	2.39 <sup>b</sup> ± 0.186	2.89 ± 0.21	0.99	Highly toxic
	5 <sup>th</sup>	2.75 <sup>a</sup> ± 0.162	2.57 ± 0.38	0.98	Highly toxic
	2 <sup>nd</sup>	0.72 <sup>d</sup> ± 0.013	2.31 ± 0.14	0.99	Highly toxic
	3 <sup>rd</sup>	1.24 <sup>c</sup> ± 0.029	2.26 ± 0.28	0.96	Highly toxic
4 <sup>th</sup>	2.20 <sup>b</sup> ± 0.196	2.21 ± 0.38	0.99	Highly toxic	
5 <sup>th</sup>	2.96 <sup>a</sup> ± 0.216	3.64 ± 0.52	0.99	Highly toxic	

IMT, imidacloprid technical grade; IMF, imidacloprid formulation; Pb, lead.

**Table II. Values of  $LC_{50}$  (Dm), dose-mortality curve coefficient (m) and combination index of Lead and imidacloprid (Technical and formulation) mixture against different instars of silkworm *B. mori* after 48h of exposure.**

Chemi- calcs	Instar	Dm (mg/L)	m	r	Combination Index values (CI)		
					$LC_{10}$	$LC_{50}$	$LC_{90}$
Pb + IMT	2 <sup>nd</sup>	0.29 <sup>d</sup> ± 0.13	1.48 ± 0.43	0.99	0.11	0.17	0.26
	3 <sup>rd</sup>	0.74 <sup>c</sup> ± 0.24	1.25 ± 0.27	0.91	0.25	0.50	1.00
	4 <sup>th</sup>	2.19 <sup>b</sup> ± 0.47	2.05 ± 0.65	0.97	0.44	0.70	1.11
	5 <sup>th</sup>	3.55 <sup>a</sup> ± 0.42	2.35 ± 0.71	0.98	0.72	0.90	1.22
Pb + IMF	2 <sup>nd</sup>	0.12 <sup>c</sup> ± 0.06	1.86 ± 0.34	0.95	0.09	0.12	0.20
	3 <sup>rd</sup>	0.37 <sup>c</sup> ± 0.19	1.71 ± 0.23	0.94	0.15	0.23	0.35
	4 <sup>th</sup>	1.77 <sup>b</sup> ± 0.26	2.30 ± 0.59	0.99	0.53	0.55	0.61
	5 <sup>th</sup>	2.83 <sup>a</sup> ± 0.74	2.79 ± 0.64	0.96	0.56	0.68	0.83

For abbreviations see Table I.

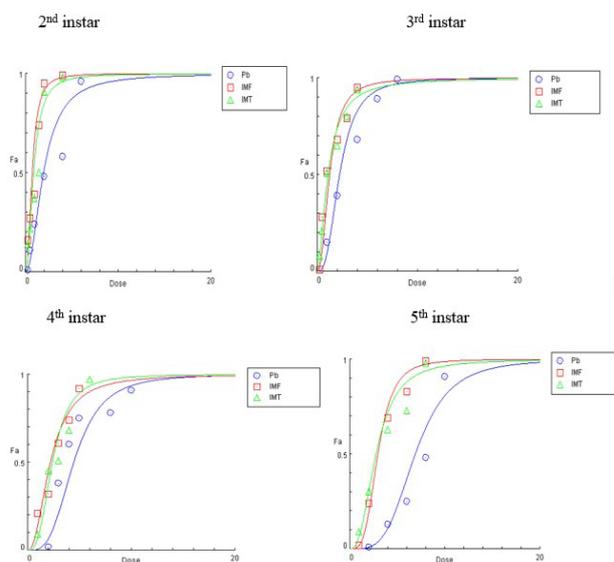


Fig. 1. Dose-effect curves of Lead (Pb), imidacloprid commercial formulation (IMF), imidacloprid technical grade (IMT) on different instars of silkworm after 48 h exposure.

According to the toxicity grades, all chemicals were highly toxic against all instar of silkworm on the basis of 48 h of exposure. The binary mixture of IMF+Pb was extremely toxic for 2<sup>nd</sup> and 3<sup>rd</sup> instars and highly toxic for 4<sup>th</sup> and 5<sup>th</sup> instars of silkworm. While, the binary mixture of IMT+Pb was extremely toxic for 2<sup>nd</sup> instar and highly toxic for 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> instars of silkworm.

## DISCUSSION

In recent years, mulberry leaves have been contaminated with different types of xenobiotics including different insecticides and heavy metals. Insecticide Imidacloprid is frequently used in mulberry orchards to control infestation of different insect pests and reach to silk worm through contaminated mulberry leaves (Sun *et al.*, 2012; Yu *et al.*, 2015). During the study, both commercial formulations and technical grade imidacloprid was used to assess the toxicity against *B. mori*. The formulation of an insecticide contains both active ingredients and inert ingredients like surfactants, stabilizers, dyes, etc. These inert ingredients make formulated insecticides safer, more effective and easier to handle. Sometime, these inert ingredients cause increase in the acute toxicity of insecticides (Puglis and Boone, 2011). So, to fully understand the toxicity of insecticides against nontarget organisms, it is imperative to study their formulations also. In our study, both imidacloprid technical grade and

formulation showed high toxicity against all instars of *B. mori*. Luo *et al.* (2011) reported 0.256 mg/liter LC<sub>50</sub> value of imidacloprid against silkworm after 96-h exposure. Yu *et al.* (2016) also reported imidacloprid as highly toxic insecticide against *B. mori*. In their experiment LC<sub>50</sub> value of imidacloprid against 2<sup>nd</sup> instar larvae of *B. mori* after 48h exposure was 2.66 (2.50–2.84) mg/liter. However, in our study, LC<sub>50</sub> value of same instar was more than reported values of Luo *et al.* (2011) and less than Yu *et al.* (2016). In present study, we also recorded high toxicity of Pb against all instars of *B. mori*. Zhou *et al.* (2015) reported no mortality in *B. mori* larvae that consumed

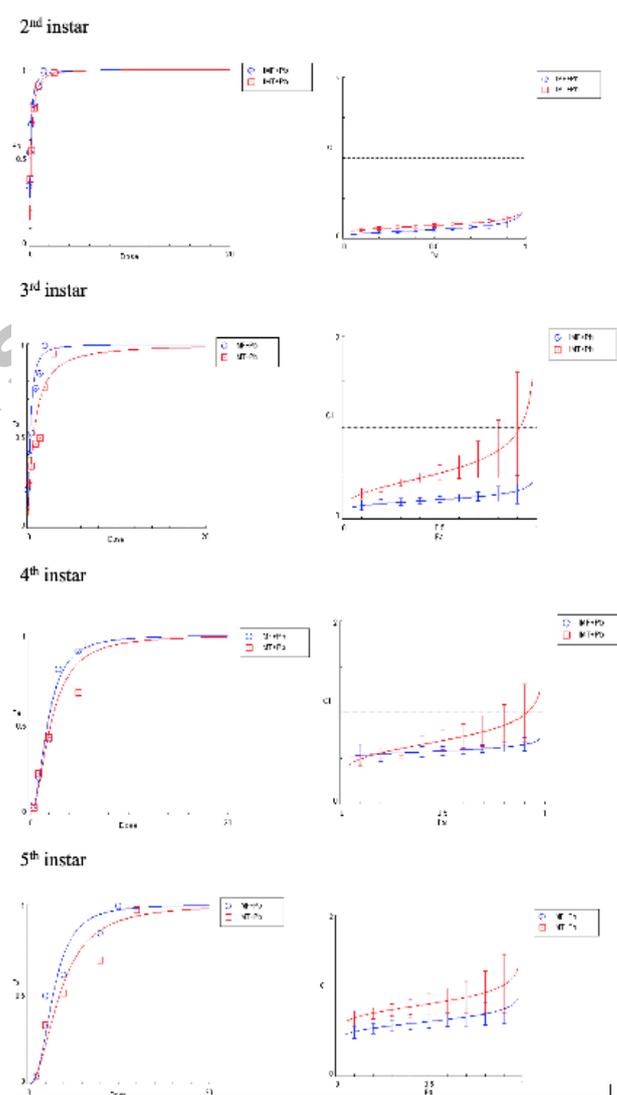


Fig. 2. Plot of the combination index versus the effect produced by Pb-imidacloprid binary mixtures against different instars of silkworm.

high Pb (60 mg/kg) contaminated mulberry leaves. Most of the Pb consumed by silkworm larvae removed through feces and a small quantity absorbed through gut epithelium (Jiang *et al.*, 2012). Pb assimilated through gut stored in different organs and removed from the body through molting and spinning (Zhou *et al.*, 2015). These variations in susceptibility of imidacloprid and Pb could be explained due to difference of silkworm species, physiological conditions of studied organisms and environmental factors (Zhang *et al.*, 2008).

Present study showed dose dependent increase in the mortality of *B. mori* larvae when exposed to different concentrations of imidacloprid and Pb. High concentrations of insecticides or any other xenobiotic is more toxic to organisms because their accumulations in body inhibit activities of different detoxification enzymes (Zhu *et al.*, 2014). The age dependent increase in LC<sub>50</sub> values indicated that toxicity of imidacloprid and Pb was inversely proportional to the age of larvae. Many studies have reported higher susceptibility of early instar larvae compared with older instars against different xenobiotics (Hornby and Gardner, 1987; Prabhaker *et al.*, 1989; Prabhaker and Toscano, 2007). Less susceptibility of neonicotinoid thiamethoxam in fifth instar larvae of the Indian meal moth, *Plodia interpunctella*, than second instar larvae were recorded by Yue *et al.* (2003). In many studies, body size was positively related with the capacity of organisms to withstand the stress caused by the xenobiotics. Smaller organisms have higher feeding and metabolic rate and accumulate more xenobiotics than larger ones (Vesela and Vijverberg, 2007).

In nature organism is mostly exposed to a mixture of xenobiotics such as pesticides, heavy metals, and other chemicals. The combinations of different compounds modify each other's effects that interfere erroneously and undesirably with the activities of biological system. The modulated behavior of combination compounds can be additive, synergistic and antagonistic. Some studies have reported joint toxicity of different insecticides (Zhang *et al.*, 2008, 2010; Tatum *et al.*, 2011; Yu *et al.*, 2016; Yang *et al.*, 2017) and heavy metals (Wu *et al.*, 2016) to non-target organisms. However, no study was performed to assess the combination effect of insecticides and heavy metals against silkworm larvae. Our results showed that binary combination of imidacloprid and Pb exerted synergistic effect on all instars of silkworm. Synergistic effect in this combination of compound may be due to their mode of action on different biological systems, as both chemicals differ in their structures (Thany *et al.*, 2006). The related variables may include synthesis of toxic metabolites, inhibition of detoxifying mechanism, changes in the distribution, enhanced uptake via biological membranes,

and decrease in excretion (Wu *et al.*, 2016). Another possible explanation would be reduced protein formation in the treated silkworms that consequently decreased the production of particular detoxifying enzymes like GST and Cytochrome P450 (Wang *et al.*, 2013) ultimately making the silkworms more vulnerable to the xenobiotics. However, the synergistic effect might be explained by the fact that toxicants may somehow combine to increase toxicity by an unknown way of action (Rodea-Palomares *et al.*, 2010). The combination of IMT+Pb showed moderate synergistic effect at low toxicity values and moderate antagonistic at higher toxicities values in 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> instar larvae. It is possible that these chemicals competed for uptake through same binding sites and suppressed the toxic effect of each other (González-Pleiter *et al.*, 2013).

## CONCLUSIONS

Our results indicate that combination of different compounds present in nature can increase the risk to silkworm industry. Mulberry plants can uptake many heavy metals and chemicals from the soil or air that ultimately accumulate to mulberry leaves. These contaminated leaves effect the population of silkworm and reduce their production of silk. For risk assessment, it is necessary to estimate acute and residual toxicity of different combinations of compounds present in the environment to revise the existing standards of pollution management.

## ACKNOWLEDGEMENTS

We thank the Head, Sericulture Research Lab, Ravi Road, Lahore for providing necessary facilities for this research.

### Statement of conflict of interest

The authors have declared no conflict of interest.

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