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Original Article

THE ACUTE ORAL TOXICITY OF COMMONLY USED PESTICIDES IN IRAN, TO HONEYBEES (*APIS MELLIFERA MEDA*)

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Abstract

The honey bee is credited with approximately 85% of the pollinating activity necessary to supply about one-third of the world's food supply. Well over 50 major crops depend on these insects for pollination. The crops produce more abundantly when honey bees are plentiful. Worker bees are the ones primarily affected by pesticides. Poisoning symptoms can vary depending on the developmental stage of the individual bee, and the kind of chemical employed. The oral toxicity of these insecticides: (phosalone and pirimicarb), acaricide (propargite), insecticide and acaricide (fenpropathrin), fungicides, and bactericides (copper oxychloride and the Bordeaux mixture), were evaluated for the purposes of this research. The results showed that fenpropathrin had high acute oral toxicity (LC_{50}-24h and LC_{50}-48 were 0.54 and 0.3 ppm, respectively). Propargite had 7785 ppm (active ingredient) for LC_{50}-24h and 6736 ppm (active ingredient) for LC_{50}-48h in honeybees and is therefore, non-toxic to *Apis mellifera*. On the other hand, copper oxychloride had minimum acute oral toxicity to honeybees (LC_{50}-24h and LC_{50}-48 were 4591.5 and 5407.9 ppm, respectively) and was therefore considered non-toxic. Also, the Bordeaux mixture was safe to use around honeybees. Phosalone and primicarb were considered highly and moderately toxic to honeybees, respectively.

Keywords: acute oral toxicity, *Apis mellifera meda*, LC_{50}, pesticide.

INTRODUCTION

The honeybee *Apis mellifera* is valuable for the economy due to its hive by-products (honey, pollen, royal jelly) which generate considerable income for beekeepers. Honeybees also contribute to plant biodiversity by pollinating wild plants. Honeybees and their products are potentially exposed to several contaminants present in the environment, such as chemical products released into the hive to fight diseases and parasites, and pesticides used in agriculture against pests (Aliouane et al., 2009). As a response, multiple studies were conducted to assess pesticide residues in the field. The results were dramatic. For example, a study of apiaries in North American orchards recovered 121 agrochemicals in honeybees, pollen, and the wax (Mullin et al., 2010). However, the impact of the agricultural landscape is not limited to honeybee colonies. In fact, other pollinators also suffer. In the last 40 years, non-*Apis* species, such as bumblebees, are decreasing in great quantities (Goulson et al., 2008). Honeybees are estimated to provide annual pollination services worth US $4.1 billion to agriculture. Every year tens of beekeepers with hundreds of bee colonies move to various hills and valleys of Himachal Pradesh to provide pollination services to apple farmers. In return, the beekeepers get paid for their services. Farmers pay beekeepers IRs 800 (US $18) as a pollination fee for one colony of honeybees during each flowering season (Hepburn and Radloff, 2011).

Chemicals may elicit various effects in biological organisms through their interaction with numerous molecular targets that can induce lethal and adverse sublethal effects (Sattelle and Yamamoto, 1988; Soderlund and Bloomquist, 1989). This can be exemplified by the neurotoxic pyrethroid, carbamate, and organophosphate pesticides which can trigger not only more of less severe neural effects but also reprotoxicity through a mechanism independent of

their neural action (Yousef, 2010; Zhang et al., 2010; Joshi et al., 2011). Previous studies have shown that low-dosage deltamethrin will delay the return time (Vandame et al., 1995) and reduce foraging activity (Decourtye et al., 2004). In addition, cypermethrin leads to the extinction of bees (Bendahou and Bounias, 1999) while parathion influences communication between bees (Schricker and Stephen, 1970). Direct spray plays a significant role in the contamination of flower nectar. Nectar and other sugar sources (e.g. extra floral nectaries and aphid honey dew) are used as an energy source. Spray applications at or close to the flowering period, pose the greatest likelihood of acute exposure for bees. This can cause direct contamination of flower nectar (Alex and Miles, 2011). Nectar sugar is most important for attracting honeybees. The direct effects of nectar sugar concentration were positive and negligible (Hepburn and Radloff, 2011).

In the assessment and evaluation of toxic characteristics of substances, it may be required to determine the acute oral toxicity in honeybees, e.g. when it is likely that bees will be exposed to a given chemical. The acute oral toxicity test is carried out to determine the inherent toxicity of pesticides and other chemicals to honeybees. Simulation of flower nectar and aphid honeydew is done by mixing pesticides with a sucrose solution. In particular, this method can be used in step-wise programs for evaluating the hazards of pesticides to bees, based on sequential progression from laboratory toxicity tests to semi-field and field experiments (EPPO/Council of Europe, 1993; OECD, 1998; Laurino et al., 2010; 2011; 2013). Therefore, both active substances (a.s.) and formulated pesticides are currently undergoing various tests. The tests assess the risk posed by a.s. and formulated pesticides to honey bees, before the a.s. and pesticides are allowed to be used in agriculture. In the European Union, the European and Mediterranean Plant Protection Organization guidelines No. 170 (OEPP/EPPO, 2010a) and the relative risk assessment scheme (OEPP/EPPO, 2010b) are usually followed. Such procedures substantially rely on Median Lethal Dose (LD_{50}) or another similar toxicity index-determination to ascertain if risk levels associated with the tested a.s. are acceptable for honey bees.

To test the toxicity of insecticides, animal experiments are used to estimate the half-lethal dosage (LD_{50}) or lethal concentration (LC_{50}), and thus, estimate the possible harm to humans and non-target organisms. For non-target organisms, insecticides not only can cause direct poisoning or death of bees, it can also influence the bee larvae, division of labor, foraging as well as the development of bee colonies while subjecting all of the above to a lower lethal dose (Thompson, 2003).

Since there is no published information on the precise acute oral toxicity (LC_{50}) of the six pesticides: fenpropathrin, pirimicarb, propargite, phosalone, copper oxychloride, and Bordeaux mixture on *Apis mellifera meda*, evaluations of LC_{50}-24h, and LC_{50}-48h were conducted to address this lack. The aims of this study were the calculation of LC_{50}-24h, and LC_{50}-48h of the aforementioned six commonly used pesticides, and the significant evaluation between LC_{50}-24h and LC_{50}-48h in Iran.

MATERIAL AND METHODS

Commonly used formulations available in Iran were implemented in the study. The formulations contained: fenpropathrin (Danitol®, 10% Emulsion), pirimicarb (Pirimor®, 50% wetable), propargite (Omite®, D-014®, BPPS®, Comite®, 57% Emulsion), phosalone (Zolone® 35.0% Emulsion), copper oxychloride (Cupravite, 355®, 35% wetable), and Bordeaux mixture (Bordeaux Fix®, 18% Emulsion). Forager adult worker bees of the same species were used for oral toxicity. Honeybees were obtained from adequately fed, healthy, disease-free, and queen-right colonies. Treated honeybees were held in plastic cages. The cages were 30 cm high and 20 cm wide. Mesh-like nets for ventilation were used in parts of the cage. A sleeve-like net was used to transfer the treatment petri dish into the cage (Fig. 1). Treatment doses were mixed with a sucrose solution in water (25% w/v). The honeybees were starved for up to 2 hours before the initiation of the test. Lethal experiments were conducted using 315 honeybee adults for each pesticide and there was also a control. Three cages (the repetitions) were used in each treatment and the control. In each cage, 15 worker honeybees were placed. Worker honeybees under anesthesia, were mechanically transferred to each cage. A pretest experiment was conducted then six concentrations and the control were designed using the six pesticides. After a 1 hour treatment, all honeybees were fed with a non-toxic sucrose solution in water (50% w/v). The mortality rates were logged at 24, 48, and 72 h after the start of the test. The tests were performed in a dark room at 25 - 30° C; 45-55% relative humidity (Laurino et al., 2010; 2011; 2013). The pretest experiments were conducted for designing the concentrations. Concentrations causing 10% and 90% mortality

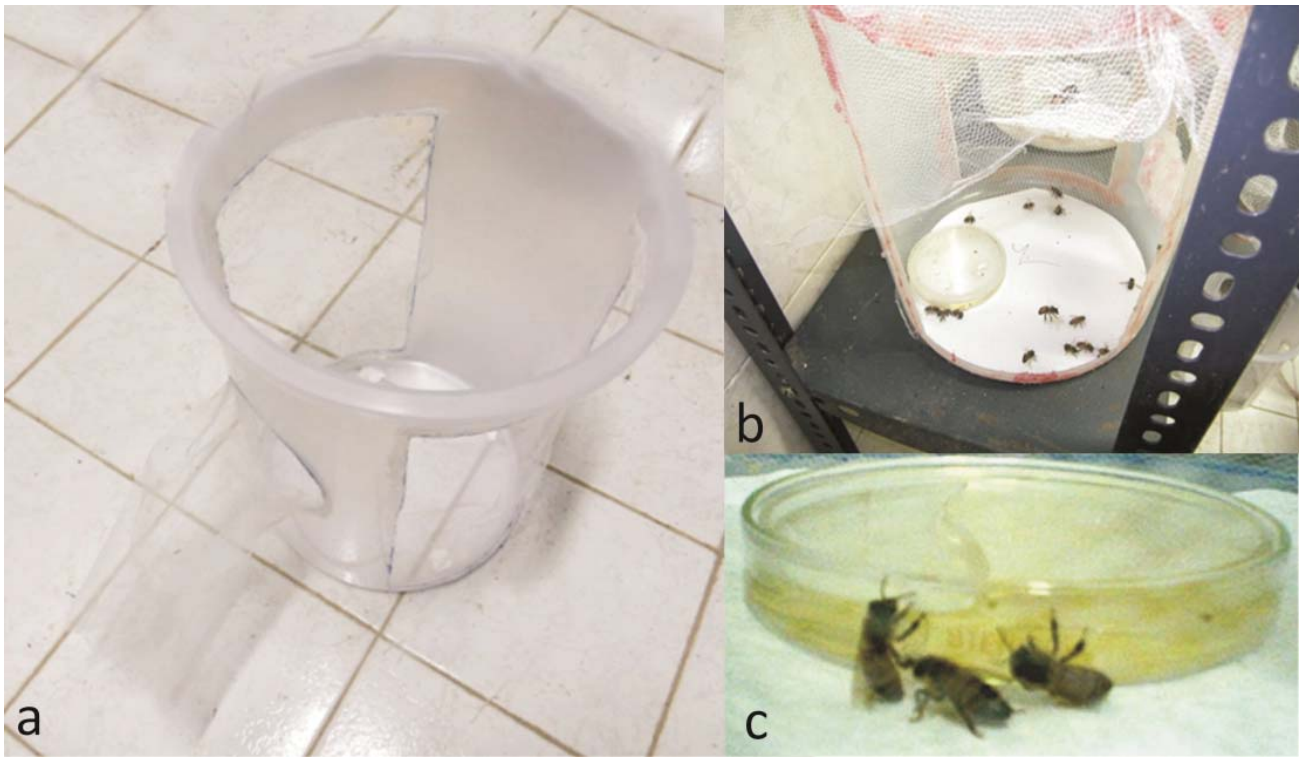

Fig. 1. Cage with a sleeve-like net was used to transfer the treatment petri dish into the cage (a); Cage prepared for the ingestion trials with treatment doses mixed with a sucrose solution (25% w/v) in water (b); detail of the feeder pointing out the narrow space where the bees could feed (c).

were obtained, then concentrations between them were designed. The experiments were prolonged until the time (day) that the control mortality did not exceed 10 percent (≤10%) (OECD, 1998; Laurino et al., 2013). In our experiment, the control mortality exceeded 10 percent after 72 hours (≥10%), therefore we did not continue our observation records to 96 hours. Our acute oral toxicity experiment continued up to 72 hours. Polo-PC software was used to do the calculation of the potential toxicity presented as LC_{50}-24h, LC_{50}-48h, LC_{50}-72h. Probit regressions were plotted by SPSS ver. 18. A significant comparison between LC_{50}-24h and LC_{50}-48h was conducted with the lethal dose ratio method (Robertson and Preisler, 1992). Concentrations used for determining the LC_{50} were: fenpropathrin- 0.1, 0.3, 0.5, 0.6, 0.8, and 1 ppm (active ingredient); primicarb- 50, 90, 150, 300, 400, and 480 (active ingredient); phosalone- 10, 50, 100, 200, 300, and 400 ppm (active ingredient); propargite- 5000, 6000, 7000, 8000, 9000, and 10000 (active ingredient); Bordeaux mixture - 2500, 3300, 4200, 5000, 6000, and 6800 (active ingredient); copper oxychloride- 3500, 4000, 4800, 5600, 6500, and 7000 ppm (active ingredient).

RESULTS

In our experiment, the control mortality exceeded 10 percent after 72 hours (≥10%), therefore we did not continue our observation records to 96 hours. Our experiment continued to up to 72 hours. The results of the fenpropathrin concentrations indicated that the concentration of 0.8 ppm caused mortalities of 82.2, 95.5, and 97.7% at 24h, 48h, and 72h, respectively. Fenpropathrin had the highest toxicity rate when compared to the other pesticides (Fig. 3a). The results showed that for fenpropathrin, LC_{50}-24h was 0.54 ppm, whereas LC_{50}-48h and LC_{50}-72h were 0.3 and 0.28 ppm, respectively (Tab. 1). There were correlations of 0.97, 0.99, and 0.98 between the logarithm of concentrations and mortality probit at 24h, 48h, and 72h, respectively (Fig. 2). There was a significant difference between LC_{50}-24h and LC_{50}-48h (lethal dose ratio = 1.5 - 2.2). There was no significant difference between LC_{50}-48h and LC_{50}-72h (lethal dose ratio = 0.8 - 1.3) (Tab. 2). There was a significant difference between LC_{50}-24h and LC_{50}-48h (lethal dose ratio = 1.2 - 1.8) (Tab. 2). The results of primicarb concentrations indicated that a concentration of 400 ppm caused mortalities of 77.7, 93.3, and 97.7% at 24h, 48h, and 72h, respectively (Fig. 3b). The results indicated that the

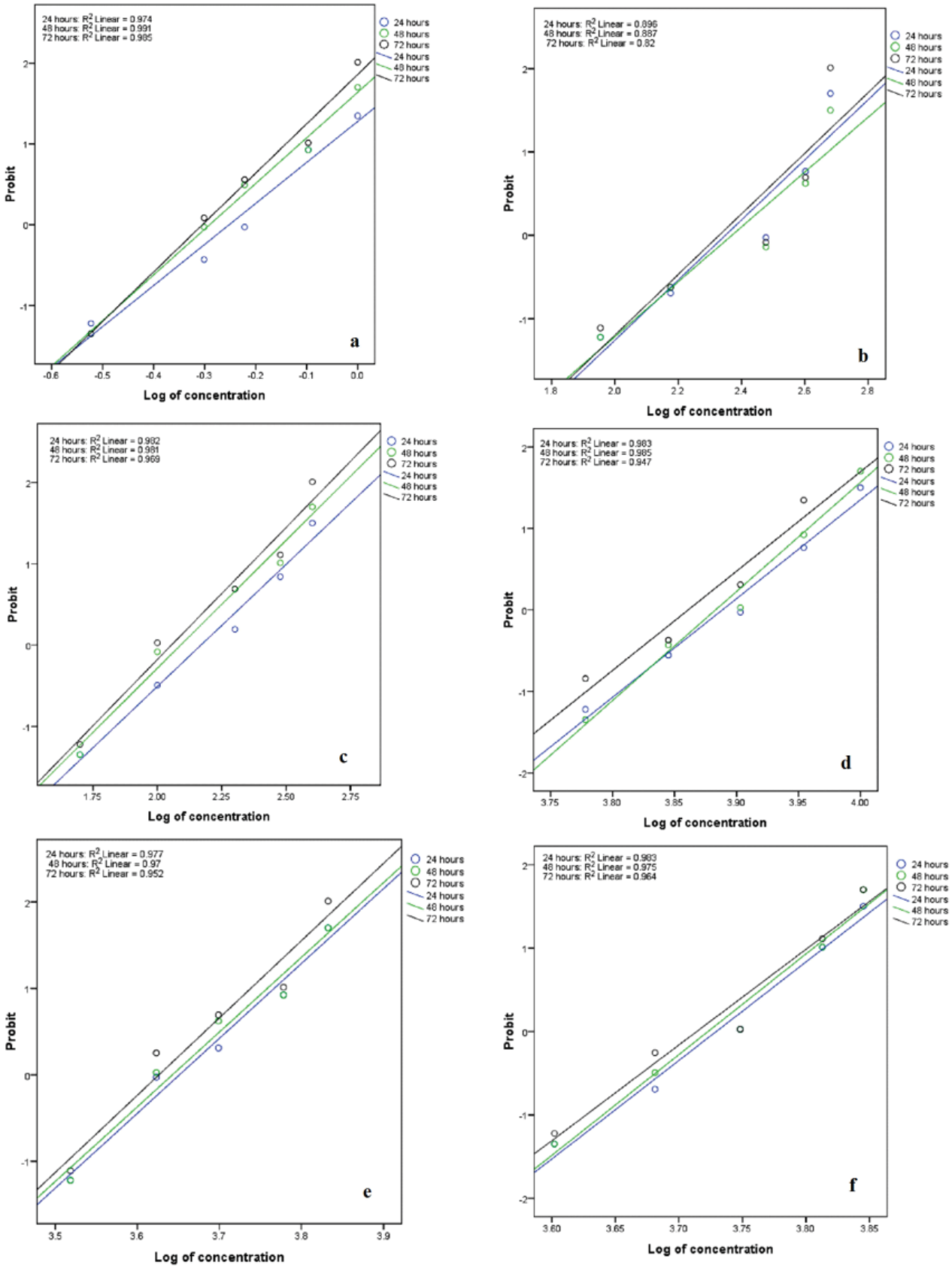

Fig. 2. Probit regression lines of pesticides commonly used in Iran, in 24h and 48h; a - fenpropathrin, b -primicarb, c - phosalone, d - propargite, e - Bordeaux mixture, f - copper oxychloride.

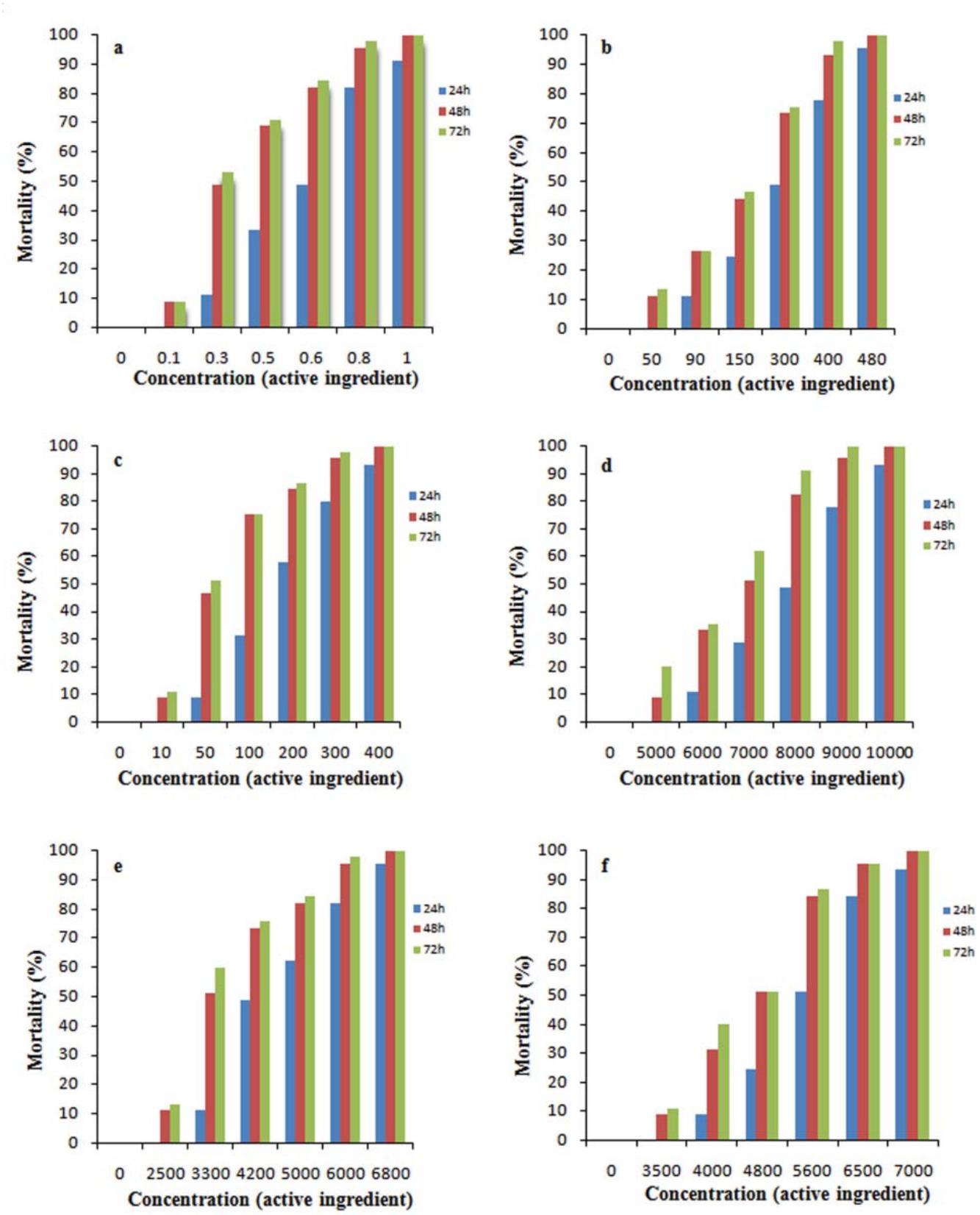

Fig. 3. Mortality comparisons of honeybee adults when different concentrations of: a - fenpropathrin, b -primicarb, c - phosalone, d - propargite, e - Bordeaux mixture, f - copper oxychloride were used at 24h, 48h, and 72h.

LC_{50}-24h of Pirimicarb was 220.8ppm. Also, the LC_{50}-48h and LC_{50}-72h were 153.7 and 143.4 ppm, respectively (Tab. 1). Furthermore, there were correlations of 0.89, 0.88, and 0.82 between the logarithm of concentrations and mortality probit at 24h, 48h, and 72h, respectively (Fig. 2). There was a significant difference between the LC_{50}-24h and LC_{50}-48h (lethal dose ratio = 1.2 - 1.8). Also, There was no significant difference between the LC_{50}-48h and LC_{50}-72h (lethal dose ratio = 0.8 - 1.3) (Tab. 2).

The phosalone concentration of 300 ppm had mortalities of 80, 95.5, and 97.7% at 24h, 48h, and 72h, respectively (Fig. 3c). In our study, the LC_{50}-24h, LC_{50}-48h, and LC_{50}-72h of phosalone were 151.1, 55.8 and 48.2 ppm, respectively. There was a significant difference between the mortalities of the applied concentrations at 24h (F = 134.88, P = 0.00). Probit regression was plotted at 24h and 48h. The results illustrated the correlations of 0.98, 0.98, and 0.96 between the logarithm of the concentrations and mortality probit at 24h, 48h, and 72h, respectively (Fig. 2). There was a significant difference between the LC_{50}-24h and LC_{50}-48h (lethal dose ratio = 2.02 - 3.8). Additionally, there was no significant difference between the LC_{50}-48h and LC_{50}-72h (lethal dose ratio = 0.73 - 1.6) (Tab. 2).

A propargite concentration of 9000 ppm caused mortalities of 77.7, 95.5 and 100% at 24h, 48h, and 72h, respectively (Fig. 3d). In our study, the LC_{50}-24h for propargite was 7785 ppm but this was reduced to 6736 and 6349.9 ppm at LC_{50}-48h and LC_{50}-72h, respectively (Tab. 1). There was a significant difference between the mortalities of the applied concentrations at 24h (F = 116.15, P = 0.00). There was a significant difference between the LC_{50}-24h and LC_{50}-48h (lethal dose ratio = 1.09 - 1.22). There was no significant difference between the LC_{50}-48h and LC_{50}-72h (lethal dose ratio = 0.9 - 1.1) (Tab. 2). Correlations between the logarithm of the concentrations and mortality probit were 0.98, 0.98, and 0.94 for 24h, 48h, and 98h, respectively (Fig. 2).

The Bordeaux mixture concentration of 6000 ppm caused mortalities of 82.2, 95.5, and 97.7% at 24h, 48h, and 72h, respectively (Fig. 3e). The LC_{50}-24h, LC_{50}-48h and LC_{50}-72h of the Bordeaux mixture were correspondingly 4469, 3519, and 3363.4 ppm (Tab. 1). The results indicated that at 24h, 48h, and 72h, there were correlations of 0.97, 0.97, and 0.95 between the logarithm of the concentrations and the mortality probit, respectively (Fig. 2). There was a significant difference between LC_{50}-24h and LC_{50}-48h (lethal dose ratio = 1.1 - 1.3). Also, there

Table 1.

Comparisons of lethal concentrations LC_{10}, LC_{50}, and LC_{90} of commonly used pesticides in Iran

Pesticide	LC_{50} (ppm)			Slop ± SD			Intercept ± SD			Chi-square			LC_{10} (ppm)			LC_{90} (ppm)		
	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h
Phosalone	151.1	55.8	48.2	2.9 ± 0.33	2.1 ± 0.2	2 ± 0.27	-6.4 ± 0.73	-3.8 ± 0.73	-3.4 ± 0.5	1.6	1.3	1.399	55.6	12.8	11.5	410.6	225.3	202.3
Pirimicarp	220.8	153.7	143.4	3.5 ± 0.39	2.7 ± 0.3	0.23 ± 0.1	-8.4 ± 0.95	-5.9 ± 0.7	-6.1 ± 0.7	2.4	2.8	5.7448	100.1	52.3	51.1	558.4	451.6	401.9
Propargite	7785	6736	6349.9	11.8 ± 1.4	11.8 ± 1.5	12.1 ± 1.7	-46.1 ± 5.4	-45.5 ± 5.9	-46.2 ± 6.6	1.6	1.8	5.5203	6087	5246	4982.5	9955.8	8633	8092.6
Fenpropathrin	0.54	0.3	0.28	5.1 ± 0.62	3.0 ± 0.3	0.59 ± 0.14	-1.35 ± 0.17	-1.6 ± 0.18	1.7 ± 0.18	2.06	2.5	3.3980	0.32	0.11	0.11	1	0.78	0.71
Copper oxychloride	5407.9	4591.5	4483.9	11.9 ± 1.3	10.8 ± 1.2	-5.03 ± 1.3	-44.4 ± 4.9	-39.7 ± 4.4	-37.07 ± 4.2	1.9	1.8	4.5961	4221	3498	3353	6928	6026	5996.2
Bordeaux mixture	4469	3519	3363.4	8.3 ± 0.9	7.1 ± 0.8	7.2 ± 0.89	-30.3 ± 3.6	-25.4 ± 3.1	-25.4 ± 3.1	2.3	2.7	4.7806	3132	2332	2234.8	6375	5309	5061.9

Table 2.

Significant evaluation and parallelism hypothesis between lethal concentrations LC_{50}-24 hours and LC_{50}-72 hours with LC_{50} - 48 hours of pesticides

Pesticide	LC_{50}			Parallelism (Chi-square)		df	Lethal Dose Ratio (lower-upper limits)	
	24 hours	48 hours	72 hours	24 hours with 48 hours	72 hours with 48 hours		24 hours with 48 hours	72 hours with 48 hours
Phosalone	151.1	55.85	48.2	4.2* (P<0.05)	5.5 (P>0.05)	2	2 - 3.8**	0.73 - 1.6
Primicarp	220.8	153.7	143.4	1.8 (P>0.05)	2.05 (P>0.05)	2	1.2 - 1.8	0.8 - 1.3
Propargite	7785	6736	6349.9	0.01 (P>0.05)	9.1 (P<0.05)	2	1 - 1.2	0.9 - 1.1
Fenpropathrin	0.54	0.3	0.28	8.6* (P<0.05)	9.7 (P<0.05)	2	1.5 - 2.2	0.8 - 1.3
Copper oxychloride	5407.9	4591.5	4483.9	0.35 (P>0.05)	1.01 (P>0.05)	2	1.1 - 1.2	0.96 - 1.09
Bordeaux mixture	4469	3519	3363.4	0.74 (P>0.05)	0.91 (P>0.05)	2	1.1 - 1.3	0.94 - 1.1

* Parallelism hypothesis is rejected in P<0.05

** If 95% confidence interval includes 1, then LC_{50}-24 hour and LC_{50}-72 hour are not significantly different with LC_{50}-48 hours.

df – degrees of freedom

was no significant difference between LC_{50}-48h and LC_{50}-72h (lethal dose ratio = 0.94 - 1.1) (Tab. 2).

The copper oxychloride concentration of 6500 ppm caused mortalities of 84.4, 95.5, and 95.5% at 24h, 48h, and 72h, respectively (Fig. 3f). As can be seen in Table 1, the LC_{50}-24h, LC_{50}-48h, and LC_{50}-72h of copper oxychloride were 5407.9, 4591.5, and 4483.9 ppm, respectively. There was a significant difference between the mortalities of the applied concentrations at 24h ($F = 136.15, P = 0.00$). There was a significant difference between the LC_{50}-24h and LC_{50}-48h (lethal dose ratio = 1.1 - 1.2). There was no significant difference between the LC_{50}-48h and LC_{50}-72h (lethal dose ratio = 0.96 - 1.09) (Tab. 2). Probit regression was plotted at 24h and 48h. The results showed that at 24h, 48h, and 72h, there were correlations of 0.98, 0.97, and 0.96 between log concentrations and mortality probit, respectively (Fig. 2).

DISCUSSION

In our research, the risk assessments of the six studied pesticides have only been limited in some fragmented data. In the past, systemic compounds like neonicotinoids were recovered in pollen. Recently, large studies in Europe and North America showed the presence of pesticide residues in pollen collected by honeybees (Skerl et al., 2009; Mullin et al., 2010; Wu et al., 2011). Pesticides have been known to induce behavioral changes in adult bees (Thompson, 2003). To date, several studies have demonstrated that ingestion of small amounts of pesticides (e.g. imidacloprid, deltamethrin) by adult honeybees (Colin et al., 2001; Decourtye et al., 2003) interferes with the honeybees' learning and orientation capacity.

Our research showed that the LC_{50}-24h and LC_{50}-48h of phosalone were 151.1 and 55.8 ppm (active ingredient), respectively. Additionally, the LC_{90}-24h was 410.6 ppm (active ingredient). In the usage instructions for the trade formulation of phosalone, 35% is from 0.525 to 0.91 kg/ha active substance for controlling pests. Thus, this insecticide is highly toxic to honeybees. It was reported that the LD_{50} of phosalone was 89000 µg/kg on *Apis mellifera* (Thompson, 2012). Assessment of acute contact toxicity of phosalone on *Megachile rotunda* showed that the usage of 1kg/ha caused 95% mortality at 24h (Tasei et al., 1987). Oral toxicity of phosalone on *Bombus terrestris* was evaluated by Marletto et al. (2003). They found that the LD_{50}-24h was 3.98 ppm. There is only fragmented data about moderately toxic phosalone used around honeybees (Mayer et al., 1999; Sanford, 2009; Adams and Bartholomew, 2012).

Pirimicarb is a carbamate compound that acts as a selective insecticide. The mode of action of carbamates is somewhat similar with organophosphate compounds. Our research showed that the LC_{50}-24h and LC_{50}-48h of pirimicarb were 220.8 and 153.7 ppm (active ingredient), respectively. Moreover, LC_{90}-24h was 558.4 ppm (active ingredient). The usage instructions for the trade formulation of pirimicarb 50%, is from 0.25 to 0.35 kg/ha active substance for controlling pests. Therefore, this insecticide is moderately toxic to honeybees. Using 0.28 kg of the active ingredient per hectare caused an 8% mortality in honeybees. The median lethal dose (LD_{50}) of acute contact toxicity and oral toxicity were 54 µg/bee and 3.2 µg/bee, respectively (Stevenson, 1978). It has been reported that LD_{50} of phosalone was 89000 µg/kg on *Apis mellifera* (Thompson, 2012).

Propargite 57% is an acaricide that uses from 0.57 kg to 0.85 kg/ha of active substance for control of mites in Iran. This acaricide had 7785 ppm (active ingredient) for the LC_{50}-24h and 6736 ppm (active ingredient) for the LC_{50}-48h in honeybees and, therefore, is non-toxic to *Apis mellifera*. Fenpropathrin had the highest toxicity compared to the other pesticides (LC_{50}-24h and LC_{50}-48h, 0.54 and 0.3 ppm (active ingredient), respectively). Some researchers have reported that farmers should not use fenpropathrin when fruit trees are blossoming (Mayer et al., 1999; Riedl et al., 2006).

There is no precise information about acute contact toxicity in fungicides and bactericides of copper oxychloride, and the Bordeaux mixture. Usage of the Bordeaux mixture is; 1.8 kg/ha active substance. Also, from 0.35 to 1 kg/ha of the active substance of copper oxychloride is used in Iran. Our research showed that the LC_{50}-24h of copper oxychloride, and the LC_{50}-24h of the Bordeaux mixture were 5407.9 and 4469 (active ingredient), respectively. Therefore, these pesticides were considered non-toxic to honeybees. Tesoriero et al. (2003) showed that 1 µL copper oxychloride did not have a toxic effect on adult *Osmia cornuta* (Latreille), but this pesticide (1 µL/egg) did cause 40% mortality in eggs.

CONCLUSIONS

Risk assessments of the six pesticides showed that phosalone (insecticide) and fenpropathrin (acaricide and insecticide) are highly toxic to honeybees and they must not be used when honeybees are foraging. Additionally, propargite (acaricide), copper oxychloride, and the Bordeaux mixture (fungicides and bactericides) are non-toxic when used around honeybees. Therefore, propargite (acaricide), copper oxychloride, and the Bordeaux mixture (fungicides and bactericides) can be used safely when honeybees are foraging (*Apis mellifera*).

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