

Original Article

IMIDACLOPRID TOXICITY AND ITS ATTENUATION BY AQUEOUS EXTRACT OF *MORINGA OLEIFERA* LEAF IN ZEBRA FISH, *DANIO RERIO*

VINEETA YADAV^{1*}, SHADAB AHMAD², KANEEZ ZAHRA³

^{1,2,3}Department of Zoology, Bipin Bihari College, Bundelkhand University, Jhansi, U. P., India
Email: vineetayadav6987@gmail.com

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ABSTRACT

Objective: Imidacloprid (IMC) is a systemic chloro-nicotinyl insecticide that is applied in crop fields to control a wide variety of harmful insects and pests. The objective of the present study was to evaluate the mitigating effects of Moringa leaf extract (MLE) against Imidacloprid (IMC) induced mortality after acute toxicity bioassay in zebrafish, *Danio rerio*.

Methods: The fishes were exposed to various concentrations of IMC to estimate the LC50 values at different exposure periods using the probit analysis.

To assess the attenuating effect on mortality fishes were exposed to different concentrations of MLE along with 24 and 96 h LC50 of IMC. Mortality rate of fishes was recorded during all experimental periods.

Results: The LC50 values were found to be 0.423, 0.352, 0.297 and 0.270 ml/l after 24, 48, 72 and 96 h respectively. The upper confidence limits were 0.491, 0.408, 0.334 and 0.302 ml/l and lower confidence limits were 0.355, 0.296, 0.260 and 0.238 ml/l for 24, 48, 72 and 96 h respectively.

The fishes remain alive at 12 ml/l and 10 ml/l of MLE against 24 and 96 h LC50 respectively. Some behavioral responses such as violent, sluggish and erratic movement, respiratory distress, mucous secretion all over the body, discoloration were also observed in IMC treated fishes in comparison to MLE treated fishes.

Conclusion: These findings demonstrated that MLE has mitigating effects against the toxic influence of IMC on mortality in zebra fishes which is attributed to its antioxidant effects.

Keywords: Imidacloprid, MLE, Acute toxicity, Zebrafish

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INTRODUCTION

In developing world environmental pollution has become a major problem which is responsible for the unbalanced ecosystem. Agrochemicals such as pesticides, fertilizers contaminate water significantly when they enter into the water reservoir through runoff. The contamination by pesticides finally absorbed by aquatic organisms causes detrimental effects. Most of the synthetic pesticides are non-biodegradable and has longer life-time. Pesticides remain in the water for a considerable period cause adverse effects on non-target organisms including fishes [1, 2].

Imidacloprid [1-6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylidene-amine] is widely used systemic chloronicotinyl insecticide for crop protection to control sucking insects, termites, tick and mites. It has high selective toxicity and exceptional potential against insects and pests showing a high affinity to acetylcholine receptors of insects. Therefore, it acts as a neurotoxin for insects which attack their central nervous system, resulting in the impairment of nerve functions. On reaching into the aquatic environment, it causes toxicity to fishes by affecting their physiology, behavior, hematology and biochemistry of fishes [3-6].

Toxicity is the degree at which a chemical substance or a particular mixture of substances can cause destruction of an organism. Acute toxicity is expressed as the median lethal concentration (LC50) that is the concentration of toxicant in water which kills 50 % of a test population of any test organism within a short exposure period. LC50 test is the best measure of the species susceptibility and survival potential to the particular toxicant. Probit analysis is most preferred statistical method for calculating the LC50 of the toxicant. It is a type of regression which is used to determine binomial response [7]. In addition to mortality test, behavioral responses of animals are the most favourable, sensitive indicator of ecotoxicology [8]. Different studies revealed that wide range of pesticide exposure showed abnormal behavioral and morphological alterations [9, 10].

Polyphenolic compounds are present in several plants which protect against the generation of free radicals. Free-radicals play an important role in the pathogenesis of several human diseases such as cancer, rheumatoid arthritis, cardiovascular diseases. Plant sources have potential to reduce Reactive Oxygen Species [11, 12]. *Moringa oleifera* is a member of family Moringaceae. It is native to northern Europe and India especially south of Himalaya Mountain [13]. *Moringa oleifera* is commonly known as "Miracle tree" or "Sehjana". It is a good source of therapeutically active compounds having 46 natural antioxidants and over 92 nutrients. Some important are Kaempferol, rhamnetin, rutin, quercetin, chlorogenic acid, apigenin, also enriched in ascorbic acid, carotenoids, polyphenols, flavonoid glycosides, thiocarbamate glycosides, amino acids like methionine, lysine, cysteine, tryptophan [14-17]. Moringa leaves have antibacterial, anti-inflammatory and hepatoprotective properties [18]. Leaf extract cures fevers, bronchitis, eye and ear infections, inflammation of mucus membrane, gastric ulcers and severe diarrhea [19, 20]. Several workers have been carried out to determine the protective effect of *Moringa oleifera* leaves extract against heavy metal such as arsenic, cadmium and drugs like acetaminophen-induced toxicity in rats [21-23]. Khalil and Korn (2017) have also been reported that *Moringa oleifera* leaves extract helps in boosting growth, immune system by mitigate oxidative stress in fishes [24]. Previous studies reported the attenuating effect of Moringa leaf extract against pesticides in rats and fishes [25, 26].

Toxicological studies have been conducted in the aquatic ecosystem of a variety of pesticides but less information is available on the toxicity of neonicotinoids such as Imidacloprid in fishes. Fishes are quite sensitive to the wide range of pollutants and serves as best bio-indicator to assess water quality of aquatic ecosystem. In present study, zebra fishes were selected as an experimental model as recommended by International Organisation for Standardization and Organisation for economic co-operation and Development because of the sequenced genes of zebra fishes are just similar to human beings [27]. Therefore, the current study was carried out to determine the LC50 of Imidacloprid and its attenuation by MLE in zebrafish, *Danio rerio*.

MATERIALS AND METHODS

Experimental fishes

Live, healthy and mature individuals of zebra fishes were purchased from the aquarium shop of Jhansi district, U. P. India, weighing from 2-4g. Fishes were brought carefully in polythene bags to the laboratory and transferred into a well-aerated glass aquarium. They were subjected to a prophylactic treatment by bathing in 0.1% potassium permanganate (KMnO₄) for 2 min to prevent possible dermal infections. Fishes were allowed to acclimatize for 10-15 d under laboratory conditions before the commencement of the experiments. The physicochemical characteristics of water were maintained throughout the acclimatization as Temperature 25 °-28 °, dissolved oxygen (DO) 5.0-6.5 mg/l, pH 7.2±0.2, hardness 220 mg/l. Fishes were fed twice daily with commercial food. The water was renewed and nitrogenous waste products were siphoned off daily. The feeding was stopped 24 h prior to acute toxicity bioassay. All the experiments were carried out by keeping acclimatized fishes in rectangular glass aquaria of 2'×1'×1' size separately according to groups.

Test chemical

Commercial Imidacloprid (1-6-chloro-3-pyridyl methyl-N-nitroimidazolidin-2-ylidene-amine) 17.8% SL (Trade name-Crocodile) was purchased from local pesticide shop, manufactured by Pioneer Pesticide Pvt. Ltd. Samba district, J and K, India.

Acute toxicity bioassay

To determine the lethal concentration of 50% of Imidacloprid for zebrafish, *Danio rerio*, toxicity assays were conducted in the laboratory.

Preparation of stock solution

According to the experimental design stock solution was prepared by dissolving crocodile in distilled water as 1 ml solution contained 0.1 ml crocodile.

Direct interpolation method

In direct interpolation method two exploratory and one definitive tests were carried out. To determine the mortality of fishes within the range of 0% to 100%, 1st exploratory experiment was conducted which required two random concentrations of 0.05 ml/l and 1.0 ml/l. Both lower and higher concentrations were employed in glass aquaria separately containing 5 fishes each. Subsequently 2nd exploratory test was done using five concentrations of IMC (0.10, 0.20, 0.30, 0.40 and 0.50 ml/l) and 10 fishes were exposed to each concentration. For definitive test, the acclimated 10 fishes were exposed separately in glass aquaria with 7 concentrations of crocodile as 0.22, 0.26, 0.30, 0.34, 0.38, 0.42 and 0.46 ml/l. Mortality was recorded after 24, 48, 72 and 96 h exposure periods and dead fishes were removed immediately from aquaria.

Probit analysis

To assess the actual LC₅₀ values through probit analysis, the concentrations obtained from the definitive test were converted into log concentrations, % mortalities into correct percentage and their empirical value of probit were determined using Finney's table. Correct percent for 0% and 100% mortality were evaluated as follows [28].

For 0% mortality = 100(0.25/n)

For 100% mortality = 100(n-0.25/n)

Where, n= number of fishes

A graph (regression line) was plotted between the log concentrations and probit values for different exposure periods. Log concentrations were obtained from the regression line by drawing a perpendicular at 5 probit corresponding to the 50% mortality for 24, 48, 72 and 96 h intoxication and inverse of these log concentrations were the actual LC₅₀ values. The standard error of LC₅₀ was computed by the following formula-

$$\text{Standard Error (SE) of LC}_{50} = \frac{(\text{Log LC}_{84} - \text{Log LC}_{16})}{\sqrt{2N}}$$

N= Number of fishes (10)

Probit of log LC₈₄ and log LC₁₆ were taken from Finney's table [29] which are 5.99 and 4.01 respectively and approximately equal to 6 and 4 probit. After calculation the antilog values were the SE of LC₅₀.

Assessment of effective concentrations of moringa leaf extract (MLE) after both exposure periods

Collection and preparation of aqueous leaf extract

The leaves of *Moringa oleifera* were collected from the Bundelkhand region, cleaned up with tap water and air-dried in the shaded area. The dried leaves were grinded into fine powder. 25 g of powder was mixed with 250 ml hot (98 °C) distilled water, stayed for 24 h, then filtered with filter paper [24].

Effects on mortality

For standardization of effective concentration of Moringa leaf extract (MLE) against 24 h LC₅₀ of Imidacloprid, 70 fishes were randomly divided into 7 groups of 10 fishes each. Fishes of group Ist, IInd, IIIrd, IVth, Vth, VIth and VIIth were treated with 2, 4, 6, 8, 10, 12 and 14 ml/l of MLE respectively in combination with 24 h LC₅₀ concentration of crocodile. To assess the effective concentration of MLE against 96 h LC₅₀, 60 fishes were randomly divided into 6 groups of 10 fishes each. The fishes of group Ist, IInd, IIIrd, IVth, Vth, and VIth groups were exposed with 2, 4, 6, 8, 10 and 12 ml/l of MLE respectively in combination with 96 h LC₅₀ concentration of crocodile (IMC).

RESULTS

Acute toxicity bioassay

During the evaluation of acute toxicity of imidacloprid (IMC) all fishes were survived at a lower concentration (0.05 ml/l) whereas 100% mortality was observed at high concentration (1.0 ml/l) after 24 h in 1st exploratory experiment. In 2nd exploratory test, five concentrations were employed in which minimum mortality rate about 20% was observed at lower concentration (0.10 ml/l) when zebra fishes exposed to 96 h while at higher concentration (0.50 ml/l) 100% mortality occurred after 48 h exposure period of IMC. On the basis of IInd range-finding test, the selected seven concentrations and their respective mortality rate for crocodile (Imidacloprid 17% SL) during different exposure periods are shown in table 1 (Definitive test). The concentrations of the definitive test were converted into log concentrations and % mortalities into the correct percentage and their empirical probits by Finney's table [29]. The values of log concentrations at 5 probit were -0.373, -0.453, -0.527 and -0.567 ml/l and their actual LC₅₀ were obtained as 0.423, ml/l for 24 h, 0.352 ml/l for 48 h, 0.297 ml/l for 72 h and 0.270 ml/l for 96 h by taking antilogs (fig. 1, 2, 3 and 4). The values of LC₈₄ and LC₁₆ for probit 6 and 4 were evaluated to analyze Standard Error of LC₅₀ for different intervals from fig. 1, 2, 3 and 4.

Table 1: Definitive test for direct interpolation method

S. N.	Conc. (ml/l)	No. of fishes	24 h		48 h		72 h		96 h	
			M	% M	M	%M	M	%M	M	%M
1.	0.22	10	0	0	1	10	1	20	1	30
2.	0.26	10	1	10	1	20	1	30	1	40
3.	0.30	10	2	20	1	30	2	50	1	60
4.	0.34	10	3	30	1	40	2	60	1	70
5.	0.38	10	3	30	3	60	1	70	2	90
6.	0.42	10	5	50	2	70	2	90	1	100
7.	0.46	10	6	60	2	80	2	100		

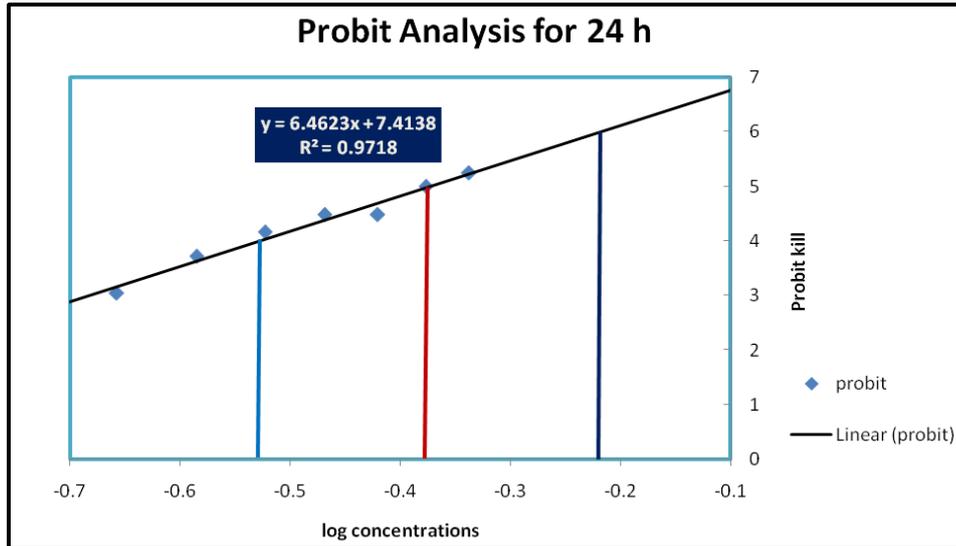


Fig. 1: Plot of log concentrations versus probit kill after 24 h exposure

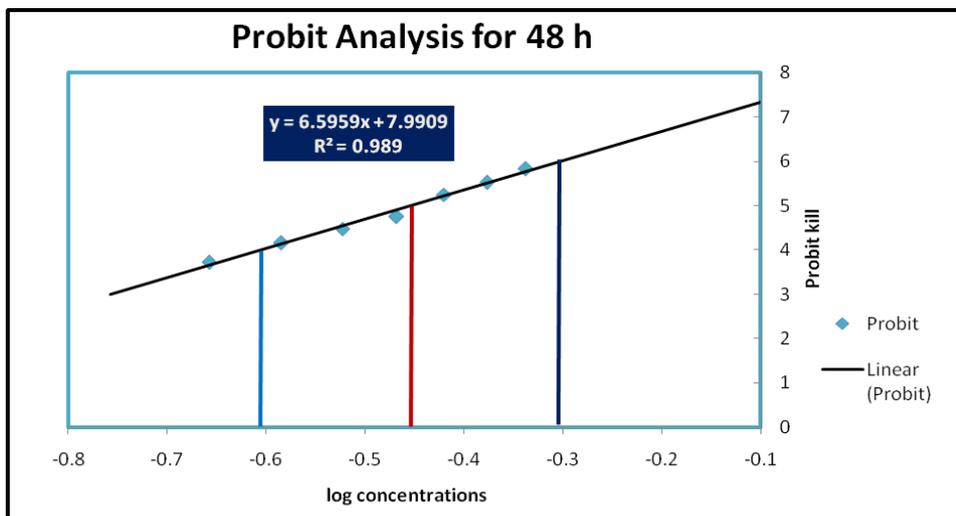


Fig. 2: Plot of log concentrations versus probit kill after 48 h exposure

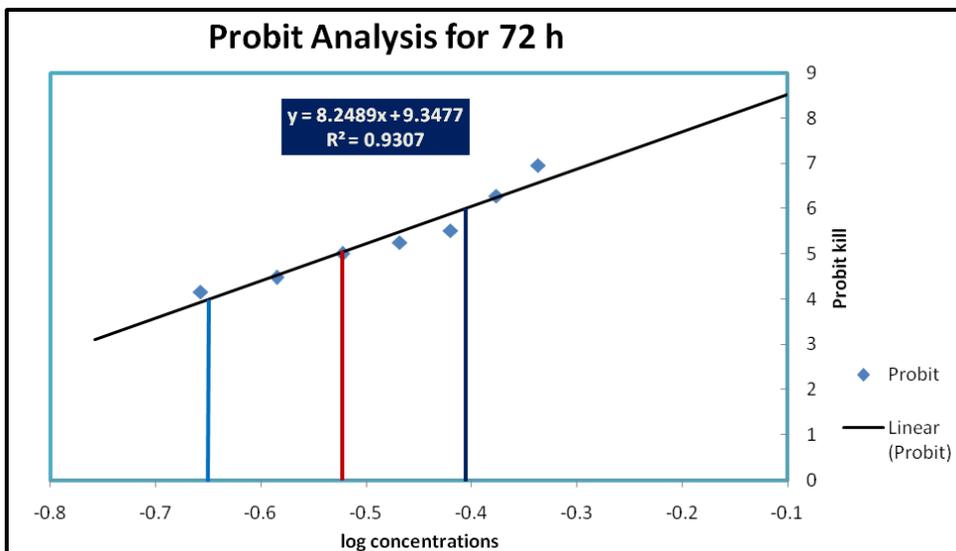


Fig. 3: Plot of log concentrations versus probit kill after 72 h exposure

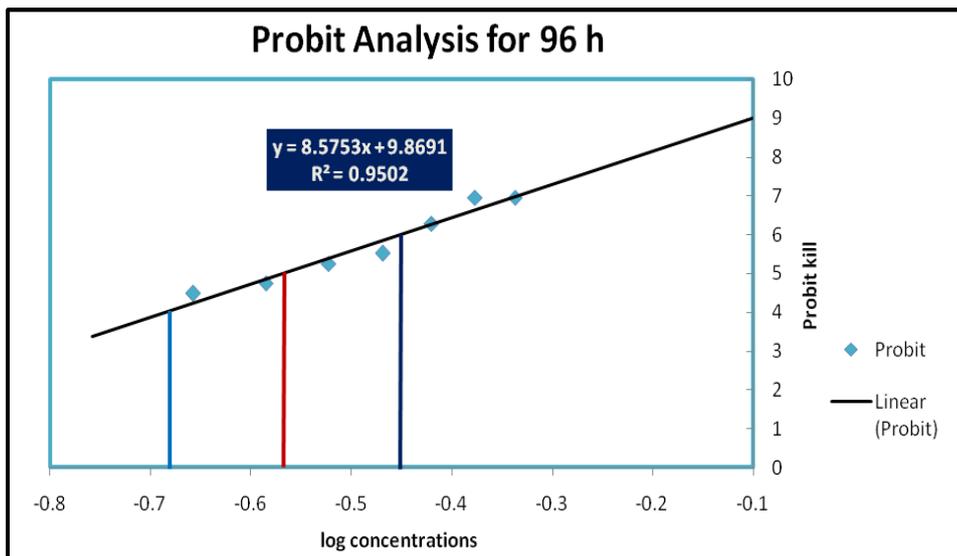


Fig. 4: Plot of log concentrations versus probit kill after 96 h exposure

Table 2: Estimation of 95% confidential limit after 24, 48, 72 and 96 h imidacloprid (IMC) intoxication

S. No.	Exposure periods (in hours)	LC50	LC84	LC16	SE of LC50	95% Confidential limit	
						Lower limit	Higher limit
1.	24	0.423	0.604	0.296	0.068	0.355	0.491
2.	48	0.352	0.499	0.248	0.056	0.296	0.408
3.	72	0.297	0.392	0.224	0.037	0.260	0.334
4.	96	0.270	0.353	0.206	0.032	0.238	0.302

The calculated SE of LC50, LC84, LC16, SE of LC50 and 95 % confidence interval are also illustrated in table-2. During imidacloprid intoxication, influential changes were also observed in fish behavior such as violent and sluggish swimming, respiratory distress, mucous secretion all over the body, discoloration, jumping and hitting against the wall of aquarium due to irritation prior to death.

Assessment of effective concentrations of MLE

The mortality rate against toxicant with MLE is represented in fig. 5 and 6. When the 24 and 96 h LC50 concentrations of IMC were

administered along with 2 ml/l MLE 50% mortality occurred. On increasing the concentrations of MLE the mortality rate was decreased. No mortality occurred at 12 ml/l of MLE in combination with LC50 concentration of 24 h (0.423 ml/l) whereas all fishes remain alive at 10 ml/l of MLE with LC50 concentration of 96 h (0.270 ml/l) exposure period. Increased death rate was observed on further increasing concentrations of MLE after both toxicant exposure periods (fig. 5 and 6). Therefore the effective concentrations of MLE were found to be 12 ml/l and 10 ml/l after 24 and 96 h respectively.

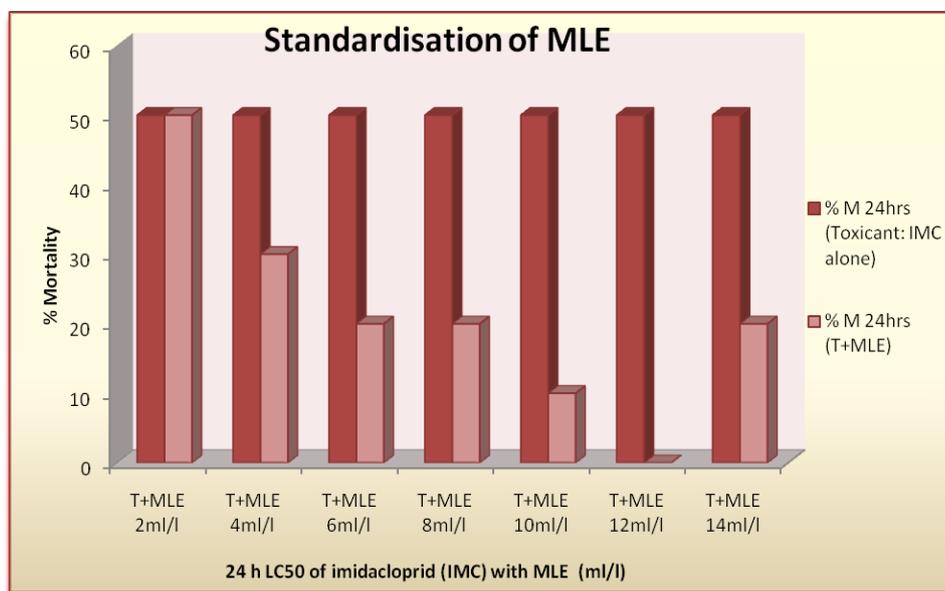


Fig. 5: Assesment of effective concentration of MLE against 24 h LC50 concentration (0.423 ml/l) of IMC

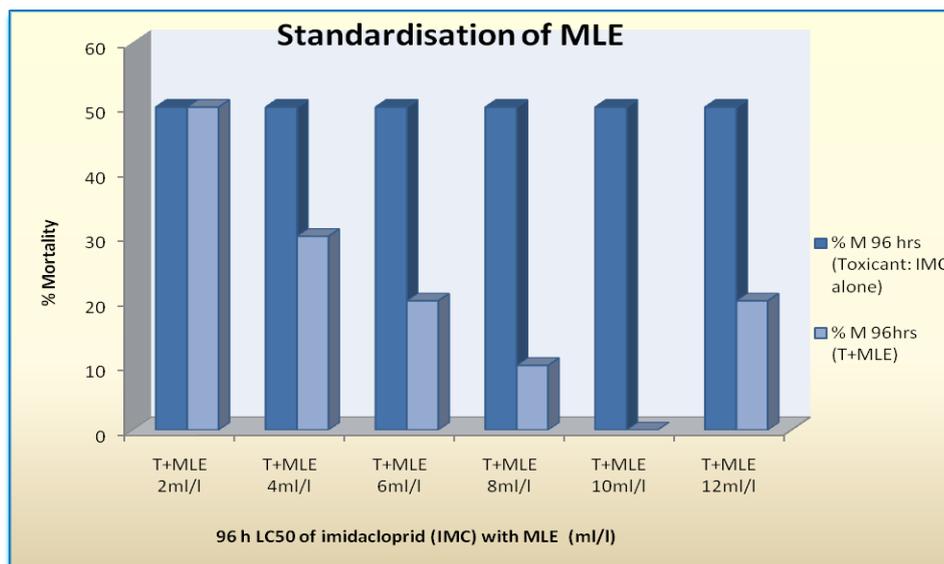


Fig. 6: Assesment of the effective concentration of MLE against 96 h LC50 concentration (0.270 ml/l) of IMC

DISCUSSION

In the present investigation, the LC50 values of Imidacloprid (IMC) in zebra fishes were found to be 0.423, 0.352, 0.297 and 0.270 ml/l at different exposure periods (24, 48, 72 and 96 h). It was observed that LC50 value after 24 h was 0.423 ml/l which decreased to 0.270 ml/l after 96 h exposure. Hence IMC shows time-dependent action. The toxicity of imidacloprid in zebra fishes was assessed by Ge *et al.* (2015) at various concentrations (0.3, 1025 and 5 mg/ml) after different exposure periods [30]. The acute toxicity of Imidacloprid to zebra fishes was assessed by several other researchers [31, 32]. Wu *et al.* (2018) reported 19.04 to 38.01 mg/l LC50 during acute exposure in imidacloprid induced zebra fishes [33]. The median lethal concentration of the commercial formulation of Imidacloprid (confidor SL200®) was reported by Tisler *et al.* (2009) as 241 mg/l for 96 h exposure period to adult zebra fishes [34]. Shukla *et al.* (2017) found that acute toxicity of Imidacloprid to zebra fishes was 27.5 mg/l for 24 h [35]. The above studies show that IMC is less toxic as compared to the present investigation. More studies were carried out previously in other fishes using Imidacloprid. Wast *et al.* (2016) have shown the toxicity of Imidacloprid to *Poecilia reticulata* which were 68.443 ppm, 90.056 ppm, 180.593 ppm and 117.614 ppm in juvenile, male, female and mixed population respectively showing the age and sex-dependent toxicity of imidacloprid [36]. Some authors also reported the median lethal concentration of Imidacloprid in freshwater fishes which is more toxic as compared to our study [37, 38]. Tyor and Harkrisna (2016) reported the LC50 concentration of Imidacloprid for the embryo of *Cyprinus carpio* as 78 ppm of 48 h shows more toxicity to the embryo than adult [5]. The results of 96 h LC50 of Imidacloprid to *Labeo rohita* was found to be 550 mg/l by Qadir *et al.* (2014) [39]. The acute toxicity for golden orfi and rainbow trout were found as 237 mg/l and 21 mg/l when exposed to Imidacloprid, showed much variation to each other [40]. Anthony *et al.* (2015) observed the acute toxicity of Confidor (Imidacloprid) in fresh water fish, *Labeo rohita* which were 14, 260 mg/l for 24 h, 13,710 mg/l for 48 h and 11,900 mg/l for 96 h [41].

Toxicity of Imidacloprid can be compared to organophosphate and Carbamate compounds as a toxic effect of these compounds to zebra fishes have been investigated by several authors [42, 43]. Reports by Al Safi (2016) to Malathion and Atrazine toxicity in zebra fishes were found to be 5.292 mg/l and 9.567 mg/l respectively [44]. The LC50 values observed in the present study are comparable to the studies of Singh *et al.* (2017) whose results were 289µg/l after 96 h chlorpyrifos intoxication in zebra fishes [45]. Similar results were also observed by Sethuraman (2015) in which estimated LC50 in zebrafish was 0.32 mg/l after 96 h triclosan poisoning [42].

Moringa oleifera leaf extract (MLE) possesses anticancer, anti-inflammatory, bacteriocidal, hypocholesterolemic, antioxidant, neuro- and hepatoprotective properties [46-48]. Ameliorative effects of *Moringa oleifera* have been studied against many chemicals by several researchers in fishes and mammals [21, 49, 50]. In the present study, no mortality occurred even though the fishes received LC50 concentration of IMC. As already stated earlier that IMC increases the oxidative stress in fishes due to which free radicals are generated. *Moringa oleifera* is known for its free radical scavenging property. In the present investigation decreased mortality may be due to boosting up scavenging activity of free radicals by *Moringa oleifera*. Administration of MLE restored the activities of AST, ALT, ALP, LDH, GGT and total protein toward the normal level induced by pesticides as shown by many workers [26, 51, 52]. The reversal of increased transaminases by MLE supplement may be due to the healing of hepatic parenchyma and regeneration of hepatocytes. *Moringa* may have induced the repairing effect on the damaged liver by its essential amino acid such as methionine and cysteine boosting the total protein contents as evidenced by several authors [53, 54]. Some authors reported that among whole plant of *Moringa oleifera* leaves are responsible for the greater production of antioxidants and have the capacity to act as a protective shield during oxidative damage [55-57]. Our previous work on MLE against bio-pesticide also showed a decrease percent death rate in zebra fishes [25]. Amelioration and rejuvenation in several tissues especially liver by MLE may be another reason for decreasing the death rate. Mortalities of fishes were decreased when concentrations of MLE were increased. When it reached on effective concentrations i.e. 12 ml/l for 24 h and 10 ml/l for 96 h there was no mortality occurred. But as the concentrations increased above these levels mortality occurred again on 14 ml/l and 12 ml/l for 24 and 96 h. This may be due the fact that besides being beneficial impact flavonoids can cause toxicity as evidenced earlier [25, 58, 59].

CONCLUSION

The present investigation has provided the best evidences that the toxicant such as Imidacloprid (IMC) can cause toxicity followed by heavy mortality in zebrafish, *Danio rerio* after acute exposure inducing oxidative stress. The use of aqueous extract of *Moringa oleifera* leaf (MLE) attenuates IMC induced toxicity. Hence, it can be stated that co-administration of MLE diminish the hazardous impact of IMC by reducing the generation of reactive oxygen species.

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AUTHORS CONTRIBUTIONS

Vineeta Yadav: Critical reviews, literature search, draft writing, animal caring and dosing, biochemical and statistical analysis. Shadab Ahmad: Materials, animal caring and dosing, literature search and biochemical analysis. Kaneez Zahra: Concept, Supervision and Critical reviews.

CONFLICTS OF INTERESTS

The authors confirm that there are no conflicts of interest.

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