# CHAPTER-6

## 6.0 TOXICITY AND ITS METHODS OF EVALUATION

### 6.1 Toxicity

It is the degree to which a substance can damage an organism. Toxicity can refer to the effect on a whole organism, such as an animal, bacterium, or plant, as well as the effect on a substructure of the organism, such as a cell (cytotoxicity) or an organ such as the liver (hepatotoxicity) (Stellman, 1998). By extension, the word may be metaphorically used to describe toxic effects on larger and more complex groups, such as the family unit or society at large. Sometimes the word is more or less synonymous with poisoning in everyday usage.

### 6.2 Bioassays

It is an experiment in which a living organism is used as a test subject with the intention to estimate the relationship between the response and quantity or intensity of the stimulus (toxicant) under standard set of conditions (Agatonovic-Kustrin et al., 2015).

#### 6.2.1 Prerequisites of bioassay

##### 6.2.1.1 Insects

Mass capturing of the insects should be done using lures/pheromone-baited traps, sweep nets, light traps and aspirators etc. at adult stage (Levi-Zada et al., 2017). Immatures like larvae or pupae may also be collected manually. After collection, the insects should be reared in the laboratory under standard conditions of temperature and humidity on a suitable source of food to obtain a uniform population which is most important prerequisites of bioassay. However, field collected insect population of same stage can also be exposed directly after observing its natural mortality due to damages during collection by maintaining its population for 8-24h. It is very important to expose healthy and active insects in the bioassay. Extent of robustness of data set for a bioassay depends upon the numbers of insects exposed. It is mandatory to expose more number of insects in a concentration is necessary to achieve more dynamic results. Ten-twenty five insects should be exposed in one concentration but the numbers could be reduced depending upon the availability of insects. However, the number of replicates should not be less than three per concentration. Random selection of insects for each concentration of a bioassay is of paramount importance to obtain valid results. For example, never use all healthy individuals in one concentration or all weaker individuals in one concentration. Instead assure the exposure of blend of both weaker and healthier individuals by random selection from the pool to get reliable results.

##### 6.2.1.2 Determining a range of concentrations

Establishing a range of concentrations causing <100% and >0% response is difficult task. Normally 5-8 different levels of a toxicant are made by serial dilution method like 10, 20, 40, 80 and 160µg/mL etc. Dilution is normally made in water but other diluents may be used depending upon availability or suitability of diluents with toxicant. Several repetitions are done to select a desired range of concentration on hit and trial basis. This range may vary over time with changes in the level of susceptibility. Several factors such as species, stage, previous exposure of insects, and size of insect as well as the class of insecticide also influence the range of concentrations for a bioassay. Previously published bioassay results assist in developing the desired range of concentration at initial stage (Sayyed & Crickmore, 2007; Shah et al., 2007; Shah et al., 2015a; Shah et al., 2015b; Shah et al., 2016).

Bioassays have been published for families like Brentidae (Smith & Hammond, 2006), Miridae (López et al., 2008), Thripidae (López et al., 2008), Pentatomidae (Nielsen et al., 2008), Aphididae (Foster et al., 2002), Curculionidae (James, 2003), Coccinellidae (Smith & Cave, 2006), Cybocephalidae (Smith & Cave, 2006); Encyrtidae (Smith & Cave, 2006), Culicidae (Shah et al.,

2016), Tortricidae (Sial et al., 2010), Noctuidae (Ahmad et al., 2008; Lai & Su, 2011), Braconidae (Shi et al., 2004), Ichneumonidae (Cordero et al., 2007), and Aleyrodidae (Bi & Toscano, 2007).

#### 6.2.1.3 Stock solution preparation

In serial dilutions, highest concentration of any testing material or chemical is known as Stock Solution. Stock solution is prepared by mixing insecticide in a solvent like water or alcohol. Quantity of insecticide is determined by following formulae (Kranthi, 2005).

$$\text{Dose (µl or mg)} = \frac{\text{PPM required} \times \text{Water/solvent required}}{\%\text{F} \times 10}$$

Generally, (%F indicates the percentage of active ingredient in the formulated product) formulations are either solid or liquid and measured in mg/µl. Parts per million (PPM) tells about the quantity/strength of insecticide in stock solution. Water is most easily available and inexpensive solvent that is capable to dissolve most of the insecticide formulations. Quantity of the solvent is calibrated prior to performing bioassay e.g. amount of solvent needed to dip a filter paper is different from that required for a leaf. In case of diet incorporation bioassays, amount of the solvent required is always higher as compared to the other types of bioassays because the paste is prepared in such cases.

#### 6.2.3.3 Safety Statement

Using proper safety precautions is important when dealing with insecticides. Consult the material safety data sheet (MSDS) i.e. Gain an understanding of the hazards and precautions necessary for the safe use of chemical products (WP, 2015). An MSDS is normally a prescription regarding identification (name, composition of ingredients, supplier), recommended usage, possible hazards, first aid measures, fire fighting measures, accidental release measures, handling and storage, personal protection, physical and chemical properties, stability and reactivity of product, toxicological information and transport measures (Fait et al., 2001). It is mandatory to wear the personal protective equipment (PPE) before handling pesticides (Perry et al., 2002). PPE refers to protective clothing, helmets, goggles, or other garments or equipment designed to protect the wearer's body from injury or infection. Specific laboratory training on handling pesticides should be required by personnel before attempting any bioassays (Paramasivam & Selvi, 2017).

### 6.2.2 Types of bioassay

#### 6.2.2.1 Topical application method

It is one of the most commonly employed method in which insecticides are directly applied to the body surface of the insects (Galdino et al., 2011). The insecticide is mixed in a volatile solvent like acetone and applied to insect using a micro syringe. Although, it is one of the most effective method of direct application of insecticides but has some serious flaws including intensive labor, size and behavior of the insects (Matthews, 2008).

#### 6.2.2.2 Injection method

Although the topical application method is useful to assess the effects of contact toxicity but could not determine the actual amount of the toxicant entering into the insect body. Actual amount of the insecticides entering the insect body could be accurately assessed by injection method (Nuringtyas et al., 2014). First of all, insecticide is dissolved in a carrier solvent such as acetone, propylene glycol and injected into the body cavity. Inter segmental region or the abdominal sterna is usually selected for injection. Insects should make unconscious. The needle should not be injected into the body of insects in longitudinal position so that the nerve card should be protected from harm. Furthermore, needle should be held in a position for a while and pulled away to avoid bleeding. There are some serious limitations of the injection method including the finding of a solvent that is non-toxic as well as vigorously dissolve the insecticide (Paramasivam & Selvi, 2017). Sometime insect behavior or size is also an important factor in limiting the applicability of this method.

#### 6.2.2.3 Dipping method

In insecticide dipping or immersion method of bioassay, whole insect or its desired life stage is dipped into the prepared insecticide solution (Miller et al., 2010). Normally forceps, screened containers or dipping nets are used for the purpose of dipping and holding the insects for a few seconds in toxic solutions (Chandrasena et al., 2011). Different concentrations of the insecticide solutions could be prepared by serial dilution method (Shah et al., 2015b). Insects treated with the insecticides are placed in clean containers. Data of mortality is assessed after the specified intervals (Shah et al., 2016; Shah et al., 2017).

#### 6.2.2.4 Contact method

In this method, insecticide is mixed with a volatile solvent and applied to the surface of a glass jar (Snodgrass, 1996). The solvent evaporates and the insecticide residues film will remain on the surface of the glass jar. Insecticides solution could also be applied to the filter paper by dipping it in the toxic solution which is placed in the petri dish (Ullah et al., 2015).

#### 6.2.2.5 Fumigation method

This is an efficient method employed to evaluate the toxic vapors of a particular insecticide against the stored product pests. The insecticide is introduced into a sealed container containing the insect pest infested stored grain (Kim et al., 2003). The mortality is recorded at different intervals.

#### 6.2.2.6 Feeding method

It is a bioassay method in which insecticide is mixed in the diet. Insects being exposed are starved for few hours before exposure. Mostly immatures are exposed in this method to check the susceptibility to a particular insecticide (Abbas et al., 2012; Shah et al., 2015a). For phytophagous insects, leaf disc is treated with the toxic solution (Afzal et al., 2015). The mortality data is assessed after different intervals of post treatment.

#### 6.2.2.7 Toxicity testing for higher animals

It is almost impossible to perform the traditional toxicity tests as used for insects in case of higher animals. The availability of hundreds of higher animals for using in a single bioassay is impractical. Therefore, several adjustments are done to evaluate the toxicity of pesticides with higher animals. There are three different methods including acute, sub-acute and chronic used which are mandatorily required in the pesticide industry (Simon, 2014).

#### 6.2.2.8 Acute toxicity bioassay

In this type of bioassay, oral feeding, dermal injection or inhalation intake methods are employed to determine the oral, dermal and inhalation $LC_{50}$ or $LD_{50}$ (Pandey et al., 2009).
The acute oral toxicity is determined by administering single dose by normal feeding or forced feeding i.e. stomach tube or capsule (White & Bradnam, 2015). Mortality data is assessed after 24h of post treatment. To assess the safety of insecticide to workers acute dermal toxicity tests are performed. For this purpose, an albino rabbit is shaved and painted with the chemical in question. The median lethal dose 50 is determined after 24 h of post treatment. For the determination of acute inhalation toxicity, tests are performed in an exposure chamber to assure the nose or head only exposure and minimize the oral entry of chemicals by licking of fur by the animals. Insecticides to be tested are employed in the form of aerosol, dust or mist formulations. In acute inhalation testing, individuals are exposed for 4 h and then shifted to other container for next 14 days period (Matsumura, 2012).

#### 6.2.2.9 Sub-acute toxicity and chronic toxicity tests

To evaluate the primary chronic toxic effect on the tissues and organs as well as the secondary toxic effect such as carcinogenicity, teratogenicity, mutagenicity and no effect levels. These tests are performed to assess the response of the animals for prolonged time periods as compared to the acute toxicity tests. Data is observed for 90 d for sub-acute toxicity tests while for whole life time in chronic toxicity testing. The amount of toxicant received by an animal varies

depending upon its species, age and size. No effect level of the compound is determined by conducting these tests.

**6.2.2.10 Probit Analysis**

Probit analyzes the relationship between the stimulus i.e. dose of a toxicant and quantitative response. In an insecticide bioassay, mortality is recorded at different levels of the toxicant. This data is then subjected to probit analysis to estimate the lethal concentration/lethal dose at which 50 percent population ($LC_{50}$) is killed, $x^2$ (for testing heterogeneity), 95% fiducial limit of the $LC_{50}$(FL) and slope value (±SE).

**Steps for manual calculation of $LC_{50}$ values**

Normally five concentrations of a toxicant excluding control (untreated insects group) can be chosen for determination n of $LC_{50}$ starting from no death to 100% mortality. At least thirty individuals should be exposed at each concentration and the number may vary depending upon the availability of the specimen. The mortality data is assessed after 24h, 48h or 72h depending upon the method of bioassay, type of chemical used and purpose of study.

**Step1.**

First of all range of exposure concentrations is decided based on the hit and trial basis or consulting the literature or both.

**Example:**

Let us consider that we have conducted the bioassay of spinosad on fourth instar *Culex quinquefaciatus* Say larvae. The 8mg/L was used as highest dosage, 30 individuals were exposed at each concentration and mortality data was assessed after 24h.

**Table 6.1**. Dose response or mortality data (Shah et al., 2015a)

| Conc.(mg/L) | Total Number exposed | Number dead (24 hours) |
|---|---|---|
| 0.0 (control) | 30 | 1 |
| 8.0 | 30 | 25 |
| 4.0 | 30 | 20 |
| 2.0 | 30 | 15 |
| 1.0 | 30 | 11 |
| 0.5 | 30 | 7 |

**Step2**

**Abbot's correction:**

The control mortality is corrected by using the following formulae:

$$\text{Corrected Mortality (\%)} = \frac{\text{M(obs)} - \text{M(control)}}{100 - \text{M(control)}} \times 100$$

Where "M (Obs)" represents percent mortality in response to a concentration and "M (Control)" is used for percent mortality in control groups.

**Example**

If control mortality is 1 out of 30 i. e, 3.3% and observed treatment mortality is 15 out 30 (50%).the Abbott correction would be

$$\text{Corrected} = (\frac{50 - 3.3}{100 - 3.3}) \times 100$$
$$= 48\%$$

**Table 6.2. The corrected mortality % by using Abbots formula**

| Conc.(mg/L) | Total No. | No dead (24 hours) | Corrected mortality % |
|---|---|---|---|
| 0.0(control) | 30 | 1 | - |
| 8.0 | 30 | 25 | 83 |
| 4.0 | 30 | 20 | 66 |
| 2.0 | 30 | 15 | 48 |
| 1.0 | 30 | 11 | 35 |
| 0.5 | 30 | 7 | 21 |

**Step3**

Data is transformed by consulting the Probit values table for %corrected mortality. In case of 1st treatment where corrected mortality (%) is 83, we can see it from transformation table, vertically at 80 and moving up to 3 horizontally ahead of 80 i. e, 5.95. The arrow below 80 shows the method to see the Probit value for 83% mortality.

**Table 6.3**. Transformation of the percentage mortalities to probits

**Transformation of percentage mortalities to probits**

| % | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| **0** | - | 2.67 | 2.95 | 3.12 | 3.25 | 3.36 | 3.45 | 3.52 | 3.59 | 3.66 |
| **10** | 3.72 | 3.77 | 3.82 | 3.87 | 3.92 | 3.96 | 4.01 | 4.05 | 4.08 | 4.12 |
| **20** | 4.16 | 4.19 | 4.23 | 4.26 | 4.29 | 4.33 | 4.36 | 4.39 | 4.42 | 4.45 |
| **30** | 4.48 | 4.50 | 4.53 | 4.56 | 4.59 | 4.61 | 4.64 | 4.67 | 4.69 | 4.72 |
| **40** | 4.75 | 4.77 | 4.80 | 4.82 | 4.85 | 4.87 | 4.90 | 4.92 | 4.95 | 4.97 |
| **50** | 5.00 | 5.03 | 5.05 | 5.08 | 5.10 | 5.13 | 5.15 | 5.18 | 5.20 | 5.23 |
| **60** | 5.25 | 5.28 | 5.31 | 5.33 | 5.36 | 5.39 | 5.41 | 5.44 | 5.47 | 5.50 |
| **70** | 5.52 | 5.55 | 5.58 | 5.61 | 5.64 | 5.67 | 5.71 | 5.74 | 5.77 | 5.81 |
| **80** | 5.84 | 5.88 | 5.92 | 5.95 | 5.99 | 6.04 | 6.08 | 6.13 | 6.18 | 6.23 |
| **90** | 6.28 | 6.34 | 6.41 | 6.48 | 6.55 | 6.64 | 6.75 | 6.88 | 7.05 | 7.33 |

**Table 6.4.** Probit values for our data are as follows

| Conc.(mg/L) | Total number exposed | Number dead (24h) | Corrected mortality % | Probit values |
|---|---|---|---|---|
| 0.0(control) | 30 | 1 | - | |
| 8.0 | 30 | 25 | 83 | 5.95 |
| 4.0 | 30 | 20 | 66 | 5.41 |

| | | | | |
|---|---|---|---|---|
| 2.0 | 30 | 15 | 48 | 4.95 |
| 1.0 | 30 | 11 | 35 | 4.61 |
| 0.5 | 30 | 7 | 21 | 4.19 |

**Table 6.5.** The $Log_{10}$ values of concentrations

| Conc.(mg/L) | Total No. | No dead (24h) | Corrected mortality % | Probit values | Log10 concentration |
|---|---|---|---|---|---|
| 0.0 (control) | 30 | 1 | - | | |
| 8.0 | 30 | 25 | 83 | 5.95 | 0.90309 |
| 4.0 | 30 | 20 | 66 | 5.41 | 0.60206 |
| 2.0 | 30 | 15 | 48 | 4.95 | 0.30103 |
| 1.0 | 30 | 11 | 35 | 4.61 | 0 |
| 0.5 | 30 | 7 | 21 | 4.19 | -0.30103 |

**Why Probit transformation?**

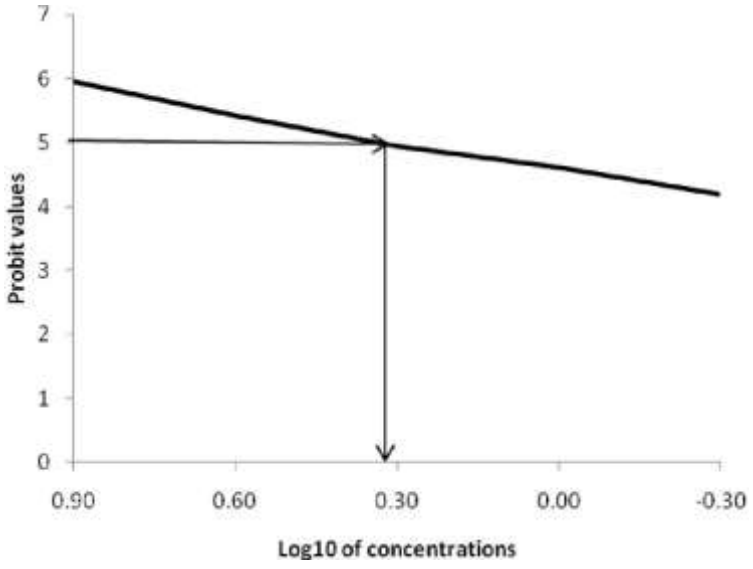
If we plot a graph between the corrected percent mortality and log10of concentration we would not get a straight line. For the purpose to get the straight line we plot a graph between the Probit value and log10 of concentration and estimation of $LC_{50}$ would be easier.

**Step4**

**Graphical Method**

A graph between the Probit value and log10 of concentration (See Table 5) is plotted to assess the $LC_{50}$.

**Graphical method**




X =0.32
$LC_{50}$ =antilog (0.32)
=2.1mg/L

**Figure 6.1** Plot of $log_{10}$ concentrations versus Probit values

**Calculation of standard error of $LC_{50}$**

Following formula is used to assess the SE of the $LC_{50}$that is needed for the estimation of the FLs of the $LC_{50}$

$$\text{Approx. SE of } LC_{50} = \frac{(\text{Log}LC_{84} - \text{Log}LC_{16})}{\sqrt{2N}}$$

Where N is the total number of subjects in each treatment

Consult the Probit value table to see the probit value of the 84 and 16.The Probit values for 84 and 16 are 6 and 4, respectively after rounding off to single significant digit.

$$\text{Approx. SE of } LC_{50} = \frac{[(\text{Log}(6) - \text{Log}(4)]}{\sqrt{2N}}$$

While the log-concentration values corresponding to these Probit units are 0.9 and -0.3, respectively (Figure 6.1). The antilog values for 0.9 and -0.3 are 7.94 ($LC_{84}$) and 0.50 ($LC_{16}$), respectively.

$$\text{Approx. SE of } LC_{50} = \frac{(7.94 - 0.5)}{\sqrt{2(30)}}$$

$$= \frac{7.44}{7.74}$$

=0.9612

Hence the SE is 0.9612.

**Estimation of 95% FL of $LC_{50}$**

The 95% FL of the $LC_{50}$ can be calculated by using SE = 2.1±0.9612=2.1 (1.138-3.06)

**Step7**

**Estimation of standard deviation ($\sigma$)**

$$\sigma = \frac{1}{2}\left(\frac{LC84}{LC50} + \frac{LC50}{LC16}\right)$$

$$\sigma = \frac{1}{2}\left(\frac{7.94}{2.1} + \frac{2.1}{0.5}\right)$$

=3.99

**Step8**

**Estimation of slope ($\beta$)**

$$\beta = \frac{1}{\sigma}$$

$$\beta = \frac{1}{3.99}$$

=0.250

**Step9**

**Estimation of chi-square ($\chi^2$)**

$$\chi^2 = \sum \frac{(E - O)^2}{E}$$

Where **E** is the expected mortality and **O** is the observed mortality.

The expected mortality for each treatment is calculated by following formula

Expected mortality =Sum of mortalities of the all the treatments of bioassay/Number of treatments

**Table 6.6** Chi-square values of the bioassay

| Conc.(mg/L) | Total Number | Number dead (24h) | Corrected mortality % | Observed Mortality (corrected) | Expected mortality | Chi-square |
|---|---|---|---|---|---|---|
| 0.0(control) | 30 | 1 | | | | |
| 8.0 | 30 | 25 | 83 | 24.9 | 15.18 | 6.22 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4.0 | 30 | 20 | 66 | 19.8 | 15.18 | 1.40 |
| 2.0 | 30 | 15 | 48 | 14.4 | 15.18 | 0.04 |
| 1.0 | 30 | 11 | 35 | 10.5 | 15.18 | 1.44 |
| 0.5 | 30 | 7 | 21 | 6.3 | 15.18 | 5.19<br>Sum=14.29 |

$\chi^2$=14.29

**Step10**

Goodness of fit test was used to determine probability

P = CHIDST (df, $\chi^2$)(performed using Microsoft X – cel)

P = 0.120

**6.2.2.11 Comparison of Toxicity**

Generally, toxicities of different insecticides are compared based on their respective values of the $LC_{50}$. Generally, lower the $LC_{50}$ value and higher will be the toxicity of the considered insecticide. However, CI of the $LC_{50}$ are the considered the base to compare the toxicity of the different insecticides.

**Example:**

Below is given the $LC_{50}$ values of insecticides with their respective 95% CI in "()"

1. Clothianidin 0.31 (0.19–0.45) vs Emamectin benzoate 0.27 (0.17–0.42)
   In above mentioned example, toxicities are similar because of the overlapping 95% FL.
2. Fipronil 0.92 (0.72–1.18) vs Clothianidin 0.31(0.19–0.45)
   In second example, $LC_{50s}$ of the mentioned insecticides are similar because of overlapping 95% FL.

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