Chapter-3
Toxicity Study
Introduction

The term toxicology derived from the word ‘toxicon’ means poison and ‘logos’ means science. Toxicology is the science that deals with the adverse effect of chemical and drug on living organisms. Medicinal plants are natural resources yielding valuable phytoc constituent, which are often used in the treatment of various diseases. A substantial part of the population in developing countries uses folk medicines for their daily healthcare. Some of the traditional medicine involves the use of crude plant extracts, which may contain an extensive diversity of molecules, often with indefinite biological effects. However, most of the information available to the consumer with regard to the medicinal herbs is not backed by credible scientific data. For this reason, research is carried out to determine the toxicity of medicinal plants (Konan et al., 2007).

Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and cells. This interaction may vary depending on the chemical properties of the toxicants and the cell membrane, as it may occur on the cell surface, within the cell body, or in the tissues beneath as well as at the extracellular matrix. The toxic effects may take place prior to the binding of the toxicants to the vital organs such as liver and kidneys (Jothy et al., 2011). Hence, evaluation of toxic properties of a substance is crucial when considering for public health protection because exposure to chemicals can be hazardous and results to adverse effects on human being (Figure 3.1).

![Figure 3.1: Fate and effect of toxicant on physiological system](image-url)
Most of the toxicity studies are conducted to assess the degree to which substances are toxic (poisonous) for humans or animals to investigate the mechanism of toxic chemicals or to develop new or improved tests for specific types of chemically induced effects. The toxicological studies include: acute toxicity studies where as the examination of adverse effects that may occur on first exposure to a single dose of a substance, studies that seek to assess the potential of substance to interact with genetic materials (genotoxicity) and tests that aim to identify whether toxicity occurs after continuous exposure to a substance (repeated dose toxicity studies), tests that are undertaken to find out whether cancers may develop as a result of exposure to certain chemicals and studies to ensure the safety of medicines (Konan et al., 2007).

The measurement of toxicity is also complex. Toxicity may be acute or chronic and may vary from one organ to another as well as with age, genetics, gender, diet, physiological condition or the health status of the organism. As opposed to experimental animals, which are highly inbred, genetic variation is a most important factor in human toxicity since the human population is highly outbreed and shows extensive genetic variation. Even the simplest measure of toxicity, the LD$_{50}$ (the dose required to kill 50% of a population under stated conditions) is highly dependent on the extent to which the above variables are controlled. The LD$_{50}$ is one way to measure the short term poisoning potential of a material (Crossland, 1980).

Current testing regimes have evolved significantly over the past three decades. Existing practices have changed and new methods have been added. A major influence on these developments has been the Test Guidelines Programme of the Organization for Economic Cooperation and Development (OECD), which has developed standardized methods of testing chemicals are periodically reviewed in the light of scientific progress or changing assessment practices. The result of the extensive review and discussions on the significance of the LD$_{50}$ value and the concomitant development of alternative procedures is that authorities today do not usually demand classical LD$_{50}$ test involving a large number of animals. The limit test, fixed dose procedure, the toxic class method and the up and down methods all represent simplified alternatives using only few animals (OECD guideline 420, 2001). However, efforts are being made to develop in vitro system; e.g., acute systemic toxicity can be broken down into a number of biokinetic, cellular and molecular elements, each of which can be identified and quantified in appropriate models (Walum, 1998).
Up and down method is the maximally used procedure that is used for acute toxicity testing of a drug with the minimum number of animals. The principle of up and down method is based on a stepwise procedure with the use of a minimum number of animals per step; sufficient information is obtained on the acute toxicity of the test substance to enable its classification. The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex (normally females). Absence or presence of compound related mortality of the animals dosed at one step will determine the next step, i.e. either no further testing is needed, dosing of three additional animals, with the same dose or dosing of three additional animals at the next higher or the next lower dose level (OECD guideline 420, 2001).

Material and methods

Plant materials

Toxicity study was performed with methanol extracts of *Anthocephalus cadamba* (MEAC) and β-sitosterol glucoside (BSSG). All the test drugs were diluted with purified water to prepared desired doses (Babu et al., 2003) for toxicity studies.

Animals

Swiss albino mice of about 8 weeks of age with an average body weight of 20-25 g were used for the experiment. The mice were housed in poly acrylic cages (38 cm × 23 cm × 10 cm) with not more than six animals per cage. The animals were maintained under standard laboratory conditions (temperature 25-30 °C and 55-60% relative humidity with dark/light cycle 14/10 h) and were allowed free access to standard dry pellet diet and water ad libitum. The mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment.

Experimental model

1. Acute toxicity test

Acute oral toxicity of MEAC and BSSG was carried out by up and down procedure as laid down by OECD guidelines for the testing of chemicals (OECD guideline 420, 2001). Before administration of dose the mice were kept fasting for 3-4 hrs and food may be withheld for further 1-2 hrs after dosing.

After administration of dose, the animals were observed individually at least once during the first 30 minutes, periodically for first 24 hrs, with special attention given during the first 4
The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed (Cahn and Hayes, 1994).

Parameters of observation should include changes in skin, fur, eyes and mucous membranes and also respiratory, circulatory, autonomic, central nervous systems, somatomotor activity and behavior pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. All the observations were systematically recorded for each animal.

2. Brine shrimp lethality bioassay

The brine shrimp (Artemia salina Leach) is a simple zoologic organism (an arthropod). The brine shrimp lethality test (BSLT) as a tool to measure general bioactivity in chemical and natural products for cytotoxicity and pesticidal activities. The BSLT is also useful method for evaluating toxicology studies because, availability of the eggs, the ease of hatching them into larvae, the rapid growth of the nauplii and the relative ease of maintaining a population under laboratory conditions. Combined with a reference standard, the brine shrimp test offers a bioassay that can be rapid, simple, bench-top and more importantly, inexpensive and reproducible. BSLA is used as an indicator for general toxicity and also as a guide for the detection of cytotoxic and antitumor compounds (Tawaha, 2006).

![Figure 3.2: Brine shrimp larvae](image)

**Collection and hatching of Brine shrimp**

The brine shrimp eggs were purchased from Tuty Fifhes hatchery, Villipuram, Tamilnadu, India. Brine shrimp eggs were hatched in a shallow rectangular dish filled with artificial seawater (about 38 g of salts per liter). A plastic divider with several 2 mm holes was clamped in the dish to make two unequal compartments. The eggs were sprinkled into the larger compartment which was darkened (covered side of the divided tank), while the smaller compartment was illuminated. After 48 h, the phototropic nauplii were collected by pipette from the lighter side, having been separated by the divider from their shells. Lamp positioned above
the uncovered side attracts hatched shrimp. The lamp provides direct light and warmth (about 25 °C) throughout embryogenesis (Amara and ElMasry, 2008; Kumar et al., 2011).

**Procedure**

About 4.5 ml of brine solution was taken into each test tube. MEAC and BSSG were diluted as per the diluted concentrations (10-500 µg/ml). The 0.5 ml diluted solution was added to the test tube and 10 active shrimps were introduced into each test tube by drawing them with glass capillary tube. The negative control solution is simply the solvent without test sample. The test tubes were left uncovered under the lamp. The number of surviving shrimps were counted and recorded after 24 hours (Figure 3.3).

The toxicity rate was estimated on the basis of the number of dead nauplii or the mortality rate that was estimated using the following equation:

\[
\% \text{ mortality or death rate} = \left( \frac{d_{\text{test}} - d_{\text{control}}}{A_{\text{control}}} \right) \times 100
\]

Where, \( d_{\text{test}} = \) the average number of dead nauplii in the experimental groups, \( d_{\text{control}} = \) the average number of dead nauplii in the control group, and \( A_{\text{control}} = \) the average number of living nauplii in the control group.

*Figure 3.3: Brief outline brine shrimp lethality bioassay*
Results

Acute toxicity test

The acute oral toxicity MEAC and BSSG were safe on Swiss albino mice up to the dose of 2000 mg/kg and 500 mg/kg body weight respectively.

Brine shrimp lethality bioassay

Experimental results reveal that the lethality concentration (LC$_{50}$) of MEAC and BSSG were 158 and 150 µg/ml respectively after 24 hrs (Table 3.1). The degree of lethality was directly proportional to the concentration of the test drug. Maximum mortalities (100 %) were observed at a concentration 500 µg/ml.

Discussion

Phytotherapeutic products from medicinal plants have become universally popular in primary healthcare, particularly in developing countries and some have been mistakenly regarded as safe just because they are a natural source. Nevertheless, these bioactive products from medicinal plants are presumed to be safe without any compromising health effect, and thus widely used as self medication (Vaghasiya et al., 2011). Acute oral toxicity study is vitally needed not only to identify the range of doses that could be used subsequently, but also to reveal the possible clinical signs elicited by the substances under investigation. It is also a useful parameter to investigating the therapeutic index of drugs.

Oral acute toxicity testing in mice could be used to evaluate natural remedies for different pharmacological activities, taking into account the basic premise that pharmacology is simply toxicology at a lower dose. A toxic substance might elicit interesting pharmacological effects at a lower nontoxic dose. Toxicity results from animals will be crucial in definitively judging the safety of medicinal plants if they are found to have sufficient potential for development into pharmacological products (Sasidharan et al., 2008). As use of medicinal plants increases, experimental screening of the toxicity of these plants is crucial to assure the safety and effectiveness of those natural sources.

In this oral acute toxicity study, the Swiss albino mice were employed to observe the toxicity effects of MEAC and BSSG. The acute oral toxicity MEAC and BSSG were found to be safe up to the dose level of 2000 mg/kg and 500 mg/kg body weight respectively. The present experimental data suggest that the MEAC and BSSG are safe for therapeutic utilize.
### Table 3.1: Lethal concentration of MEAC and BSSG in brine shrimp bioassay.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentrations (µg/ml)</th>
<th>Number of Surviving Nauplii After 24 h</th>
<th>Mortality (%)</th>
<th>LC$_{50}$ (µg/ml)</th>
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<tr>
<td></td>
<td></td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
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<tr>
<td>Methanol extract of <em>Anthocephalus cadamba</em> (MEAC)</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>9</td>
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<tr>
<td></td>
<td>50</td>
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<td>150</td>
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<td></td>
<td>200</td>
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<td></td>
<td>500</td>
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<tr>
<td>β sitosterol glucoside (BSSG)</td>
<td>10</td>
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<td>8</td>
<td>9</td>
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<tr>
<td></td>
<td>50</td>
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<td>3</td>
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<tr>
<td></td>
<td>500</td>
<td>0</td>
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*T=Trial, n = 10, T1+T2+T3 = Total shrimps.

BSLT is a useful screening system in medicinal plants for toxicity and may be discovered new bioactive compounds. BSLT was included to next step for more effective and sensitive refining of toxicants in order to find a minute change on living organism. The MEAC and BSSG were found to be concentration dependent which indicated the presence of potent cytotoxic and probably antitumor components of these plants. According to Meyer et al., 1982, crude plant extract is toxic (active) if it has an LC$_{50}$ value of less than 1000 µg/ml while nontoxic (inactive) if it is greater than 1000 µg/ml. Experimental results suggest that MEAC and BSSG are not toxic and can further explore for the development of pharmaceutical products.

**Conclusion**

From the above toxicity study, it was shown that MEAC and BSSG in acute oral toxicity study and brine shrimp bioassay model are non toxic at an optimum dose level. Based on this toxicological study these test drugs were taken for further pharmacological research work.
References


