CHAPTER - 4

ACUTE TOXICITY STUDIES

4.1. Introduction

Toxicology is the science that deals with the study of potential harmful effects of chemicals and drugs on living organisms. The word toxicology is derived from two words toxicon (poison) and logos (discourse) mean, study of poisons. The toxicologist, a specialist attained pharmacologist involved in the study of poisons, adverse or toxic effects of chemicals and drugs on animals and human beings. The scope of toxicology encompasses the qualitative determination of poison/chemicals, their deleterious (injurious) effects on the living organism, their incidence, mechanism, factors modifying them and reversibility (treatment) of such adverse effect[1].

Toxicology studies using animals and *in vitro* cellular or tissue preparations have been used to study the toxic effects and mechanism of action of drugs and chemicals and to determine the effective and safe dose of drugs in humans and the risk of toxicity from chemical exposures. Special attention should be paid to the solvent or dispersing agent of the drug as the toxic effect may arise from the solvent and animal care during the period of the toxicity tests is of paramount importance. Drugs that survive the initial screening and profiling procedure must be carefully evaluated for potential risks before clinical testing is begun[2].

The purpose of National Toxicology Program (NTP) is to identify toxic effects resulting from exposure to a particular chemical or agent and to characterize dose-response relationship[3]. Although chemicals and drugs can be evaluated for their toxic potential by using *in vivo* animal studies and *in vitro* models, it should be recognized that these testing procedures are only component in the process of evaluating the potential toxic risk of drugs and chemicals. The evaluation of the toxicity of drugs and chemicals should include, when possible, data obtained from a number of investigative approaches[4,5], i.e. secular trend or ecological trend analysis[6], animal studies[7], pharmacokinetic, toxicokinetic, pharmacodynamic, and toxic dynamic studies[8], mechanism of action (MOA) studies[9], and basic science studies that pertain specifically to the studies, which include receptor affinity, cytotoxicity, genotoxicity, organ toxicity, neurotoxicity[10,11] etc.

The index of the acute toxicity is LD_{50} (median lethal dose at which 50 percent of the population dies), which should not be regarded as a biological constant, since different results are observed on different sets of tests or when the investigations are carried out in

different laboratories. This has been indicated very clearly in multicentric study carried out in the European community with five substances[12]. Historically, the LD_{50} was determined with high degree of precision and was used to compare toxicities of compounds relative to their therapeutic doses. It is now realized that high degree of precision may not be necessary to compare toxicities[13]. Therefore, the median lethal dose is now estimated from the smallest number of animals possible[14].

In screening drugs determination of LD_{50} (the dose which has proved to be lethal to 50% of the tested group of animals) is usually an initial step in the assessment and evaluation of the toxic characteristics of a substance. It is an initial assessment of toxic manifestations (provides information on health hazards likely to arise from short term exposure to drugs), and is one of the initial screening experiments performed with all compounds. Data from the acute study may

(a) serve as the basis for classification and labeling.

(b) provide initial information on the mode of the toxic action of a substance.

(c) help to arrive at a dose of a new compound

(d) help to determine LD_{50} values that provide many indices of potential types of drug activity.

Aim of acute toxicity test is to determine the therapeutic index i.e. ratio between the lethal dose and the pharmacologically effective dose in the same strain and species (LD_{50}/ED_{50}).

Greater the index, safer is the compound. LD_{50} with confidence limits is to be established on one common laboratory species such as mouse/rat using the standard method. The LD_{50} dose thus found was administered to guinea pigs, rabbits, cats or dogs on weight basis (on basis of relative surface area gives better results).To determine the absolute dose for a species in the column, the absolute dose given to the species in a row was multiplied by the factor given at intersection of the relevant row and column. Because of species variation, several species of animals (one rodent and one non rodent) were used to determine LD_{50}. When a clearly different response was observed in any of these species, a larger number of that species needs to be tested to establish the approximate LD_{50} value. Thus we suggest a method of acute toxicity testing and calculation of LD_{50}

that has to go through the entire process of validation with different categories of substances before its final acceptance by the regulatory bodies[15].

4.1.1. The median lethal dose (LD_{50})

The median lethal dose or LD_{50} is the dose which would be expected to kill one-half of a group of test animals of same species and strain. The LD_{50} dose is usually expressed as milligrams or grams of material per kilogram of animal body weight (mg/kg or g/kg). The material may be administered by mouth or applied to the skin. The median effective dose (ED_{50}) is the dose (mg/kg body weight), which produces a desired therapeutic action in 50 percent of the test population[16]. In this present study lethal dose (LD_{50}) of the methanol extracts of *K. rotunda* rhizome and *E. cannabinum* leaves has been determined by oral route of administration on mice.

4.2. Materials and methods

4.2.1. Plant materials

Methanol extracts of rhizomes of *Kaempferia rotunda* Linn. and leaves of *Eupatorium cannabinum* were used as test drug in these experiments and sodium CMC solution was used as control vehicle.

4.2.2. Experimental animals

Swiss albino mice of either sex weighing between 20-25 g were used for the study. They were housed in polypropylene cages maintained under standardized environmental conditions and had free access to food and water. Ethical clearance was obtained from Institution Animals Ethical Committee for using animals in the present study method. Experiment was conducted in accordance to the guidelines provided by committee for the control and supervision of experiments on animals.

4.2.3. Methods of evaluation

Ten groups of animals were used taking ten animals in each group. Different doses of methanol extract suspended in1% w/v sodium CMC solution was administered orally to nine groups of animals. One group of animal was treated as control and was fed with 1% solution of sodium CMC. The method of Lorke[17] was followed to determine the acute toxicity of the extracts. The animals were kept under observation in open field condition

for 72 hrs after the administration of extracts of rhizome and leaves in oral route as mentioned earlier, and the number of deaths and signs of clinical toxicity were recorded. The toxicological effect was assayed on the basis of mortality, which was expressed as an LD_{50} value, which was calculated by the graphical method of Miller and Tainter[18].

In the groups with no dead animals and the groups with only dead animals, the obtained percentages were corrected using the following formula:

Correction formula for 0% dead group = 100 (0.25/n)
Correction formula for 100% dead group = 100 [(n-0.25)/n]
where 'n' represents the number of animals in the group.
After correction, the percentages were converted into probits referring to the probit table. The values thus obtained were plotted against log dose. The dose corresponding to 50% or probit 5 was taken as LD_{50}. Probit 5 on the Y-axis is interpolated to the X-axis to get log LD_{50}, the antilogarithm of which gives LD_{50} (Fig. 4.1. and 4.2.).

Similarly acute toxicity studies were carried out for isolated compounds of *K. rotunda* and *E. cannabinum* with six groups of animals (four animals in each group) by using UP down or Stair case method[19]. Different doses of isolated compound I (150-325 mg/kg b.w) and compound II (150-250 mg/kg b.w) suspended in1% w/v sodium CMC solution were administered orally to five groups of animals. One group of animal was treated as control. The numbers of death in each group within 24h were recorded. The LD_{50} was estimated by the graphical method of Miller and Tainter[18].

4.3. Results

The results of the acute toxicity studies of methanolic extracts of rhizomes of *Kaempferia rotunda* and leaves of *Eupatorium cannabinum* have been presented in table & fig. 4.1, and 4.2 respectively. The LD_{50} of methanol extract of rhizomes of *K. rotunda* was found to be 5g/kg body weight where as the LD_{50} of the methanol extract of *E. cannbinum* leaf was found to be 4.5g/kg body weight by oral route. The LD_{50} of isolated compound I was found to be 213.7mg/kg body weight (Table & Fig. 4.3) where as the LD_{50} of the isolated compound II was found to be 196.3mg/kg body weight (Table & Fig. 4.4) by oral route. The doses were arbitrarily chosen which, was less than $1/10^{th}$ of the median lethal dose[20,21].

Table 4.1. Determination of LD_{50} of methanol extract of *K. rotunda* rhizome after oral administration in mice

No. of Group	Dose (mg/kg body wt.)	Log dose (X)	No of animals used	No of animals dead	Dead (%)	Corrected dead (%)	Probit (y)
1	500	2.69	10	0	0	2.5	3.04
2	1500	3.17	10	1	10	10	3.72
3	2500	3.39	10	2	20	20	4.16
4	3000	3.47	10	3	30	30	4.48
5	4000	3.60	10	4	40	40	4.75
6	5000	3.69	10	5	50	50	5.00
7	5500	3.74	10	7	70	70	5.52
8	6000	3.77	10	8	80	80	5.84
9	6500	3.81	10	10	100	97.5	6.96

Corrected formula: for 0% dead = 100 × 0.25/n; for 100% dead = 100 (n – 0.25)/n, where n is the number of animals in each group.

Table 4.2. Determination of LD_{50} of methanol extract of *E. cannabinum* leaves after oral administration in mice

No. of Group	Dose (mg/kg body wt.)	Log dose (X)	No of animals used	No of animals dead	Dead (%)	Corrected dead (%)	Probit (y)
1	500	2.69	10	0	0	2.5	3.04
2	1000	3.00	10	1	10	10	3.72
3	2000	3.30	10	2	20	20	4.16
4	3000	3.47	10	3	30	30	4.48
5	4000	3.60	10	4	40	40	4.75
6	4500	3.65	10	5	50	50	5.00
7	5000	3.69	10	7	70	70	5.52
8	5500	3.74	10	9	90	90	6.28
9	6000	3.77	10	10	100	97.5	6.96

Corrected formula for 0% dead = 100 × 0.25/n; for 100% dead = 100 (n – 0.25)/n, where n is the number of animals in each group.

Table 4.3. Determination of LD_{50} of Isolated Compound I after oral administration in mice

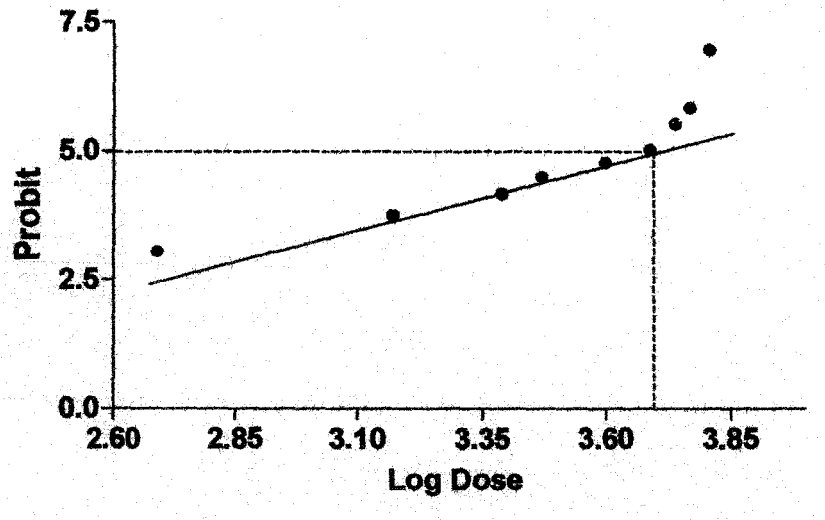
No. of Group	Dose (mg/kg body wt.)	Log dose (X)	No of animals used	No of animals dead	Dead (%)	Corrected dead (%)	Probit (y)
1	150	2.17	4	0	0	6.25	3.45
2	175	2.24	4	1	25	25	4.33
3	225	2.35	4	2	50	50	5.00
4	275	2.43	4	3	75	75	5.67
5	325	2.51	4	4	100	93.75	6.55

Corrected formula for 0% dead = 100 × 0.25/n; for 100% dead = 100 (n – 0.25)/n, where n is the number of animals in each group.

Table 4.4. Determination of LD_{50} of Isolated Compound II after oral administration in mice

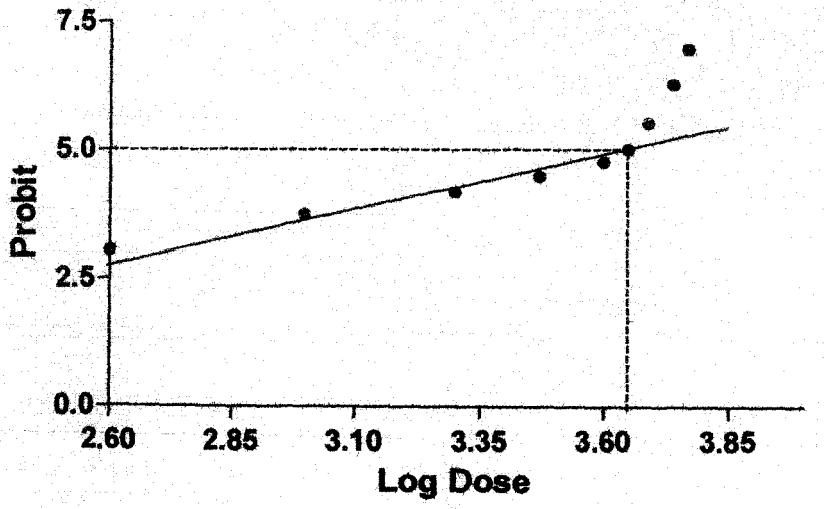
No. of Group	Dose (mg/kg body wt.)	Log dose (X)	No of animals used	No of animals dead	Dead (%)	Corrected dead (%)	Probit (y)
1	150	2.17	4	0	0	6.25	3.45
2	175	2.24	4	1	25	25	4.33
3	200	2.30	4	2	50	50	5.00
4	225	2.35	4	3	75	75	5.67
5	250	2.39	4	4	100	93.75	6.55

Corrected formula for 0% dead = 100 × 0.25/n; for 100% dead = 100 (n – 0.25)/n, where n is the number of animals in each group.



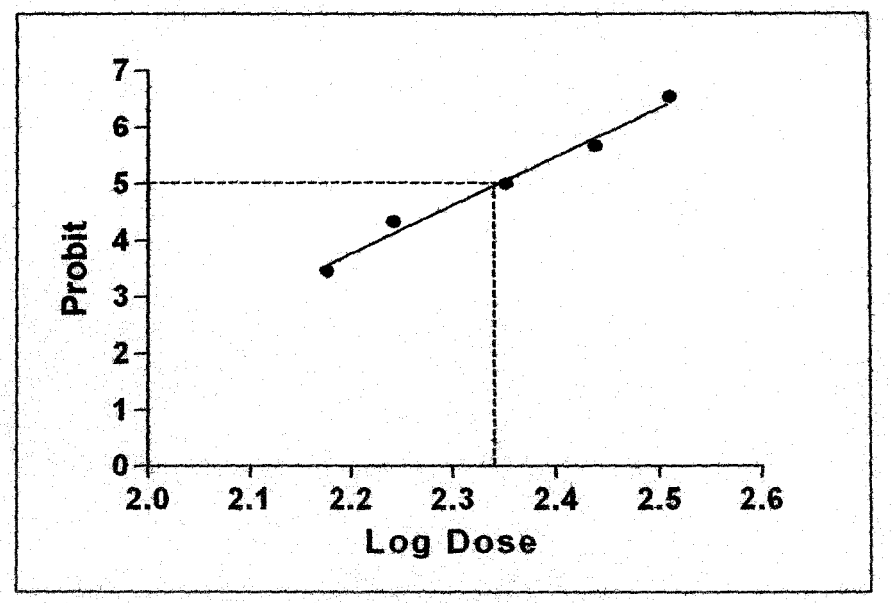
LD_{50} = Log 3.699 = 5000 mg/kg

Fig. 4.1. Probit vs Log dose plot for methanolic extract of *K. rotunda*



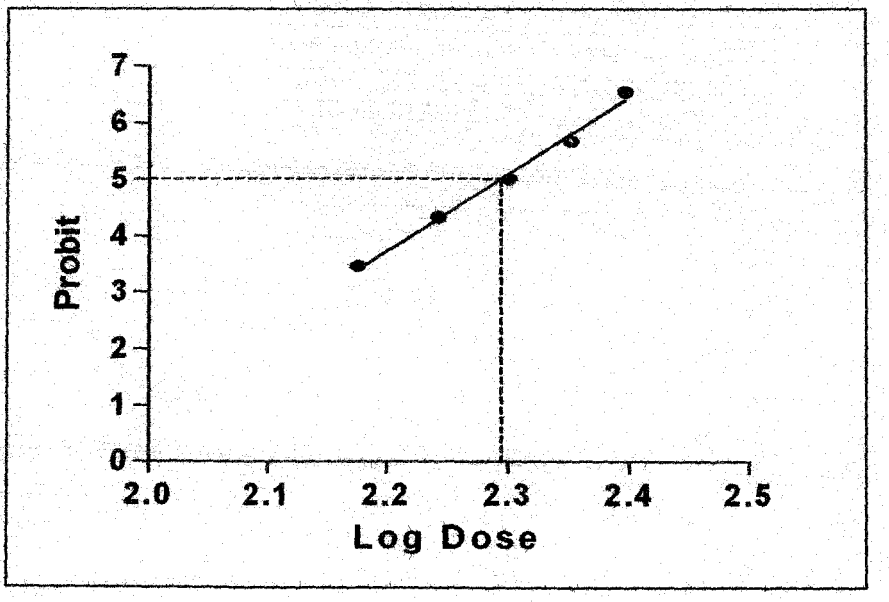
LD_{50} = Log 3.653 = 4497.79 mg/kg

Fig. 4.2. Probit vs Log dose plot for methanolic extract of *E. cannabinum*



LD_{50} = Log 2.33 = 213.7 mg/kg

Fig. 4.3. Probit vs Log dose plot for Compound I



LD_{50} = Log 2.29 = 196.3 mg/kg

Fig. 4.4. Probit vs Log dose plot for Compound II

4.4. References

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