CHAPTER 4

ACUTE TOXICITY STUDY

4.0 INTRODUCTION

Toxicology (Sood 1, 1999) is the study of the adverse effect of the chemicals on living organisms. It is unique form of science because it has more than a single discipline with one objective. It is concerned with the assessment and subsequent management of potential chemical hazards to man, animals, and the environment. In order to achieve this objective, the toxicologist need a detail knowledge, not only a chemical's primary and cumulative toxicity, but also of its 'no observed effect' level, as well as knowledge concerning its' teratogenic, mutagenic and carcinogenic potential.

4.1 Objective

I. Hazard/risk assessment

(A) Detection of causative factor of both acute and chronic illness due to chemicals.

(B) Exclusion or minimization of adverse effects to maintain balance between economic benefits against the risk to protect the manufacturer, worker, consumer, environment and public.

II. Aid the selection and development of therapeutic agents.

III. Aid basic science or Knowledge of life processes.

4.2 Duration of Toxicity Studies

Essentially three types of study have become mandatory in the course of safety evaluation of chemical. These are as follows:

4.2.1 Acute toxicity studies:

Acute studies demonstrate the adverse effects occurring within a short time, usually up to 2 months, following administration of a single dose of a substance or multiple doses given within 24 hr.

4.2.2 Repeated-dose (subacute/subchronic) studies: The definition of subchronic toxicity is confusing, as opinions differ as to the length of exposure that constitutes a sub acute study. However, their purpose is the same– namely to demonstrate adverse effect occurring as a result of repeated daily dose of a chemical for part, not exceeding 10 percent, of the life span of animal. Thus, 14, 21, and 28 day studies in rats are generally referred to as 'subacute' studies, while 90 days and upto 150 days' study constitute 'subchronic' tests.

4.2.3 Chronic toxicity study:

Chronic Toxicity Test or Long term toxicity test is defined as study of longer than 3 months duration, i.e., approximately 10% of life span in the laboratory rats. These types of studies are conducted in all species of laboratory animals and in some economically important animals, wild and domestic. The results of a chronic toxicity study should suggest signs and symptoms of adverse reactions to look for in man. With the exception of idiosyncratic reaction and hypersensitivity, many of the systematic and organismic response are predictable from laboratory animal to man (Hayes, 1982). Ideal duration for different types of toxicity studies are given in the **Table 4.1** (Witthawaskyl *et al.*, 2003).

Table-4.1 Duration for various toxicity tests (OECD guidelines)

TYPE OF STUDY	OECD GUIDELINE	MAX STUDY DURATION
ACUTE TOXICITY STUDY		
Acute oral toxicity study in rats	423	2 Months
Acute dermal toxicity study in rats	402	2 Months
Acute skin irritancy study in rabbits	404	2 Months
Acute oral toxicity study in mice	401	2 Months
Acute inhalation toxicity study in rats	403	2 Months
SUB CHRONIC TOXICITY STUDY		
Repeated Dose-28 Days Sub-acute Oral Toxicity Study in rats	407	5 Months
Repeated Dose-28 Days Sub-acute Oral Toxicity Study in Rabbit	410	5 Months
Repeated Dose-28 Days Sub-acute Oral Toxicity Study in dogs	409	5 Months
CHRONIC TOXICITY STUDY		
Repeated Dose-90 Days Chronic Oral Toxicity Study in rats	408	7 Months
GENOTOXICITY STUDY		
Ames test	471	2 Months
In vitro human lymphocyte	473	2 Months
Micronucleus test in mice	474	4 Months
Chromosomal aberration in mice	475	
In vitro mammalian gene mutation test	476	2 Months
REPRODUCTION		
Teratology/prenatal development in Rats	414	-
Teratology/prenatal development in Rabbits	414	-
CARCINOGENICITY		
Carcinogenicity in rats	451	-
Carcinogenicity in mouse	451	-
Combined chronic carcinogenicity in rats	453	-

4.3 Animal Husbandry and Observations

The most important facet of any toxicological experiment is the condition of the animals. Accordingly, all toxicity studies should be conducted in a controlled environment, at a temperature of 22 ±3 °C with adequate ventilation (i.e. 10 changes of air per hour), relative humidity between 30% and 70 %, and a twelve hours light/dark cycle. The diet and quality of drinking water should be of standard and maintained through out the experiment and this

should be carried out to Good Laboratory Practice standards to ensure reproducibility/validity of data.

4.4 Protocol Design

The protocol design (Sood 2, 1999) depends upon the type of chemical substances and the country in which it is used. Often both sexes of two species, employing a rout of exposure, which is anticipated to be the most probable rout of exposure for man is necessary for regulatory purposes. The laboratory mice and rats are the species typically selected. Additional species are required by some regulations and in these cases, probe studies are often used to select an appropriate dose range and species.

The experimental design for acute systemic toxicity assessment has for many years been a modification of the Travan approach of interval dose levels applied to groups of experimental animals such that an incidence of response can be achieved varying from zero incidence to 100 percent response, and median lethal dose can be derived (LD_{50}). The number of replicates and size of sample population will dictate whether the experimentally derived curve reflect the actual response. A wide variety of intrinsic and extrinsic factors can influence the outcome of a test. Many investigations in to the source of variability of acute toxicity testing have been conducted recently. In order to establish a dose response relationship, the same species / strain, sex and age should be divided in to equivalent size groups, with the different subgroups and the different subgroups treated at the same time of day with different doses by the same route and observed for a set and consistent period of time.

All protocols should state the ceiling or limit doses. Small differences in protocols are probably the major cause of the considerable laboratory to laboratory variations in result achieved (Lorke, 1983). There is some question concerning the utility of extensive pathological assessments as part of an acute study. Gross necropsies are the minimum requested by most regulatory bodies. Protocols include necropsies on all animals found dead and those killed following the two week post-dosing observation period. Body weights are determined on day 1 (prier to dosing), day 7 and day 14, as required by most regulatory guidelines. Animals should not differ in age by more than 15 percent.

Toxicological data help to make decision whether a new drug should be clinically used or not.

4.5 Acute toxicity

Acute toxicity is the toxicity produced by a pharmaceutical when it is administered in one or more doses during a period not exceeding 24 hours. The objective of an acute study are to define the intrinsic toxicity of a chemical, to assess the susceptible species, to identify the target organ of toxicity, to provide information for risk assessment after acute exposure to the chemical, and to provide information for the design and selection of dose levels for more prolonged studies. In the absence of data on the toxicity of a chemical, acute study also help in

formulating safety measure/ monitoring procedures for all workers involved in the development and testing of a chemical. Accordingly, a battery of tests under different conditions and exposure routes should be conducted.

From a regulatory viewpoint, acute toxicity data are essential in the classification, labeling and transportation of chemical (van den Hauvel *et al.*, 1987). Investigation of acute toxicity has led to the identification of selective toxic action and the beneficial use of substances as pesticides in controlling the environment and as drugs for therapeutic use in domesticated animals and man. The most frequent performed test is an acute systemic toxicity assessment, the number of animals used in the protocol designs is far fewer than for chronic studies and form a relatively small proportion of the total number of animals used in experimental studies. Traditionally, the emphasis in these types of studies was on determining the LD_{50}, time to death, the slope of the lethality curve, and the prominent clinical sings; however, non lethal parameters of acute toxicity testing should also be considered. Acute lethality testing designed to determine the amount of a chemical that cause death as the only end point, has come under extensive criticism. Acute toxicity studies have achieved a level of notoriety in the public domain due to the efforts of animals welfare groups. A primary focus has been the 'LD_{50}' test. While acute toxicity generally deals with the adverse effects of single doses, delayed effect may occur due to accumulation of the chemical in tissues or other mechanism, and it is important to identify any potential for these by repeated dose testing. Dosing periods distributed between single dose and 10% of life span doses are often called subacute.

The term 'subchronic' has been use to embrace the toxic effects associated with repeated doses of a chemical over greater than a 10% part of an average life- span of experimental animals.

4.5.1 Objective of acute toxicity test

(i) 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}).

(ii) The 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).

(iii) 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 (Table 4.2). Because of species variation, several species of animals (one rodent and one non-rodent) were used to determine LD_{50}.

(iv) 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 (Ghosh, 1984).

Table 4.2 Surface area ratios of some common laboratory species and man (Paget *et al.*, 1964)

	Mouse 20 g	Rat 200 g	Guinea-pig 400 g	Rabbit 1.5 kg	Cat 2 kg	Monkey 4 kg	Dog 12 kg	Man 70 kg
20 g Mouse	1.0	7.0	12.25	27.8	29.7	64.1	124.2	357.9
200 g Rat	0.14	1.0	1.74	3.9	4.2	9.2	17.8	56.0
400 g Guinea-pig	0.08	0.57	1.0	2.25	2.4	5.2	10.2	31.5
1.5 kg Rabbit	0.04	0.25	0.444	1.0	1.08	2.4	4.5	14.2
2 kg Cat	0.03	0.23	0.41	0.92	1.0	2.2	4.1	13.0
4 kg Monkey	0.016	0.11	0.19	0.42	0.45	1.0	1.9	6.1
12 kg Dog	0.008	0.06	0.10	0.22	0.24	0.52	1.0	3.1
70 kg Man	0.0026	0.018	0.031	0.07	0.076	0.16	0.32	1.0

4.6 Parameters in acute Systemic Toxicity Assessments

Establishing a dose- response relationship for exposures at which the probability of a known fraction of a population of a species under study will show lethality will not the objective of acute systemic toxicity studies. In summary, acute studies establish the following:

- Dose range for subsequent studies.
- Potency, ranking from extreme to non-toxic.
- Identifying probable physiological systems/target organs being affected.
- Extent or degree of effect—e.g. subdued behavior, coma and death.
- Minimal regulatory guideline requirements.

The following illustrates the additional data that can be obtained with appropriate protocol design:

Clinical sign: Time of onset, duration and recovery.

Morbidity: agonal changes; reflexes; pharmacological effects; dose responses curve (ED_{50})

Lethality: dose response (LD_{50} with confidence limit); shape and slop of dose- response curve; estimation of median lethal dose (LD_{50}); estimation of minimum lethal dose (LD_{01}); estimation of certain lethal dose (LD_{100}).

Body weight: decreased body weight gain; body weight loss; reduced food consumption.

Target organ identification: necropsy and gross tissue examination; histological examinations; blood clinical chemistry; hematology.

Physiological function: immunology; neuromuscular reflexes; behavioral screening; electrocardiogram; electroencephalogram.

Pharmacokinetic: therapeutics index; bioavailability (AUC, volume of distribution, half life).

Pharmacodynamics: relationship between plasma and tissue levels and occurrences of clinical sign.

Identification of the probable physiological systems and target organs involve in acute systemic toxicity are important objecting when conducting these types of assessments. Often in the

past, only a small selected amount of data from an acute toxicity study has been reported and recorded on informational databases. The data selected are often only the median lethal dose (LD_{50}), or median lethal concentration (LC_{50}) (Sood 2, 1999).

4.7 LD_{50}

LD stands for "Lethal Dose". LD_{50} is the amount of a material, given all at once, which causes the death of 50% (one half) of a group of test animals. The LD_{50} is one way to measure the short-term poisoning potential (acute toxicity) of a material. Most often acute toxicity test is done with rats and mice. It is usually expressed as the amount of chemical administered (e.g., milligrams) per 100 grams (for smaller animals) or per kilogram (for bigger test subjects) of the body weight of the test animal. The LD_{50} can be found for any route of entry or administration but dermal (applied to the skin) and oral (given by mouth) administration methods are the most common.

4.7.1 LC_{50}

LC stands for "Lethal Concentration". LC values usually refer to the concentration of a chemical in air but in environmental studies it can also mean the concentration of a chemical in water. For inhalation experiments, the concentration of the chemical in air that kills 50% of the test animals in a given time (usually four hours) is the LC_{50} value.

4.7.2 Reason for study of LD_{50} and LC_{50}

Chemicals can have a wide range of effects on our health. Depending on how the chemical will be used, many kinds of toxicity tests may be required. Since different chemicals cause different toxic effects, comparing the toxicity of one with another is hard. We could measure the amount of a chemical that causes kidney damage, for example, but not all chemicals will damage the kidney. We could say that nerve damage is observed when 10 grams of chemical A is administered, and kidney damage is observed when 10 grams of chemical B is administered. However, this information does not tell us if A or B is more toxic because we do not know which damage is more critical or harmful. Therefore, to compare the toxic potency or intensity of different chemicals, researchers must measure the same effect. One way is to carry out lethality testing (the LD_{50} tests) by measuring how much of a chemical is required to cause death. This type of test is also referred to as a "quantal" test because it is measures an effect that "occurs" or "does not occur". The information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity. Acute toxicity studies may also aid in the selection of starting doses for Phase 1 human studies, and provide information relevant to acute overdosing in humans.

4.7.3 Some other toxicity dose terms in common usage

LD_{nt} Lethal dose for 1% of the animal test population
LD_{100} Lethal dose for 100% of the animal test population

LD_{LO} The lowest dose causing lethality

TD_{LO} The lowest dose causing a toxic effect

4.8 Acute toxicity studies

4.8.1 Methods

The bioassays can be conducted according to the World Health Organization guideline for the evaluation of the safety and efficiency of herbal medicines and other drugs (WHO, 1992). The visual observations included skin changes, mobility, aggressiveness, sensitivity to sound and pain, excitation, tremors, twitches, motor coordination, righting reflex as well as respiratory movements and the number of survivors is noted after 24 hr. Also the animals are observed for next 14 days where their weights are recorded. The LD_{50} was then determined at the end of the experiment. The acute toxicity can be evaluated by Lorke method (1983), Turner method, Karber method, Lichtfield and Wilcoxon method (Lichtfield-Wilcoxon, 1949) etc.

4.8.2 Testing Procedures

The test compound should be administered to animals to identify doses causing no adverse effect and doses causing major (life-threatening) toxicity. The use of vehicle control groups should be considered. For compounds with low toxicity, the maximum feasible dose should be administered. Acute toxicity studies in animals should ordinarily be conducted using two routes of drug administration: The route intended for human administration, and intravenous administration, if feasible. When intravenous dosing is proposed in humans, use of this route alone in animal testing is sufficient. Studies should be conducted in at least two mammalian species, including a non rodent species when reasonable. The objectives of acute studies can usually be achieved in rodents using small groups of animals (for instance, three to five rodents per sex per dose). Where non rodent species are appropriate for investigation, use of fewer animals may be considered. Any data providing information on acute effects in non rodent species, including preliminary dose-range finding data for repeat-dose toxicity studies, may be acceptable.

4.8.3 Observation

Animals should be observed for 14 days after pharmaceutical administration. All mortalities, clinical signs, time of onset, duration, and reversibility of toxicity should be recorded. Gross necropsies should be performed on all animals, including those sacrificed moribund, found

dead, or terminated at 14 days. In addition, if acute toxicity studies in animals are to provide the primary safety data supporting single dose safety/kinetic studies in humans (e.g., a study screening multiple analogs to aid in the selection of a lead compound for clinical development), the toxicity studies should be designed to assess dose-response relationships and pharmacokinetics. Clinical pathology and histopathology should be monitored at an early time and at termination (i.e., ideally, for maximum effect and recovery). Corrosive characteristics should not be administered in concentrations that produce severe toxicity solely from local effects.

4.8.4 Karber's method

For calculating LD_{50} by this method, find out the least tolerated (smallest) dose (100% mortality) and most tolerated (highest) dose (0% mortality) by hit and trial method. Once these two doses are determined, select at least 5 doses in between the least tolerated and most tolerated dose, and observe the mortality due to these doses. Generally, Rats are use for this purpose and each dose group should consist of 10 animals. One can determine the LD_{50} value by different rout of administration. The LD_{50} value of a new drug is determined by oral as well as by one of the parenteral routes (ip, iv or im) of administration.

4.8.5 Acute toxicity studies and determination of median lethal dose

Whenever an investigator administers a chemical substance to a biological system, different types of interactions can occur and a series of dose-related responses result. In most cases these responses are desired and useful, but there are a number of other effects which are not advantageous. These may or may not be harmful to the patients. The types of toxicity tests which are routinely performed by pharmaceutical manufactures in the investigation of a new drug involve acute, sub-acute and chronic toxicity. Acute toxicity is involved in estimation of LD_{50} (the dose which has proved to be lethal (causing death) to 50% of the tested group of animals). Determination of acute oral toxicity is usually an initial screening step in the assessment and evaluation of the toxic characteristics of all compounds. In screening drugs, determination of LD_{50} (the dose which has proved to be lethal (causing death) 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 toxic action of a substance; (c) Help arrive at a dose of a new compound; (d) Help in dose determination in animal studies; (e) Help determine LD_{50} values that provide many indices of potential types of drug activity.

4.8.6 Animal selection:

(i) Species and strain – Two species are selected, one rodent and other non-rodent, because species differ in their response to toxic agents. Animals are obtained from random breeding in a closed colony, because the aim was to discover new and unexpected effects of a drug in groups of animals of wider variability or F/1 hybrids of two inbred strains.

(ii) Number and sex of animals – At least five rodents are used at each dose level. They are all of the same sex. After completion of the study in one sex, at least one group of five animals of the other sex is dosed (Ghosh, 1984).

The females are nulliparous and non-pregnant. In acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers may be considered. A drug effect that is seen in say, both a rat and a dog, probably involves a common physiological mechanism that is likely to be present in humans. Whereas an effect seen only in one of the two species indicates that it is peculiar to that species and is less likely to be present in the third species (Ghosh, 1984).

(iii) Age – If a compound is to be administered in infants under six months of age, the LD_{50} values in newborn rats under 24 h of age, are compared with those of mature rats in order to assess any difference in sensitivity due to age.

Assignment of animals – Each animal is assigned a unique identification number. A system to assign animals to test groups and control groups randomly is required.

Housing – Animals are group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal. The biological properties of the test substance or toxic effects (e.g. morbidity, excitability, etc.) may indicate the need for individual caging.

Administration – The compound is administered once (Ghosh, 1984), orally or parenterally, to rats that have been fasted for 18 h.

Dose levels and dose selection: The substance used in the toxicity tests should be as pure as the material eventually to be given to humans. At least three to four dose levels are used, spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose-response curve and permit (Ghosh, 1984; Paget *et al.*, 1964) an acceptable estimation of LD_{50}.

- If the lethality of the groups is such that only one group has a lethality falling between 4 and 6 probits, more groups may be required.
- Solvent – Where necessary, the test substance is dissolved or suspended in a suitable solvent.

Volume: This depends on size of the test animal. In rodents (Ghosh, 1984; Turner 2, 1965) it should not exceed 1 ml/100 g body weight maximum of 50 ml/kg. Injection is given slowly and uniformly. This will avoid undue killing by a drug having predominant action on the CNS/heart (Ghosh, 1984).

Route of administration: The LD_{50} value depends on the route of administration. Usually the values are found to increase with the following sequences of routes: intravenous, intraperitoneal, subcutaneous and oral. The intravenous route is preferable to the intraperitoneal route (because many drugs get detoxified by the liver if the intraperitoneal route is employed).

The signs recorded during acute toxicity studies: These are increased motor activity, anaesthesia, tremors, arching and rolling, clonic convulsions, ptosis, tonic extension, lacrimation, Straub reaction, exophthalmos, pilo-erection, salivation, muscle spasm, opisthotonus, writhing, hyperesthesia, loss of righting reflex, depression, ataxia, stimulation, sedation, blanching, hypnosis, cyanosis and analgesia. After the test, the animal is the sole occupant of the cage, with free access to food and water during the observation period of 1–2 h, and thereafter at intervals.

At the end of the test surviving animals are weighed and sacrificed. A gross necropsy may be performed, all gross pathology changes are observed. If necropsy cannot be performed immediately after the death of the animal it should be refrigerated to minimize autolysis. Necropsies must be performed no later than 16 h after death (Ghosh, 1984).

Before the actual LD_{50} determination, a pilot study also can be done in a small group of animals, to select the dose ranges for the subsequent study.
The maximum non lethal and the minimum lethal doses are thus determined using about minimum number of animals (Ghosh, 1984). Once the approximate LD_{50} or the range between the maximum non lethal and minimum lethal doses is found, a final and more reliable LD_{50} assay can be planned using at least three or four dose levels within this range, with a larger number of animals in each group (Ghosh, 1984).

4.8.7 Arithmetical method of Karber

In this method of acute toxicity study, the interval mean of the number dead in each group of animals are used as well as the difference between doses for the same interval. The product of interval mean and dose difference is obtained. The sum of the product is divided by the number of animals in a group and the resulting quotient is subtracted from the least lethal dose in order to obtain LD_{50} value (Turner, 1965).
LD_{50} = The apparent least dose lethal to all in a group is $\Sigma (a \times b)/ N$

Where, N is the number of animals in each group, 'a' the dose difference and 'b' the mean mortality.

4.9 Subacute toxicity studies

The subacute toxicity studies can be carried out by Lorke (1983) method, Lichtfield and Wilcoxon method, where biochemical estimation of serum as well as of various orgasns, haematological parameters and histopathological changes are observed.

Liver enzymes such as Aspartate aminotransferase (AST), alanine aminotransferase (ALT); alkaline phosphatase (ALP), creatinine, protein, cholesterol, glutathione were assayed using various method used for biochemical estimation. Haematological parameters such as count of white blood cells (WBC), red blood cells (RBC) and platelets; histopathological investigation such as liver, kidney etc can be done with the help of microtomy.

4.10 Regulatory affaires

It is essential that any new method that is considered to be adequately validated as a replacement for an existing method receives as widespread international recognition as possible. For example, the OECD (The Organisation for Economic Cooperation and Development) test guidelines are particularly important in this respect, since they are used for tests conducted in member countries in Europe and North America, and in Japan, Australia and New Zealand. Furthermore, under the OECD Mutual Acceptance of Data Agreement, member countries have agreed to accept data from tests performed according to OECD test guidelines, provided that the principles of Good Laboratory Practice (GLP) are observed. The OECD has established a procedure for updating test guidelines and for the introduction of new test methods (Koeter, 1994). This takes into account both advances in science and proposals that are based on animal welfare considerations.

According to Schorderet (1992), subtances with LD_{50} values greater than 5 g/kg of body weight are considered to show low toxicity. According to the OECD guideline, if an acute toxicity test at one dose level of at least 500 mg/kg body weight produced no observable toxic effects (Witthawaskyl et al., 2003), the full study at a dose of 1000 mg/kg given once daily for 14 days can be used to evaluate subacute toxicity.

4.11 MATERIALS AND METHODS

4.11.1 Preparation of extract

The plant material used in this study i.e. leaves of *Urtica parviflora (U. parviflora), Callicarpa arborea (C. arborea)* and root bark of *Morinda citrifolia (M. citrifolia)* were collected at Majhitar, East Sikkim and were authenticated by Botanical Survey of India (BSI), Gangtok, Sikkim and the herbaria were preserved in the institutional museum (HPI / PK/ No. 131, 132 and 133).

The leaves of *U. parviflora* and *C. arborea,* free from dirt were separated and shade dried for ten days and made to powder by a mechanical grinder. The powdered drugs (500g) were

extracted with ethanol by continuous hot extraction process (soxhelation). The solvent was recovered and the extracts were concentrated under reduced pressure. In case of *Morinda citrifolia* the clean roots are shade dried for twenty days and then subjected to soxhelation using ethanol as the solvent. The extract yield was found to be 5% for *U. parviflora,* 7.5% for *C. arborea* and 11.0% for *M. citrifolia.*

4.11.2 Animal stock

Adult albino rats (Wistar strain) weighing 160-200g were used in this study. All the animals were housed in a cross-ventilated room (22 ± 2.5°C), 12h light 12h dark cycle, fed with standard Local animal feed and water *ad libitum.* The animal studies were approved by Institutional Animal Ethics Committee IAEC No. HPI/07/60/IAEC/0005.

4.11.3 Acute toxicity study

The animals were divided into five groups of six rats per cage. The plant drugs were made in to slurry by adding Sodim Carboxy Methyl Cellulose (q.s) and fed orally with the help of animal feeder needle to each animal. The animals were kept fasted for 18 hrs before the experimentation. The doses were administered once to the animals. After administration, the animals were observed for next two hours for appearance of any sign and symptoms and then observed at the end of the 24^{th} hr. The animals dead or alive were counted and the data was tabulated. The LD_{50} was determined by the formula LD_{50} = Maximum given dose – (Maximum product / N). Before the experimentation a pilot study was performed to know the nearby doses to LD_{50}. The procedure was repeated for different extracts.

4.12 Results of the acute toxicity study

The results of acute toxicity study of the ethanolic leaf extract of *U. parviflora* and *C. arborea* in rat is presented in **Table 4.3** and **4.4** respectively. The result of the acute toxicity of ethanolic root extract of *M. citrifolia* is presented in **Table 4.5**. The LD_{50} (Median Lethal Dose) of orally administered ethanolic extracts of leaves of *U.parviflora, C. arborea* and root bark of *M. citrifolia* was found to be **3500 mg/kg, 1666.67 mg/kg** and **950 mg/kg** body weight per oral respectively.

Table 4.3 LD_{50} Determination by Karber's method of the alcoholic extracts of *U. parviflora*

Sl. No	Dose mg/kg	No. of animals	Dose difference (a)	Mortality	Mean Mortality (b)	Product (a × b)
1	1050	6	0	0	0	0
2	2100	6	1050	1	0.5	525
3	3150	6	1050	2	1.5	1575
4	4200	6	1050	4	3	3150
5	5250	6	1050	6	5	5250
TOTAL						10500

LD_{50} = Maximum given dose – (Maximum product / N)

=5250-(10500/6)

= 3500 mg/kg

Where N is the number of animals in each group, "a" is the dose difference and "b", the mean mortality.

Table 4.4 LD_{50} Detrmination by Karber's method of the alcoholic extracts of *C. arborea*

Sl. No	Dose mg/kg	No. of animals	Dose difference (a)	Mortality	Mean Mortality (b)	Product (a × b)
1	500	6	0	0	0	0
2	1000	6	500	1	0.5	250
3	1500	6	500	2	1.5	750
4	2000	6	500	4	3	1500
5	2500	6	500	6	5	2500
TOTAL						5000

LD_{50} = Maximum given dose – (Maximum product / N)

=2500-(5000/6)

= 1666.67 mg/kg

Where N, is the number of animals in each group, "a" is the dose difference and "b", the mean mortality.

Table 4.5 LD_{50} Detrmination by Karber's method of the alcoholic extracts of *M. citrifolia*

Sl. No	Dose mg/kg	No. of animals	Dose difference (a)	Mortality	Mean Mortality (b)	Product (a × b)
1	650	6	0	0	0	0
2	1300	6	650	1	0.5	325
3	1950	6	650	2	1.5	975
4	2600	6	650	4	3	1950
5	3250	6	650	6	5	3250
TOTAL						6500

LD_{50} = Maximum given dose – (Maximum product / N)

=3250 -(6500/6)

= 950 mg/kg

Where N, is the number of animals in each group, "a" is the dose difference and "b", the mean mortality.

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