IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 15, Issue 2 Ser. I (Mar –Apr 2020), PP 25-32
www.Iosrjournals.Org

Mini Review:Toxicity Study Of Plant Extracts

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Abstract: *Treatment of diseasesusing plant-based medicines hadincreased significantly which waspredicted due to its fewer side effects. However, some of the medicinal plantsmight not be scientifically tested, hence their side effects remainunknown. It is necessary to carry out a toxicity study to ensure the safety ofthe plants in the animal models.This review article aims to provide comprehensive information about the toxicity study of medicinal plants. Various factors could influence the results of the assay,starting from the harvesting of the plants,the drying, the plant extraction, the determining of doses used, the animals housing and feeding conditions, the measurement of body and organ weight, the serum preparation and organ isolation, biochemical, hematology, and histopathology parameters.The most influential factor in toxicity study is the dose of the plant extracts.*

Keywords: ***acutetoxicity,sub-chronictoxicity.***

Date of Submission: 28-02-2020 Date of Acceptance: 13-03-2020

I. Introduction

Plant-based medicines have been utilized to prevent, diagnose, and treat various diseases. This therapy has been carried out for the past 20 years because it is more comfortable to access multiple regions. However, the use of doses in traditional medicine has not been scientifically studied with certainty. Therefore, toxicity testing must be done to ensure the safety of the plant extracts.

Exposure of a substance in humans can be studied by observing the cumulative effects and doses that cause toxicity such as carcinogenic, mutagenic, teratogenic, and others (OECD, 2014). Toxicity testing is essential to estimate the level of damage caused by compounds to biological and non-biological materials. This test is usually carried out on prospective products to develop new drugs and to determine the therapeutic potential of a drug molecule. Toxicity testing is generally intended to determine the unwanted effects of a drug, especially in the event of cancer, heart problems, and skin or eye irritation (Parasuraman, 2011).This review article provides comprehensive information that focuses on the obstacles encountered in toxicity testing.

II. Methods

References were obtained from the GoogleScholar database using keywords “Pitfalls” AND “Toxicity” AND “Acute” AND “Sub-chronic” AND “Plants Extract”; PubMed using keywords(("toxicity"[Subheading] OR "toxicity"[All Fields]) AND ("plants"[MeSH Terms] OR "plants"[All Fields]) AND extract[All Fields]) AND (Clinical Trial ptyp] AND (hasabstract[text] AND "latterfree full text"[sb])); ScienceDirect using the keywords "Toxicity" AND "Acute"AND”Sub-Chronic” AND “Plants Extract”. The following exclusion criteria were applied: articles < the year 2000, articles that were not in English, a study in cells, non-animal study, and clinical study. The flowchart of the literature search can be seen in Figure 1.

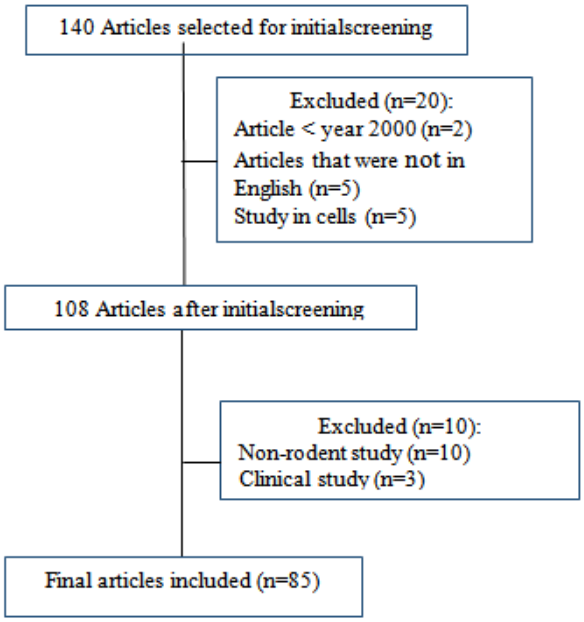

Figure 1. Flowchart of the literature search

ACUTE TOXICITY TEST

Acute toxicity testing is carried out to determine the effect of a single dose on certain test animal species. In general, critical toxicity testing was carried out on two different test species, namely rodent species and not rodents.

The principle of this method is the calculation of LD50 which does not have to be precise, but the determination of the range of toxicity has been determined where the mortality rate is estimated to occur because the death of some animals is the primary endpoint of this test. This method for determining LD50 values at two doses result in mortality higher than 0% and lower than 100% (Guideline, Testing, and Chemicals, 2001).

In the acute toxicology test, what observed is the administration of test preparations at different dosage levels, and the effect is found within 14 days. All mortality caused by the test preparation during the trial period were recorded and observed morphological, biochemical, pathological, and histological changes in the dead test animals. Acute toxicity testing allows 50% lethal dose (LD50) from the test preparation. LD50 is used as an indicator of previous acute toxicity. The determination of LD50 involves most animals, and the mortality ratio in animals is high. Because of this limitation, the method was modified into a fixed-dose procedure (FDP), the acute toxic category (ATC) method, the up-and-down (UDP) method (Parasuraman, 2011).

The fixed-dose procedure (FDP)

The extract was administered orally in a group with ten animals (5 females and five males) that had been fasted for 12 hours. The control group was given distilled water. After being treated, animals were observed in the first 12 hours, then 15, 30 and 60 minutes and every 4 hours. General toxic signs are observed and recorded. After this stage, animals were followed for 14 days and weighed on days 0, 7 and 14 days after treatment. Changes in cumulative weight (%) are calculated based on the initial load. At the end of the test, the number of deaths recorded and living animals were euthanized and autopsied (OECD, 2001). The macroscopic characteristics observed were the lungs, kidneys, intestines, liver, uterus, spleen and heart. Besides, blood was taken to be followed by the analysis of biochemical and hematological parameters (OECD, 2014).

Acute Toxicity Testing for Repeated Doses

This repeated dose toxicity test was carried out for 28 days. The test preparation is given daily for a certain period with the oral administration route. If it cannot be done through an oral administration route, the administration of the test preparation can be given parenterally. This treatment is offered regularly at the specified time. Usually, mice used male sex and ages 5-6 weeks are used for repeated dose toxicity testing. There must be a small individual variation between animals as the variation in weight of animals allowed is ± 20% (Parasuraman, 2011). The test preparation is given once a day for 28 days and observed for body weight, drinking water, and food intake every two days. Changes in cumulative weight (%) are calculated based on the initial load. At the end of the observation, it was observed the macroscopic characteristics of test animals such

as lung organs, kidneys, liver, spleen, and heart. Besides, blood is taken to analyze biochemical and hematological parameters (Wolff *et al*., 2019).

SUBCHRONIC TOXICITY TEST

The principle of thesubchronic toxicity test is an assay with various levels of concentration of the dose given daily to several groups of animals for 28 or 90 days, and if needed, a satellite group is added to see whether or not the effect is delayed or a reversible effect. During the treatment period animals are observed everyday to determine toxicity. Animals that die during testing if they have not passed rigor mortis (rigid) immediately at autopsy, organs and tissues are observed macro pathologically and histopathology. At the end of the test, all surviving animals were autopsied and then followed with macroecology in each organ and fabric, as well as the examination of hematological parameters, clinical biochemistry, and histopathology. The purpose of this sub-chronic toxicity is to obtain information about the presence or absence of undetectable toxic effects in acute toxicity. The data is likely after repeated exposure to the prescribed test period in a specified time, information that does not cause toxic effects (No Observed adverse effect level / NOAEL) and study the cumulative impact and reversibility effects of the test preparation (OECD, 2014).

IMPORTANT FACTORS OF TOXICITY STUDY

I. Extract preparation

The preparation of the plant extract should consider several aspects, *i.e*. the harvesting age of the plant, the physicochemical properties of the bioactive chemical constituents, the extraction solvent, the extraction method (heat percolation or cold maceration), etc. Several extract preparation examples are provided below:

1. *Curcuma rhizome*

This part of the plant is usually harvested at the age of 4 weeks, washed, and diced to a smaller size and then dried to a constant weight at 60°C. The dried samples are ground to powder and stored in a clean airtight container. About 0.5 g accurately weighed of each sample is added with 25 mL of 80% methanol and refluxedfor 2 hours (Alafiatayo*et al*., 2019).

2. *Pipercernuum*leaves

This part of the plant is dried at 40°C for 7 days and ground to powder. About 250 g of the powder is macerated consecutively with 90% ethanol (ratio of 1:10) at room temperature for 4 hours (Wolff *et al*., 2019).

3. *Reinwardtia indica*leaves

This part of the plant is dried with air at room temperature until constant weight and extracted in hydro-alcohol solvent (70:30) by heat percolation using a soxhlet tool or cold macerated in water. The resulted extract is then filtered, followed by evaporation to dry at 45-60°C (Upadhyay, Shukla and Kumar, 2019; Ugwah-oguejiofor and Ogbonna, 2019).

II. Doses

In general toxicity testing, the test preparation should be given in the varied dosage range. If the sample is liquid, itis not necessary to be dissolved. The maximum volume of administration of preparations in the form of liquid that can be given based on the size of the test animal used. In mice, the volume of administration should not exceed 1 mL/100 g of body weight. However, the dose must include a treatment that is equivalent to the usual dose of use in humans (Wolff et al., 2018).

III. Animals

Feedingcondition

Rodent species, *i.e.* male and female Wistar rats (SPF class) are required to be usedin sub-chronic toxicity study, while Swiss-Webster mice are often used in acute toxicity study. Animals are adapted for 14 days before testing,during this period, the animals are given sufficient standard pellet feed and clean drink water(PeraturanKepala Badan PengawasObatdan MakananRepublik Indonesia, 2014; Wang *et al.,* 2019; OECD, 2014).

Housing condition

All animals should be placed in standard environmental conditions, *i.e.*, 12 hours cycle of dark and light at room temperature and relative humidity at 25 ± 1°C (Xu *et al*., 2019). Animals placed in the metabolic enclosure are placed in well-ventilated cages with optimal temperature conditions of 22 ± 2°C (Wolff *et al*., 2019), 23 ± 1°C (Ashafa, 2010), 23 ± 2°C (Prabhatm, 2019; Mabrouk, 2018), 25 ± 2°C (Ugwah-oguejiofor and Ogbonna, 2019). Irradiation 12 hours of natural light and 12 hours of darkness, the humidity of 45–50%, cleaning of cages should be done daily (Ashafa, 2010).

The relative humidity is at least 30% and does not exceed 70%, except during room cleaning the room humidity must be 50-60%. For feeding, regular laboratory diets for animals are given unlimited drinking water.

Animals can be grouped in groups according to dosage, but the number of animals per cage should not interfere with the observations of each animal (OECD, 2014).

Measurement of Body and Organ Weight

All animals must be weighed at least once a week. Analysis of food consumption must be done at least every week. If the test preparation is given through drinking water, water consumption must also be measured at least every week (OECD, 2014).Animals should be quickly dissected and the organs, including the liver, kidneys, spleen heart, lungs, are separated from the fat, cleaned with a clean tissue and then weighed and isolated for histological studies. The ratio of organs to body weight is determined by comparing the weight of each organ with the final body weight of each animal (Ashafa, 2010; Omotayo*et al*., 2012; Li *et al.*, 2014; Wolff *et al.*, 2019; Upadhyay, Shukla and Kumar, 2019).

Serum Preparation

The animalsshould be sacrificed according to the Code of Ethics.The animals should be anesthetized and sacrificed in the neck area using a sterile sharp scalpel and one aliquot (5 mL) of the blood is collected and centrifuged for 5 minutes. The resulting serum is carefully sucked using the Pasteur pipette into the sample bottle for clinical, biochemical testing (Ashafa, 2010; Omotayo*et al*., 2012).

Biochemical Parameters

The serum is separated from heparinized blood plasma, and serum biochemical parameters include alanine aminotransferase (ALT), aspartate aminotransferase (AST) analyzed using a commercial Spectrum diagnostic kit (Aboutaleb *et al*., 2019). Blood for clinical, biochemical analysis is placed in empty bottles to avoid hemolysis of blood cells. Blood serum obtained from the centrifugation of blood samples. Clinical, biochemical studies use a standard method for aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, total protein, albumin, urea, and creatinine. Serum electrolytes (sodium, potassium, chloride and bicarbonate ions) are estimated to use an automatic ion-selective electrode machine (Audicomelectrolyte analyzer)(Ugwah-oguejiofor and Ogbonna, 2019; Uyoyoghene, 2019).

Hematology Parameters

Samples for hematological testing are put into bottles containing anticoagulants, ethylene diamine tetra-acetic acid (EDTA). The hematological analysis is performed using an automated hematology analyzer. The parameters analyzed includes total and differential leukocytes (WBC), erythrocytes (RBC), hemoglobin (Hb), hematocrit (HCT), platelet count (PLT), average blood cell volume (MCV), mean hemoglobin cell (MCH), red distribution width (MCH), red distribution width (RDW), means platelet volume (MPV) and platelet distribution width (OECD, 2014). Blood samples are collected in Vacutainer® tubes without additives or contain EDTA. The hematological analysis is carried out by cell counters. For biochemical analysis, determination of creatinine and urea by colorimetric testing with Labtest® kit (Wolff *et al*., 2018). Creatinine, urea, acid calcium vein, total bilirubin, total protein, albumin, and globulin, and activity of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT) determined in serum follow the procedure described in the kit (Ashafa, Sunmonu, and Afolayan, 2010).

Histopathology Parameters

Dehydrated tissue in alcohol, cleaned using xylene and put in paraffin wax at 60°C. The serial portion (5 mm thick) is obtained by cutting the tissue in the microtome, mounted on 3-aminopropyl trisilane. Layered slides are dried for 24 hours at 37°C. (Baravalle*et al*., 2006). The parts on the slide are deputed with xylene and hydrated in alcohol. Then the tissue was stained with hematoxylin and eosin dyes, then dried and mounted on a light microscope (X40, 100 and 200) for histological examination (Ugwah-oguejiofor and Ogbonna, 2019). At the end of the acute and sub-chronic trial, after euthanasia, the heart, spleen, lungs, kidneys and liver organs were removed, then dried with gauze, and weighed always. For each organ, the relative weight was calculated as the weight of large rat organs on the 100% sacrifice day (Wolff *et al*., 2019). Liver, kidney, and heart tissue samples were taken for a histological examination performed after going through the process of fixation, dehydration, cleaning, infiltration, planting, cutting and coloring to ensure proper fixation, the tissue was trimmed with a thickness of about 5 mm so that it got the good obsession. The masses are then fixed in 10% saline formol and then transferred to alcohol 50%, 70%, 80%, 85%, 95 and 100% for two hours (Saiprasanna*et al.*, 2017).

Table 1. Toxicity Studies of Plant Extracts

No	Name of plants	Important Factor	Assay Method	Reference
1.	Hydroalcoholic extract of *Piper cernuum*Vell. (Piperaceae)	Preparation of extract, weighing of Wistar rat, relative humidity	In vitro and In vivo	(Wolff *et al.*, 2018)
2.	Extract of Kava kava (*Piper methysticum*)	-	Ultra High-Performance Liquid Chromatography (UHPLC) and (SFE), Cell viability assays	(Petersen, Tang, and Fields, 2019)
3.	Hydro-Alcoholic Leaves Extract of *Reinwardtiaindica* in rats	Temperature room, preparation of extracts, dosage.	Hematological test, Histopathology	(Upadhyay, Shukla and Kumar, 2019)
5.	*Juglans regia green* husk extract	-	Cytotoxicity assay, X-Ray diffraction analysis	(Izadiyan *et al.*, 2018)
6.	Aqueous Extract of Aerial Parts Of *CarallumaDalzielii N. E. Brown*	Preparation of plant extract, animal weighing, Housing conditions, histopathologic	In vitro and In vivo	(Ugwah-oguejiofor and Ogbonna, 2019)
7.	Extracts of Bulbus *Fritillaria cirrhosis* and Bulbus *Fritillaria Pallidiflora*	Preparation of plant extract, animal weighing, humidity and temperature in the laboratory, dosage level,	In Vitro and In Vivo	(Xu *et al.*, 2019)
8.	Extract roots of *R. turkestanicumJanisch*	Preparation of plant extract, anti-oxidant potential,	Lipid peroxidation assay, Measurement of total thiol content, Histopathology studies	(Boroushaki *et al.*, 2018)
9.	Extracts from seeds of *Peganum harmala* Linn	Dosage	The sub-chronic toxicity and concomitant toxicokinetics of TAEP	(Wang *et al.*, 2019)
10.	Extract Of*Alysicarpusovalifolius*	Extract preparation, housing and feeding conditions, dosage, organ isolation,	Lorke's method	(Area, 2019)
11.	Leaf Extract of *BrachystegiaEurycoma*	Animal, extract preparation, dosage,	In vivo	(Ududua, Monanu, and Chuku, 2019)
12.	*Anacardium occidentale*Linn (Anacardiaceae) leaves hexane extract	Dosage, preparation extract	In vivo and In vitro	(Cam and Traditional, 2007)
13.	*Saussurealappa* root aqueous extract	Dosage	Hormone assay, Sperms morphometric analysis, Assessment of spermatozoa morphological Abnormalities, Comet assay, Histopathological examination,	(Attia *et al.*, 2018)
14.	The root bark extract of *Calliandraportoricensis* (Jacq.) Benth	Measurement body and organ weight, dosage.	Fingerprinting of methanol extract of CP by Gas Chromatography-Mass Spectrometry (GCMS)	(Adefisan and Owumi, 2018)
15.	Berry extracts of *Phytolaccadioica* L.	Animals, dosage, serum preparation, and organ isolation	In vivo and In vitro	(Ashafa, Sunmonu, and Afolayan, 2010)
16.	Ethanolic extract of *Azadirachtaindica*	Dosage,	In vivo	(Omotayo *et al.*, 2012)

III. Conclusion And Future Perspectives

This review article describes acute and sub-chronic toxicity tests in vivo and in vitro from various literature. In the toxicity test, there are various obstacles in the process. Among them are at the stage of the

extraction process starting from the selection of plants such as the part of the plant used in the test is very influential on the final results, then the process of preparing the dried plants. Obstacle factors in animals also need to be considered, namely species and sex of test animals used in toxicity test: animals used must be sensitive, healthy, species and sex must be explicit, age and weight must be suitable, for example, for mice with a minimum weight of 20 grams with an age range of 6-8 weeks, whereas for rats with a minimum weight of 120 grams and age range 6-8 minutes (OECD, 2014). The condition of the cage must be kept clean, the cage should be set at a temperature of 22 ° ± 3 ° C, with a relative humidity of 30-70%, and a cycleof 12 hours light 12 hours dark. According to the Cage Space Guidelines For Animals Used In Biomedical Research (2008), the area of the cage for mice is 77.4 cm2, 12.7 cm high, while the base of the cage for mice is 148.4 cm2, height 17.8 cm (OECD, 2014). Animals are grouped according to the test dosage preparation to facilitate observation.

The most significant factor in this test is that the dose of extract used for the test preparation for toxicity testing is the main parameter. Where the purpose of this toxicity test is to provide information on the harmful effects if the test preparation is given to humans so that a safe dose can be determined. Therefore the dose used must be equivalent to the general dose in humans. The dosage includes fixed multiplication which contains the general dose in humans until the dose required for the highest test or dose that can be given to the test animal, where the acute toxicity criteria based on dosage are ≤ 1 mg/kg very toxic, 1-50 mg toxic, 50-500 mg moderate toxic, 500-5000 mg mildly toxic, 5-15 g practically non-toxic. If up to a dose of 5000 mg/kg BB (in mice) does not cause toxic effects such as a death in animals, then the test does not need to be continued by using higher doses.

Other parameters observed were evaluating the relationship between toxic effects and doses used in each group of animals such as changes in body weight, clinical symptoms, hematology parameters, clinical biochemistry, histopathology, and other general effects or specific effects.

Acknowledgments

The author wishes to thank the Rector ofUniversitasPadjadjaran for funding the submission fee of this article via the UniversitasPadjadjaranInternalGrant.

CONFLICT OF INTEREST

None declared.

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YuniartiFalya, etal. "Mini Review:Toxicity Study Of Plant Extracts". *IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)*, 15(2), (2020): pp. 25-32.