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Toxicity Protocols for Natural Products in the Drug Development Process

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Poisonous Plants and Phytochemicals in Drug Discovery, First Edition.
Edited by Andrew G. Mtewa, Chukwuebuka Egbuna, and G.M. Narasimha Rao.

9.1 Introduction

Natural products are less toxic than synthetic compounds because of the adaption of humans and animals through exposure over time, which has enabled them to develop detoxification mechanisms [1]. Phytochemicals are extracted from plants and contain many interrelated chemical compounds that may have different pharmacological effects, when the whole plant preparation is used. Although occurring rarely compared with synthetic compounds, adverse effects of natural products have been reported in the literature [2]. Therefore, toxicity studies are necessary for medicinal products derived from natural products before humans are exposed to them.

In vitro studies, which are conducted outside the intact organism, provide important tools to develop our understanding of the hazardous effects of natural products and to enable us to predict these effects in humans. They are widely used for screening purposes [3]. Before testing the toxicity of a herbal product, the complexity of the herbal material should be considered. The product naming system (botanical, common, pharmaceutical name, or herbal drug name), botanical identity, and the relevant part of the herb to be tested should also be considered before testing [4].

9.2 In Vitro Toxicity Testing for Natural Products

Concerns for the welfare of animals have resulted in alternative methods being used for toxicological testing. In addition, the limited predictive capacity of in vivo testing for acute toxicity and the requirement for large quantities of test substance have encouraged the use of in vitro toxicity testing [5]. The three Rs method was designed to reduce unnecessary exposure of animals to experimental products. These are reduction (use the least number of animals for toxicological tests that provide full results), refinement (to improve animal research to reduce or eliminate pain and discomfort), and replacement (use of alternative toxicological tests

that do not involve the intact animal). This approach calls for alternative approaches to reduce the use of animals and replace them with in vitro toxicological testing [6]. In vitro studies can also be used to determine the mechanisms of toxicity at the cellular level, thereby allowing possible interventions with therapeutic or antidotal treatment [6]. The procedure of liver perfusion to isolate viable rat hepatocytes is used widely in toxicology testing [7]. The liver is the main target organ that is responsible for detoxifying toxic substances, and the development of this procedure has in vitro studies of toxicity. In vitro studies are a cornerstone of drug discovery and are widely applied for natural products [4].

9.2.1 Cell Culture Method for Toxicity Testing

The cell culture method was developed many years ago. Early attempts at tissue culture were carried out in the USA when scientists removed tissue explants from animals and allowed them to adhere to glass coverslips or put them in capillary tubes in clots formed from lymph or plasma. Following this, synthetic media, such as those formulated by Earle, Parker, and Eagle, were developed with different serum additives to support the growth of cells [8]. These early studies had a drawback of contamination with bacteria and fungi, which outpaced the growth of mammalian cells because of their rapid rate of mitosis. This was also addressed by the addition of liquid antibiotics to the media as well as the development of better aseptic methods, such as the use of laminar airflow hoods, autoclaves, and sterile disposable glassware, which reduced the requirement for antibiotics [9].

In vitro studies have been further modified by the development of accepted protocols, such as chemically defined culture media, the introduction of porous membranes, and filter inserts, which allow the passage of low-molecular-weight soluble substances. The use of biological safety cabinets (class II) reduced contamination of cells by microorganisms. These cabinets have a unidirectional airflow that separates the working area from the environment by blowing sterile air over the surface of the working area. Most of the supplies and plastic currently used for handling cell cultures are sterile and disposable, which reduces microbiological contamination [9].

In order to grow, cells need a liquid culture medium with defined components. The medium is usually composed of a buffered solution with physiological ion concentrations containing soluble amino acids, carbohydrates, vitamins, minerals, fatty acids, and other cofactors. Optional ingredients include a pH indicator, separate buffering systems such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and some non-essential amino acids that are incorporated when required by a particular cell type [9, 10].

The most commonly used media for cell culture are modified Eagle's medium, basal medium Eagle, Dulbecco's modified Eagle's medium, and Ham's F12

medium. These media are designed to be used with serum or serum proteins [8]. Serum (5–20%) is added to the medium formulation to promote cellular proliferation, and a balanced salt solution is used for irrigation, transport, washing, diluting fluids, and maintaining intracellular and extracellular osmotic pressure. The salt solution can also be supplemented with glucose to provide energy for cell metabolism during the washing procedure. The most commonly used prepared salt solutions include Dulbecco's phosphate-buffered saline, Earle's balanced salt solution, and Hanks' balanced salt solution [9, 11].

Most mammalian cells proliferate and differentiate at 37 °C [11]. A temperature higher than 39 °C may stimulate heat shock, which can irreversibly inhibit biological function. However, cells can tolerate falls in temperature; for example, falls up to 4 °C can reduce proliferation and differentiation but do not irreversibly affect biological function [9]. Another factor that affects cell proliferation is pH. A pH of 7.2–7.4 supports optimum cell growth. Rapidly growing cells release more acidic metabolic waste products, decrease the pH of the medium very rapidly, and need frequent washing or the addition of a buffering agent [11].

Most culture media contain bicarbonate as the buffering agent to avoid large and rapid changes in pH. To maintain an equilibrium concentration between the bicarbonate and carbonic acid, CO_2 supply needs to be controlled. This is because soluble CO_2 evaporates from the solution, thereby disturbing the equilibrium between carbonic acid and bicarbonate at 37 °C. Maintaining an increased partial pressure of CO_2 in the gas phase above the liquid is necessary to maintain this equilibrium. However, at room temperature and standard incubator pressures, bicarbonate and carbonic acid are in equilibrium. Many laboratories incorporate organic buffers such as HEPES in medium formulations to prevent pH changes when cultures are removed from a CO_2 incubator [12]. Water is also a fundamental requirement in cell culture and the quality of water used in the preparation of media and salt solutions needs to be considered. The contaminants in water, such as trace metals, divalent cations such as magnesium and calcium, and metabolic products of microorganisms, can interfere with cell growth and functional processes [9].

9.2.2 Cell Culture for Acute Toxicology Testing

The measurement of viable and dead cells in culture has a long history in toxicology [13]. Some indicators of toxicity are used to detect the effects of different natural products by measuring the number of cells that have intact membranes per unit volume to demonstrate toxic endpoints. Some indicators, for instance neutral red uptake, detect the fraction of cells with intact membranes, whereas 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is used to measure the metabolism of surviving cells [14].

The inhibition of cell proliferation is a sensitive indicator for the cellular response to the effects of a natural product, especially when coupled with measurements of metabolism. Simultaneous measurements of proliferation and viability are standard indicators of cell integrity. Together with the data from metabolic experiments, they contribute significantly to the ability of a cell culture system to predict or screen for toxicity. Altered cell proliferation, in which the effects of a natural product on the ability of cells to replicate are measured, serves as an indicator of toxicity. This is measured by the median inhibitory dose (IC_{50}), which is the concentration of the test substance at which 50% of the cells do not multiply. Cell proliferation is measured by cell counting, DNA content, protein content, and enzyme activity. Cell viability is another general index of toxicity. This endpoint is measured by using vital stains such as trypan, which enters only the compromised membranes of dead cells, and neutral red uptake, which is actively absorbed by living cells [9].

9.3 Methods Used for In Vitro Toxicity Studies

9.3.1 MTT Assay

Viable cells could be measured by using several staining methods. MTT is a water-soluble yellow dye that is absorbed by viable cells. The MTT assay is a colorimetric assay used for measuring only living cells. A tetrazolium ring is cleaved in mitochondria that are active, that is, this occurs only in living cells. MTT is absorbed into the cells and undergoes a reduction in a mitochondrion-dependent reaction to give a formazan metabolite. The formazan product accumulates within cells because it cannot pass through intact cell membranes. Dimethylsulfoxide, isopropanol, or another suitable solvent is used to solubilize the formazan product and release it from intracellular stores. The released product can be readily quantified calorimetrically. The quantity of reduced MTT is proportional to cell viability because MTT is only reduced by viable cells [9, 15].

9.3.2 Neutral Red Uptake Assay

This assay estimates the number of cells deemed viable in a culture. It is one of the most used cytotoxicity tests and has a wide range of environmental and biomedical applications based on the ability of living cells to bind to dyes such as neutral red in lysosomes. Neutral red uptake is dependent on the cell's ability to maintain a pH gradient. The dye is best absorbed at physiological pH because of the net charge of approximately zero. After uptake the lower pH inside the lysosome results in the dye becoming charged and, therefore, retained in the lysosome.

Most primary cells and cell lines from diverse origins may be successfully used in this assay. Cells are seeded in 96-well tissue culture plates and allowed to adhere for 24 hours. The plates are then incubated for 2 hours with a medium containing neutral red. The cells are washed, and then the dye is extracted in each of the reaction wells. Absorbance is determined directly by reading the specific wavelength of absorption on a spectrophotometer. This procedure is relatively more sensitive and cheaper than other cytotoxicity tests, such as those that involve enzyme leakage, tetrazolium salts, or protein content. This assay has a good throughput and can be completed in 3 hours [3, 16].

9.3.3 Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) is an enzyme that is released into the cytoplasm during cell lysis. It is a colorimetric assay that is based on the conversion of lactate to pyruvate. The level of LDH is higher in damaged cells than in normal cells. The H^+ ions formed during the reduction of nicotinamide adenine dinucleotide (NAD) to reduced NAD (NADH) catalyze the reduction reaction of the tetrazolium salt (INT) to give the colored formazan compound. The amount of formazan compound formed is directly proportional to the activity of LDH in the sample [17].

9.4 In Vitro Models for Liver Toxicity

Cell lines are commonly used to study liver toxicity because of their similarities in genotypic and phenotypic characteristics to normal liver cells that have enzymes responsible for phase I and phase II metabolism of natural products. Liver toxicity is damage derived from chemicals that leads to acute and/or chronic liver disease. Liver cell lines are the best choice for investigation of the pharmacological and toxicological effects of natural product and their cellular mechanisms of action. Commonly used immortalized liver-derived cell lines are HepG2, Hep3B, HBG, and HepaRG [18].

9.5 In Vitro Models for Nephrotoxicity Studies

Cultured cells are also used to investigate renal cellular injury that results from natural products. A primary renal culture system of rat cortical epithelial cells is one of the models used to evaluate nephrotoxicity [19]. The cortical cells stemming from the renal cortex constitute the most metabolically active cells of the

kidney. Of these, proximal tubule and distal tubule cells are more frequently used for the assessment of in vitro renal toxicity. The methods of isolating and obtaining enriched populations of tubular cells include enzymatic methods, mechanical methods, and, historically, microdissection techniques [9].

9.6 In Vitro Model for Dermal Toxicity Testing

The Draize test has been used for many years to test skin corrosivity. Because of ethical issues around using animal models, alternatives to this test have been sought. Of these alternatives, the Corrositex, EpiDerm™, Episkin™, and transcutaneous electrical resistance (TER) assays have been validated for in vitro testing by different validating organizations.

The Corrositex assay is quantitative in vitro test for assessing the skin corrosion effects of a chemical. It is based on the time required for a test chemical to penetrate a barrier membrane. The membrane used for this test is composed of a reconstructed collagen matrix, developed to mimic the physicochemical properties of rat skin. The time needed to pass through the collagen matrix is recorded. As the solution of test product passes through the bio-barrier, the chemical detection system changes color.

EpiSkin is an in vitro model that uses a three-dimensional system consisting of reconstructed human epidermis with a functional stratum corneum. The model uses topical application of the test material to the surface of reconstructed skin and assesses the viability of cells. The cell viability is assessed from formazan production as measured by the MTT assay [20]. EpiDerm is another in vitro method designed to replace the Draize test. It uses a reconstituted human epidermal model to show the cytotoxicity effects of the test product. Cytotoxicity is expressed by a reduction in mitochondrial dehydrogenase activity, as measured by formazan production from MTT [20].

9.7 Mutagenicity Testing In Vitro

Mutations are changes that occur in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) sequences. They affect the normal cell proliferation, reproduction, and physiology. Mutations can have immediate or delayed consequences and may also cause stable inherited changes in gene sequences, resulting in phenotypic alterations. The effect and the type of mutations depend on the dose, frequency, and duration of exposure of the cell to the mutagens. Currently there are different methods that are more rapid, economical, and convenient than in vivo testing [9].

9.7.1 Bacterial Cell System

Bacterial testing systems use auxotrophic organisms. These organisms depend on the presence of rate-limiting nutrient compounds in the medium. Auxotrophic organisms are mutant bacteria with a highly specific defect in a gene locus, whereas normal or wild-type prototrophic organisms lack the mutation and are capable of growing in the absence of rate-limiting amino acids in the medium.

The Ames test is a bacterial mutagenicity assay that can identify a direct-acting mutagen. The test is conducted by mixing the bacterial strain with known concentrations of a test agent. A suspension of the bacterial strain along with the natural product being tested is incubated in agar solution containing the rate-limiting component for growth of auxotrophic bacteria. In this case, the bacteria can grow freely. The mixture is then spread over the surface of an agar plate without the essential rate-limiting component in the medium. On the agar plate, auxotrophic organisms stop growing, and only those affected by the natural product being tested back-mutate to prototrophic growth. The concentration of the natural product being tested is proportional to the proportion of organisms that change from auxotrophic to prototrophic, unless there is evidence of extensive genetic changes or lethal damage to the auxotrophic bacterial genome. The method, which was developed by Ames et al. [21], uses tester strains of *Salmonella* that require histidine. The test measures the effect of test product reversal of growth on histidine-free medium. Three tester strains – TA1531, TA1532, and TA1534 – are used to test frameshift mutagens and TA1530 is used to detect mutagens that cause base pair substitution [21]. The sensitivity of the assay was later improved by the addition of other mutations, but all strains had in common some type of mutation in the histidine operon. For example, the *RFA* mutation causes loss of lipopolysaccharide surface coatings of bacteria and this increases permeability to large molecules and polar compounds that do not normally penetrate cell walls. Another improvement was mutation of *uvrB*, which greatly increases the sensitivity of the bacteria to mutagens by deleting the gene coding for DNA excision repair [9, 22].

9.8 Reproductive and Teratogenicity Studies In Vitro

The in vivo tests used today are mostly time consuming and expensive. They also require expertise, skills, and a number of laboratory animals, which are eventually sacrificed; thus, in vivo testing is surrounded by several ethical issues [23]. Therefore, over the years, several in vitro methods have been used and documented. For example, one study has demonstrated that the in vitro follicle growth (IVFG) assay is a robust, organotypic, cheap model system that can be applied to rapidly assess potential adverse reproductive outcomes following chemical

exposure of female reproductive systems. Further research on this assay has enabled in-depth studies regarding reproductive toxicities to be established [24]. Other examples of in vitro assays follow.

9.8.1 H295R Steroidogenesis Assay

This test describes an in vitro screening for chemical effects on steroidogenesis, especially the production of 17β-estradiol and testosterone [25].

The human H295R adrenocarcinoma cell line is used in this assay. It is acclimatized for a period of 24 hours in multiwell plates and then cells are exposed for 48 hours to seven concentrations of the test chemical in at least triplicate. The solvent, a known inhibitor, and an inducer of hormone production are run at fixed concentrations as negative and positive controls. Cell viability is analyzed in each well at the end of the exposure period. The concentrations of the hormones in the medium can be measured using commercially available hormone measurement kits. Data are expressed as the fold change relative to the solvent control and as the lowest observed effect concentration. If the assay is negative, the highest concentration tested is reported as the no observed effect concentration.

9.8.2 Embryonic Stem Cell Test

This test uses two cell lines – mouse embryonic stem (ES) cells and mouse 3T3 fibroblast cells – and three endpoints to predict embryotoxic chemicals. The assay endpoints are indicated by the inhibition of differentiation of the ES cells, inhibition of ES and 3T3 cell viability, and inhibition of ES and 3T3 cell proliferation [26–28].

9.8.3 Whole Rat Embryo Cultures

This assay uses isolated and cultured early postimplantation rat embryos to study the embryotoxic effects of chemicals or any test substance. The morphology of 48 hour cultured embryos exposed to the test chemical are compared with controls to determine any delays in the development of certain organ systems or the development of any malformations [29].

In addition, according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) criteria for reproductive toxicity, three hazard categories exist, as shown in Table 9.1.

In conclusion, it is important for any new or initially marketed herbal formulation or medicine to be analyzed to determine any possible effects on the reproductive systems of both males and females prior to consumption in order to avoid any side effects that affect the normal functioning of organs or cause other disorders in the body.

Table 9.1 Globally Harmonized System of Classification and Labelling of Chemicals (GHS) criteria for reproductive toxicity.

Category	Criteria
1A	Known human reproductive toxicant that is based on evidence from humans
1B	Largely based on animal studies – presumed human reproductive toxicants
	Clear evidence of adverse effects on sexual function and fertility or on development in the absence of other effects
	In the case of other toxicity effects, the reproductive toxicity is not considered to be a second non-specific consequence of other toxic effects
	If there is information that raises doubt about the relevance of effects for humans, category 2 will be more appropriate
2	Evidence from human/animal studies is limited and there is a suspected human reproductive toxicant
Effects via lactation	Toxicants may be harmful to breast-fed children, may interfere with lactation, or may be present in breast milk

9.9 In Vivo Toxicity Testing of Natural Products

9.9.1 Acute Toxicity Testing

Acute toxicity is the noxious effect produced after a single dose of a chemical [30]. The data derived are used to determine the safety of or hazards produced by the natural product with regard to humans. The effect of the administration of a product to animals mimics the effect in humans [9]. The LD_{50} has been widely used for a long time to estimate acute toxicity in experimental animals. It is defined as the estimated dose that causes the death of 50% of the test population under specific conditions. For each LD_{50} test subjects need to be exposed to at least two routes of exposure; these are mostly the oral route and parenteral routes. Based on the nature of the natural product, the route can be modified to dermal, inhalational, or other route [9].

Guidelines produced by the Organisation for Economic Co-operation and Development (OECD) recommend having all available information about the test product prior to starting the test. Such information will help in the selection of the starting dose for a test. The information includes the chemical structure if identified, its physical and chemical properties, any other in vitro and in vivo tests conducted, and toxicological data on related products. When there is no information

to estimate a preliminary LD_{50}, the OECD suggests that the starting dose should be approximately 175 mg/kg with a dose progression factor of 3.2 [31].

Experimental animals need to be acclimatized at least for 5 days before starting the test to minimize the effects of a new environment [32]. According to OECD guidelines there are two tests: the main test and the limit test. In the main test, the animals are given a single dose at a minimum of 48 hour intervals. The first animal is given a dose one step below the level of the estimated LD_{50}. If the animal survives the next dose, which is 3.2 times the previous one, will be administered, and so on. If the first animal dies, the next animal will receive a dose decreased by a similar progression factor below the lethal dose in the first animal. Each animal should be followed for 48 hours before deciding on the dose of the next animal. The decision is made based on 48 hour animal survival patterns.

The limit test is a sequential test that needs a maximum of five animals and is used to identify chemicals that are likely to have low toxicity. Testing starts at 2000 mg/kg or 5000 mg/kg [31]. In another method, which has been described by Carpejane et al. [30], animals are divided into different groups, including a control group and a treatment group for each different concentration of the test product given; animals are followed for 14 days [33, 34]. To assess toxicological effects, the animals are closely observed for behavioral change, clinical signs of toxicity, body weight, and food intake. Hematological, biochemical, and histopathological analysis on the brain, heart, lungs, liver, stomach, small intestine (section), and left kidney also conducted [35].

Some regulatory agencies require that at least two species are used: one rodent species and one non-rodent species [36]. The preferred rodent for acute toxicity testing is the rat, although other rodents could also be used. Female rats are usually used because most of the literature shows a sensitivity difference and females are slightly more sensitive than males [37]. However, if the toxicokinetic properties of a structurally related product show higher sensitivity in males than in females, then males will be used. Healthy young animals are commonly used; also, females should be nulliparous and non-pregnant. Animals should be between 8 and 12 weeks old at the start of dosing. The temperature of the experimental room should be 20 °C ± 3 °C with a humidity of 30–70%. It is also recommended that animals should be housed individually with artificial light in a 12 hour light/12 hour dark cycle and fed a conventional rodent diet with an unlimited supply of drinking water [31].

Another method for acute toxicity testing according to the OECD guidelines is the acute toxic class method. According to this guideline, a stepwise procedure using a minimum number of animals per step (usually three) is used. Animals should be fasted prior to and after dosing for 3–4 hours for rats and 1–2 hours for mice. The three animals used for each step are given a starting dose from one of the following fixed dose levels: 5, 50, 300, and 2000 mg/kg body weight. The dose

is selected based on that most likely to result in death in some of the dosed animals. In this case, when the study conducted uses doses up to 2000 mg/kg, it is unlikely that the drug will result in death. When there is no information available for the natural product to be tested, 300 mg/kg is the recommended starting dose [38, 39].

9.9.2 Subchronic Toxicity Testing

Subchronic toxicity involves the period of time between acute and chronic effects, which ranges from 1 month [40, 41] to 3 months [42]. Subchronic toxicity testing is conducted to provide information on the hazard likely occurring as a result of repeated or continuous exposure to a natural product for a long period of time [9]. It also provides information on the major toxic effects, indicates the organs that are affected, and demonstrates the possibility of accumulation of natural products. Subchronic toxicity studies can help to provide an estimate of a no observed adverse effect level (NOAEL) of exposure. NOAEL exposure is the maximum exposure of an organism for which there is no biological or statistically significant increments in toxicity. The NOAEL ascertained through subchronic toxicity testing can be used to determine the dose levels for chronic toxicity studies and for establishing safety criteria for human exposure [42].

The preferred animal for subchronic toxicity testing is the rat. Other rodent species such as mice can also be used. It is recommended by the OECD to use both male and female healthy young adult animals; the females should be nulliparous and non-pregnant. In contrast, the World Health Organization recommends that males and females of two species – one rodent species and one non-rodent species – should be used [43]. At least 10 males and 10 females for each dose level should be used. The number of animals should be increased if interim killing is planned. At the beginning of the study the weight variation should be less than 20% from the mean. According to OECD guidelines published in 2019 at least three dose levels and concurrent controls should be used. The controls should be an untreated group or a vehicle control group if a vehicle is used for administering a natural product. A limit test can be used when a test dose level of 1000 mg/kg body weight produces no observed adverse effects or if toxicity would not be expected from structurally related compounds [42].

The natural product undergoing testing is usually given orally or by the route of administration which would be used clinically [43] on a daily basis in increasing doses to different groups of animals. For each group one dose level is given for at least 90 days [44]. The volume of the natural product administered depends on the animal used for a test and should not be greater than 1 ml/100 g of animal body

weight. But for aqueous solution up to 2 ml/100 g can be used. The animal should be observed for at least 90 days and general clinical observations should be carried out at least daily until the time when the peak period is reached [42].

During the period of administration, the animals are observed for signs of toxicity. Animals that die before 90 days or humanely killed during the test are necropsied. At the conclusion of the test, the remaining animals are also humanely killed and necropsied after the full dosing period. All signs of morbidity and mortality in the animals should be recorded twice daily, preferably at the beginning and the end of a day.

The animals being tested should be weighed at least once a week. Their food consumption should also be measured before the start of the experiment, and then at least weekly. Water intake should also be followed, depending on its usefulness [43]. At the end of a test period, blood samples should be collected prior to necropsy for rodents. For non-rodents blood samples should be collected before the start of administration of the product and at least once during administration of the product, and finally before necropsy. Hematological examination, such as hemoglobin, hematocrit, erythrocyte count, reticulocyte count, white blood cell count, platelet count, and a measure of blood clotting time, should be conducted [40, 45].

Biochemical examination to investigate the toxic effects of the natural product on the kidneys and liver should be performed. The plasma or serum levels of sodium, potassium, glucose, total cholesterol, high-density cholesterol, low-density cholesterol, urea, blood urea nitrogen, creatinine, total protein and albumin, and at least two enzymes that are indicative of hepatocellular effects (e.g. alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, and sorbitol dehydrogenase) should be determined. At the end of the study, the weight of the testes and epididymis of all male animal should be recorded [42]. For histopathological examination, the viscera, that is, heart, stomach, large and small intestine, kidneys, lungs, and liver, should be immersed in fixative solution [30]. At least one epididymis from each male should be reserved for histopathological examination. At necropsy, the estrus cycle of all females should be determined by taking vaginal smears [42].

9.9.3 Chronic Toxicity Testing

Chronic toxicity is a term used to describe products that need repeated or continuous exposure to cause the toxic effects [9]. Chronic toxicity testing provides data on the possible hazards over the life span of the animal species used as a result of repeated exposure; demonstrates target organ damage due to accumulation of the natural product over long-term usage; and identifies the level of exposure of the

experimental organisms at which there is no significant increase adverse effects (NOAEL) [46].

Before beginning the study, all the available information about the product should be considered to reduce the number of animals used and the study should be designed efficiently to test the chronic safety of a natural product. Chronic toxicity testing is only conducted after the initial information on acute and subchronic toxicity is obtained. The route of administration basically depends on the route that would be used clinically. The natural product is given daily at different dose levels to a few groups of animals. The duration of testing is usually 12 months [9, 46], but 180 days can be used. The duration is chosen to sufficiently determine long-term effects and cumulative toxicity without being affected by changes in the animal [47]. The rat is the preferred rodent even though other rodents such as mice can be used. Rats and mice are preferred since the effects of the test product can be investigated over their life span. Both male and female test animals should be used, and at least 10 animals for each sex are used per dose level. For non-rodents a minimum of three animals for each sex per group are used [43]. If there is no available information on the progression of toxicological changes from previous studies, the interim killing may be needed to gather enough information. When such information is available from previous studies, interim killing is not scientifically justified [34]. Satellite groups can also be included to investigate the reversibility of any toxicological changes induced by the natural product. An additional sentinel group may also be included to monitor disease status during the study. These animals should undergo the same observations and measurements as animal in the toxicity testing study [46, 48].

In a chronic toxicity study, a minimum of three dose levels with additional control groups are used. The selection of dose level depends on the results from acute or subchronic toxicity data or any existing toxicology data on the product or structurally similar compounds. The control groups are either untreated or given the vehicle [49]. For oral administration, the animals are given the dose daily for 12 months.

The body weight of the animals should be monitored, preferably at the start of the experiment and every week for the first 13 weeks, then every month. In addition to this, food consumption and, if the product is given with water, water consumption should also be measured [50].

At the end of the experiment the animal is sacrificed and blood is collected in tubes containing ethylenediaminetetraacetic acid (EDTA) for hematological tests and in tubes without EDTA for biochemical tests. Clinical biochemistry is basically used to investigate the toxicity effects of the natural product on the major organs, especially the kidneys and liver. All surviving animals are necropsied at the end of the study. The organs and tissues are harvested for morphological examination, and fragments of these organs are fixed for histopathological investigation [47].

9.9.4 Dermal and Ocular Toxicity

The Draize skin test method has been used since the mid-twentieth century to test the safety of cosmetic agents. Draize et al. [51] published a protocol for dermal toxicity testing that used the presence of edema and erythema to quantify skin irritation. The rabbit model is preferred for this test because of the sensitivity of its skin, because these animals are easy to handle, and because their skin has high permeability.

The test area is shaved 24 hours before application. The test areas mostly frequently used are the back or the abdomen [52]. Initially, a single rabbit is exposed to 0.5 ml (liquid) or 0.5 g (solid) of the natural product for 3 minutes. If the product shows any corrosive effect the test is stopped and the test product is classified as corrosive. If it is not corrosive, two additional tests for 1 hour and 4 hours are conducted and any irritation is scored according to the Draize irritation potential classification (Table 9.2) [51, 53].

The skin sensitization study is one of the tests for allergic dermatitis that could be caused by a natural product. It involves an immunological reaction, which is the result of activation of antigen-specific T cells. The response takes 24–48 hours to develop. For skin sensitization studies, guinea pigs are preferred because of their known sensitivity to different chemical sensitizers. The most common skin sensitization methods include the guinea pig maximization test, which needs 10–20 animals in the treatment group and 5–10 animals in the control group. The study starts with an intradermal injection; this is followed after 1 week by topical application; and then after 2 weeks a topical challenge is conducted. The Buehler guinea pig skin sensitization test is another protocol in which three topical applications 1 week apart for the induction phase and 2 weeks later for the topical challenge are applied. The appearance of edema or erythema after the challenge dose greater than that of the sensitizing dose is indicative of sensitization [9, 55].

Table 9.2 Draize irritation potential classification [51, 54].

Dermal irritation score (DIS)	Classification of dermal irritability
$0 < \text{DIS} < 0.4$	Not irritant
$0.4 \leq \text{DIS} < 2$	Slightly irritant
$2 \leq \text{DIS} < 5$	Moderately irritant
$5 \leq \text{DIS} \leq 8$	Severely irritating

The score is based on observations at 1, 24, 48, and 72 hours.

$$\text{DIS} = \frac{\text{Value (erythema + edema)}}{\text{Number of animals} \times \text{Number of observations}}$$

Dermal phototoxicity and photosensitivity studies are conducted in guinea pigs or rabbits. The natural products being tested are administered orally, parenterally, or topically for 10–14 days. The challenge phase starts 2–3 weeks later with another dose along with exposure to an ultraviolet lamp. The control group is exposed to positive and negative photoallergic agents [9].

Draize published the first protocol for an eye irritancy test in 1940 [56]. The study raised many ethical issues and was, therefore, revised many times. In this test, the compound undergoing testing is placed onto the eye of conscious restrained rabbits, which were then observed for several days to see the effects of the test compound. Because the cornea is the most sensitive part of the body and is rich in nerve endings, irritation or ulceration may produce pain. OECD guideline 405 recommends that before in vivo testing all available information about a product with regard to eye corrosivity/irritancy should be evaluated and a sequential testing strategy should be used. Performing tests sequentially on one animal at a time is recommended and allows reassessment of data and avoids duplication. Generally before any in vivo eye test studies are carried out, in vitro or in vivo tests on the skin corrosive effects of a substance should be conducted [57].

Albino rabbits are the preferred laboratory animal for in vivo eye tests. According to OECD guidelines, 6 minutes before administration of the natural product a subcutaneous (SC) injection of buprenorphine 0.01 mg/kg should be given; then 5 minutes before administration one or two drops of local anesthetic such as 0.5% proparacaine hydrochloride or 0.5% tetracaine hydrochloride should be applied to give a therapeutic range of systemic analgesia. Eight hours after application of the natural product, meloxicam 0.5 mg/kg SC and buprenorphine 0.01 mg/kg SC are administered to give a sustained therapeutic range of systemic analgesia. Sixteen hours after application buprenorphine 0.01 mg/kg SC should be administered 12 hourly, alongside meloxicam 0.5 mg/kg SC 24 hourly to the point of resolution of ocular lesions and absence of clinical distress and pain signs [58]. Animal eyes need not be washed at least for 24 hours after test substance instillation, unless the substance is a solid. In the case of a solid, if the substance is still in the animal's eyes at the 1 hour observation time point, saline or distilled water can be used to rinse the eye. If appropriate, a complete washout can be conducted at the 24 hour observation time point. Follow-up of up to 21 days should be made on the animal to determine any cases of possible reversibility of the effects of natural product material. Ocular lesions must always be graded and recorded at every examination in an appropriate good laboratory practice manner [57].

9.9.5 Toxicity Testing for Fertility and Reproduction

In vivo models are known to be more reliable than in vitro models for toxicity testing for fertility and reproduction, although there are drawbacks such as

differences in biokinetic parameters [59]. The tests below suit in vivo testing of different herbal formulations and help to establish the effects on different reproductive systems over time with their daily use.

9.9.5.1 The Uterotrophic Bioassay

This is a rapid screening test that depends on the uterotrophic response or an increase in uterine weight. Its sensitivity is dependent on a test system for animals in which the hypothalamic–ovarian–pituitary axis is dysfunctional. The two estrogen-sensitive states in female rodents meeting this requirement are: (i) females prior to puberty but after weaning and (ii) females at a young adult age with adequate time for uterine tissue regress but after ovariectomy [60].

The route of test material administration is dependent on the expected route in clinical use, but test substances are mostly administered orally or SC on a daily basis. Treatment and control groups should have a minimum of six animals each. Well-regulated test material doses are administered to a minimum of two treatment groups of animals with one dose level in each group over an administration period of three consecutive days for immature animals and a minimum of three consecutive days for ovariectomized adult animals. Approximately 24 hours after the last dose, animals should be necropsied. In cases of agonists of estrogen, a significant increase in the mean uterine weight of the treated animal groups as compared with the control groups indicates a positive response to this bioassay. Information on daily body weights, the status of the animal, the wet and blotted uterine weight, and food consumption should be recorded and reported.

9.9.5.2 Hershberger Bioassay in Rats

This is an in vivo short-term screening test and evaluates the ability of a chemical to elicit biological activity consistent with androgen agonists or antagonists or 5α-reductase inhibitors. The bioassay considers changes in weight of androgen-dependent tissues, such as prostate, the seminal vesicles, and the epididymis in castrated/peripubertal male rats.

To determine the androgenic or antiandrogenic action of a test substance, two (respectively three) dose groups of the test substance as well as positive and negative controls are sufficient for this test. The test substance is administered by gavage or SC injection daily for 10 consecutive days. A minimum of six animals should be included in each treatment or control group. The antiandrogen test involves administration of the test substance together with a reference androgen agonist. Animals are to be necropsied approximately 24 hours after the last administration of the test substance. Tissues are then excised and their fresh weights determined. Results showing a statistically significant increase in weight of the five tissues indicate androgenic activity, whereas a decrease means antiandrogenic activity of the test substance [61].

9.9.6 Combined Repeated Dose Toxicity Study with Reproduction/ Developmental Testing

9.9.6.1 Toxicity Screening Test

This test describes the impacts of a test substance on female and male reproductive functioning. Endocrine disruptor endpoints, particularly measurement of anogenital distance, thyroid examination, and male nipple retention in pups, are noted in this test. This test guideline is devised for use with rats.

The test substance is administered in regular doses to several groups of females and males. Males should be dosed for a minimum of 4 weeks, whereas females are dosed for the entire length of the study, approximately 63 days. Mating of one male to one female is recommended for this kind of study. A minimum of 10 animals of each sex per group is recommended. At least three test groups and a control group should be used. Dose levels can be predicted based on information from acute toxicity tests or on results from repeated dose studies. The test substance should be administered orally and daily for the period of the study. The findings of this toxicity study should be evaluated in terms of observed effects such as body weight, food/water consumption, and necropsy and microscopic findings. Because of the short period of treatment in males, histopathology of the testis and epididymis should be considered along with fertility data for assessment of male reproductive effects [62].

9.9.6.2 Extended One-Generation Reproductive Toxicity Study

This study allows for the evaluation of developmental and reproductive effects that may occur as a result of pre- as well as postnatal chemical exposure and an evaluation of systemic toxicity in lactating and pregnant females and in young toward adult offspring.

Sexually mature female and male rodents (P generation) are exposed to regular doses of test material beginning from 2 weeks before mating and continuing through to mating, gestation, and weaning of their pups (referred to here as the F1 generation). At the weaning stage, the pups are selected and assigned to various cohorts of animals for developmental/reproductive toxicity testing (cohort 1), testing for developmental neurotoxicity (cohort 2), and testing for developmental immunotoxicity (cohort 3). F1 offspring are further treated with the test material from weaning to adulthood. Clinical observations and pathological examinations are performed on all animals to check for signs of toxicity. The integrity and performance of male and female reproductive systems as well as the health, growth, development, and function of offspring should be carefully recorded. Part of cohort 1 may be extended to include an F2 generation(cohort 1B); in this case, the procedure for F1 animals will be similar to that for the P animals [63].

9.9.7 In Vivo Carcinogenicity Testing

Transgenic rodent models have been used for many years in carcinogenic testing. In vivo carcinogenicity testing uses two species to identify trans-species carcinogens. If the natural product produces a carcinogenic effect in these two species, it may have a significant carcinogenic effect in humans [9]. The carcinogenic study is usually performed for 18–24 months in mice and for 24–30 months in rats, or for the life span of an animal if the survival rate is high [64].

Carcinogenicity testing depends on the development of neoplasia as the single endpoint of this study. In addition, morphological examination of the organs and tissues is also used to investigate any carcinogenic response. The experiment is conducted in two phases: one is a preliminary study, aimed at determining the dose level for a full carcinogenicity study. If enough data are available, the preliminary study may be omitted. Testing consists of three stages: the first is a single-dose toxicity study that is conducted on a small number of animals to determine the highest dose to be used for the second stage. The second stage is another dose toxicity study that is used to determine the maximum dose to be used in the full-scale carcinogenicity study. At least 20 animals (10 males and 10 females) should be used with three dose groups and a control group for at least 90 days [43].

The maximum tolerated dose determined from the preliminary study is the dose that inhibits the weight gain of an animal by less than 10% compared with the control group. This dose should also not result in mortality or morbidity because of toxicity and should not significantly change the laboratory findings for the animal [43]. In full-scale carcinogenicity testing a minimum of 50 males and 50 females is used. The route of administration depends on the expected route of administration of the natural product, but it is usually given with water or prepared with food. A minimum of three dose levels and a control should be used.

Hematological and blood chemistry examinations should be conducted; for rodents, this should be done on blood collected before necropsy, and for non-rodents, it should be done on blood collected before the start of administration of the natural product and at least once during administration and before necropsy. Renal and liver function tests are also important because these are the main organs where metabolism and excretion of a drug take place. At the end of the experiment the survivors are necropsied and all animals should be examined macroscopically; also histopathological examination should be performed on all those in the highest dose group and in the control group. Histopathological examination of all animals should be conducted if the incidence of neoplastic lesions in the highest dose group and in the control group are different. A natural product is considered to be carcinogenic when any of the following responses are observed: (i) if a tumor develops in the experimental groups and none are seen in the control groups; (ii) development of tumors with a greater frequency in the test group than

in the control group; (iii) a greater variety of organs and tissues involved in tumor development in the test group than in the control group; and (iv) if a tumor develops earlier in the test group even though there is no significant difference in the incidence of tumors between the test groups and the control group.

9.10 Conclusion

Claims that natural products are all safe to use is not a scientific conclusion that should preclude any investigations. The toxicity of natural plant products emanates from a number of properties, and various tests should be undertaken to verify claims on a compound, plant, or product basis and not on the whole plant. These issues should always be included in standardization processes and approvals of any herbal product.

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