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Screening of Herbal Medicines for Potential Toxicities

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Additional information is available at the end of the chapter

1. Introduction

1.1. Herbal medicines in the 21st century

Herbs and herb-derived medicines have played a crucial role in health and disease management for many centuries. Many ancient civilizations show documented evidence for the use of herbs in the treatment of different ailments; as was seen with Mesopotamian, Indian ayurveda, ancient traditional Chinese medicine and Greek unani medicine [1-5]. In Africa, knowledge of traditional medicine as part of wholistic system, was passed through generations by oral communication and indigenous practices [6]. The global demand for herbal medicinal products has increased significantly in recent years. It is estimated that, the world's population will be more than 7.5 billion in the next 10 to 15 years. This increase in population will occur mostly in the southern hemisphere, where approximately 80% of the population still relies on a traditional system of medicine based on herbal drugs for primary healthcare [7].

Use of plants for medicinal purposes is as old as human civilization [8] and continuous efforts [8-17] are being made towards its improvement. About 200,000 natural products of plant origin are known and many more are being identified from higher plants and microorganisms [18-21]. Some plant-based drugs have been used for centuries and for some like cardiac glycosides, there is no alternative conventional medicine. Therefore, medicinal plants and their bioactive molecules are always in demand and are a central point of research. As a result, there is a recent [22] surge in the demand for herbal medicine.

To date, herbs have remained useful not only as remedy for different diseases that affect humans and animals, but also as good starting points for the discovery of bioactive molecules for drug development. The scientific exploitation of herbs used ethnomedicinally for pain relief, wound healing and abolishing fevers has resulted in the identification of a wide range of compounds that have been developed as new therapies for cancer, hypertension, diabetes

and as anti-infectives [23]. The ealriest report of the toxicity of herbs originated from Galen, a Greek pharmacist and physician who showed that herbs do not contain only medicinally beneficial constituents, but may also be constituted with harmful substances. [24].

By 2003 in the United States alone, over 1500 herbal products sold were nutraceuticals which are exempt from extensive preclinical efficacy and toxicity testing by the U.S. Food and Drug Administration [25]. This has led to increased concerns about potential harmful effect of these products, which has resulted in efforts to globally harmonize standards of toxicity testing methods that can be used for herbal medicine toxicological characterization including tests for acute high-dose exposure effects, chronic low-dose toxicity tests and specific cellular, organ and system-based toxicity assays. This chapter reviews some of these tests and their applications. Recent biotechnological advancements have rapidly evolved toxicity test methods at molecular and sub cellular levels including next generation sequencing and computer-based modeling and simulation tools which have been used to predict the potential toxicity of novel drug candidates and in some cases, herbal medicine toxicities which may arise from herbs administered alone or concomitantly with other herbs and/or drugs. However, challenges still exist for testing herbal medicines in this exciting field and these will also be discussed.

2. Toxicity of herbs

Despite the growing market demand for herbal medicines, there are still concerns associated with not only their use, but their safety. Less than 10% of herbal products in the world market are truly standardized to known active components and strict quality control measures are not always diligently adhered to [26]. For majority of these products in use, very little is known about their active and/or toxic constituents. In many countries including the U.S, herbal medicines are not subjected to the same regulatory standards as orthodox drugs in terms of efficacy and safety. This raises concern on their safety and implications for their use as medicines. Toxicity testing can reveal some of the risks that may be associated with use of herbs, therefore avoiding potential harmful effects when used as medicine.

In addition, many plants produce toxic secondary metabolites as natural defence from adverse conditions. In some toxicologically and medicinally relevant plant species like *Digitalis purpurea*, *Hyoscyamus niger*, *Atropa belladonna*, *Physostigma venenosum*, *Podophyllum peltatum* and *Solanum nigrum*, these toxic substances are not distinguished from therapeutically active ingredients. Being stationary autotrophs, plants have evolved different means of adaptation to challenging environments and co-existence with herbivores and pathogenic microorganisms. Thus, they synthesize an array of metabolites characterized as 'phytoanticipins' or as general 'phytoprotectants' that are stored in specialized cellular compartments and released in response to specific environmental stimuli like damage due to herbivores, pathogens or nutrient depletion [27]. Some of the phytochemicals produced by plants against herbivorous insects also end up being harmful to humans, because highly conserved biological similarities are shared between both taxa as seen in most pathways involving protein, nucleic acid,

carbohydrate and lipid metabolism [28]. Human neurochemicals, often with similar biological functions are also reportedly present in insects [28]. These incude signalling molecules, neuropeptides, hormones and neurotransmitters [29-32]; whose functions can be mimicked or antagonized by phytochemicals like alkaloids, flavonoids, terpenoids and saponins. Ecologically, a good number of alkaloids serve as feeding deterrents via agonistic or agonistic activity on neurotransmitter systems [33]. Similarly, some lipid soluble terpenes have shown inhibitory properties against mammalian cholinesterase [34], whilst some interact with the GABAergic system in vertebrates [35]. In addition to these, saponins are potent surfactants that can disrupt lipid-rich cellular membranes of human erythrocytes and microorganisms which explains the potent antimicrobial properties of this group of phytochemicals [36]. Aristolochic acid, a nitrophenanthrene carboxylic acid in *Aristolochia* species and present in some other botanicals has also been identified as a phytochemical toxicant implicated in the development of nephropathies and carcinogenesis [37].

Another implication in the toxicity of certain herbs is the presence of toxic minerals and heavy metals like mercury, arsenic, lead and cadmium [38]. Lead and mercury can cause serious neurological impaiment when a herbal medicinal product contaminated with these metals is ingested. As shown in Table 1, the presence of high levels of arsenic in kelp seaweed may result in toxicosis in some patients [39].

3. Goals of toxicity testing of herbal drugs

The primary aim of toxicological assessment of any herbal medicine is to identify adverse effects and to determine limits of exposure level at which such effects occur. Two important factors which are taken into consideration in evaluating the safety of any herbal drug are the nature and significance of the adverse effect and in addition, the exposure level where the effect is observed. Toxicity testing can reveal some of the risks that may be associated with use of herbs especially in sensitive populations.

An equally important objective of toxicity testing is the detection of toxic plant extracts or compounds derived thereof in the early (pre-clinical) and late (clinical) stages of drug discovery and development from plant sources. This will facilitate the identification of toxicants which can be discarded or modified during the process and create an opportuinity for extensive evaluation of safer, promising alternatives [54]. For certain compounds, modifications such as dosage reduction, chemical group or structural adjustments may improve their tolerability.

3.1. Pre-clinical toxicity testing of herbs

This covers a range of toxicity tests done in non-human experimental models before conducting clinical tests for toxic effects in humans. Generally these tests are classified into non-animal tests and animal studies. Crude extracts or purified compounds obtained by fractionation of the medicinal herb can be evaluated in these tests.

Common name	Plant source/parts used	Intended indications	Potential toxicity
Ginseng	*Panax ginseng* roots	Relieves stress, promotes mental and physical activity	Central nervous system stimulation, hypertension, skin eruptions [40]
St. John's wort	*Hypericum perforatum* aerial parts	Antidepressant, mood stabilizer	Highly potent cytochrome P450 enzyme inducer which affects drug metabolism. Also causes hepatotoxicity and nephrotoxicity in pregnancy and lactation [41]
Kava kava	*Piper methysticum* roots	Sedative, anxiolytic	Hepatotoxic, cytochrome P450 enzyme inhibitor [42]
Ginkgo	*Ginkgo biloba* leaves	Impotence, vertigo, circulatory disorders, improves mental alertness	Gastric irritability, spontaeneous bleeding [43]
Danshen	*Salvia miltiorrhiza* exterior taproot	Angina pectoris, antihyperlipidemic, ischemic stroke	Bleeding, anticoagulant effects [44]
Hawthorn	*Crataegus oxycantha* Flowers, roots, berries	Mild to moderate congestive heart failure	Cardiac arrythmias, lowered blood pressure [45]
Comfrey	*Symphytum officinale* leaves	Anti inflammatory, antidiarrhoel and treatment of thrombophlebitis	Hepatotoxicity, carcinogenicity [46]
Licorice	*Glycyrrhiza glabra* roots	Antiulcer, anti inflammatory, antihypertensive	Hypokalemic myopathy, pseudoaldosteronism, thrombocytopenia [47]
Chaparral, creosote bush	*Larrea tridentata* leaves and twigs	Blood thinner, weight loss, antioxidant, anticancer, anti arthritis	Carcinogenic, nephrotoxic, hepatotoxic [48]
Mistletoe	*Phoradendron spp., Viscum album* leaves and young twigs	Digestive aid, heart tonic, sedative	Hypotension, seizures [49]
Squill	*Urginea maritima* bulbs	Anti-arthritic, bronchial expectorant	Symptoms resembling digitalis toxicity [50]
Kelp (seaweed)	*Liminaria digitata*	Metabolic tonic, thyroid tonic, anti inflammatory	Arsenic poisoning, Hyperthyroidism [39]
Ma-huang	*Ephedra*	Promotes weight loss, mental and physical alertness	Cardiotoxicity, thyrotoxicosis, seizures [51]
Senna	*Senna occidentalis* seeds	Laxative	Skeletal and cardiac muscle degeneration, hepatotoxicity, neurotoxicity [52]
Aloe	*Aloe vera* leaves	Wound healing, laxative	Cytogenetic toxicity [53]

Table 1. Potential toxic effects associated with some common herbal medicines marketed for different indications

3.2. Cell-based cytotoxicity tests

Cytotoxicity assays (CTAs) are performed to predict potential toxicity, using cultured cells which may be normal or transformed cells. These tests normally involve short term exposure of cultured cells to test substances, to detect how basal or specialized cell functions may be affected by the substance, prior to performing safety studies in whole organisms. It can also provide insight towards the carcinogenic and genotoxic dispositions of herb-derived compounds and extracts. The ability of a plant extract to inhibit cellular growth and viability can also be ascertained as an indication of its toxicity. Assessment parameters for cytotoxic effects include inhibition of cell proliferation, cell viability markers (metabolic and membrane), morphologic and intracellular differentiation markers [55]. In conducting CTAs, it is important to critically consider factors such as cell culture systems and methods which affect test outcomes. For example, some cell types maybe incompatible with the solvent used to prepare test solutions. Many plant extracts and compounds are non-polar and prepared as solutions in dimethylsulfoxide (DMSO) prior to CTAs. DMSO has been reported to be cytotoxic at certain concentrations [56] and this effect varies between cell types. Therefore, it is often necessary to pre-determine the maximum tolerable solvent concentration in CTAs especially during validatory stages, and a control using the carrier solvent alone must be used in the CTA.

CTAs are indispensible tools for medium and high throughput screens of different phytochemicals simultaneously, over wide concentration ranges. In addition, they have significant impact in the implementation of the three R's namely; the reduction of number of animals used, refinement of animal test models and replacement of animal in research.

As a herbal product may display cytotoxic effects only against specific cell types, it is important to consider the selection of a wide range of cell types for testing including normal cells of primary origin (usually from rodents), and permanent cell lines; provided they are of high quality and are reproducible over time [57].

CTAs which employ rodent cell lines like the mouse fibroblast cell line BALB/c 3T3 and the Syrian Hamster Embryo cells (SHE, pH 6.7 and pH 7) are robust models for the prediction of genotoxicity and carcinogenity. The tests have been shown to be highly predictive, as inoculation of transformed cells into x-ray irradiated mice induces tumorigenicity. Furthermore, there are no limitations with specific classes of chemicals and formulations that can be tested with these assays as it has been reported to be plausible in the assessment of nanoparticles [58]. Although the applicability of these assays in testing complex mixtures like herbal products is often hindered by non-availability of sufficient evidence in this regard, it is still useful in predicting their toxic effects so long it is makes sufficient contact with the cells [59].

In the BALB/c 3T3 assay, foci scoringis based on the level of malignant transformation, with type III classified as malignantly transformed, according to a previous classification used for cytotoxicity assays involving C3H10T1/2 cells [61]
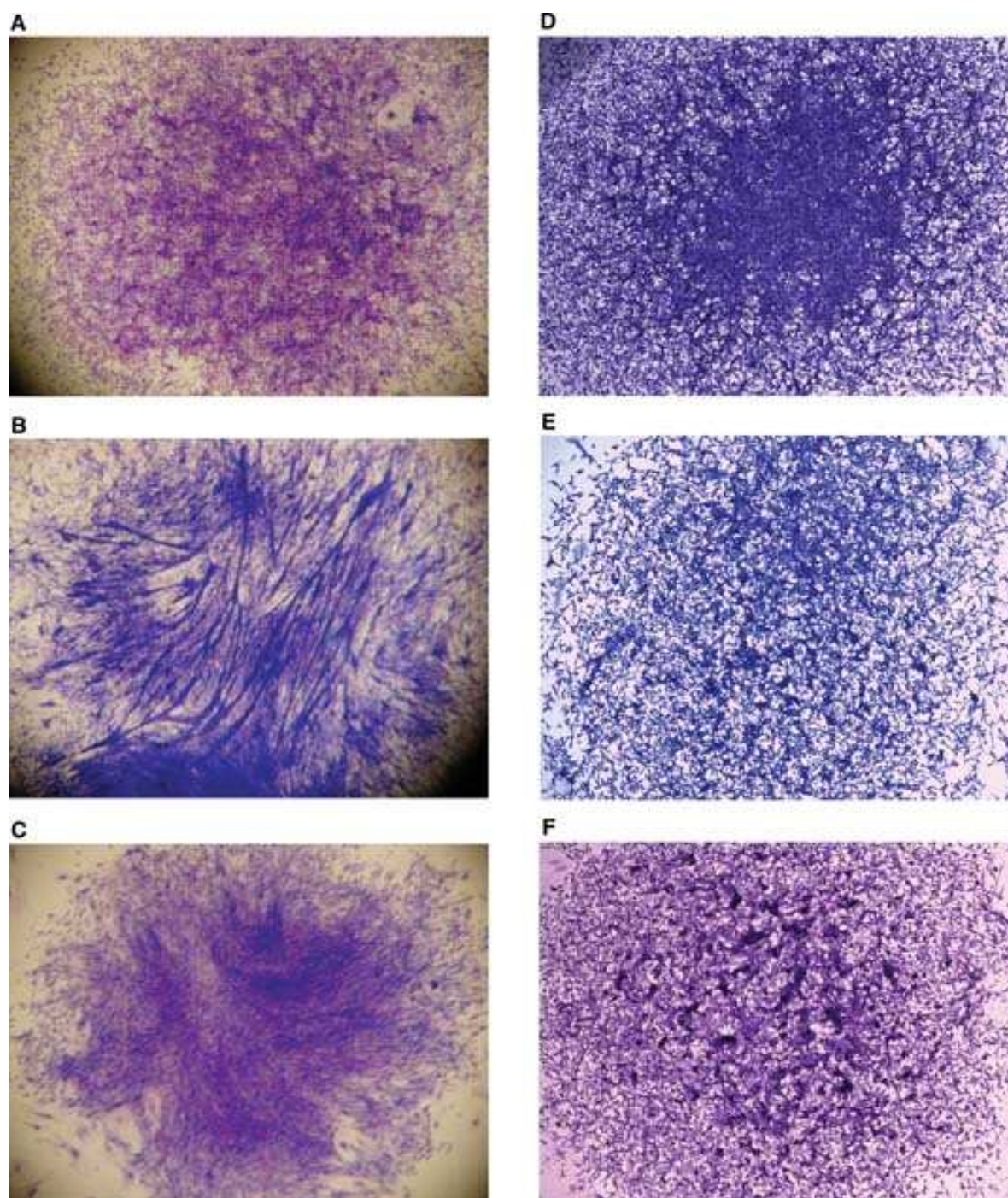
Figure 1. Examples of normal and transformed SHE cells. Plates A, B, C show normal colonies of cells organized in monolayers with no criss-crossing. Plates D, E, F show morphologically transformed colonies comprising stacked cells that are randomly oriented, three-dimensional and criss-crossed throughout; basophilic staining is usually darker. Magnification ×125 [60]
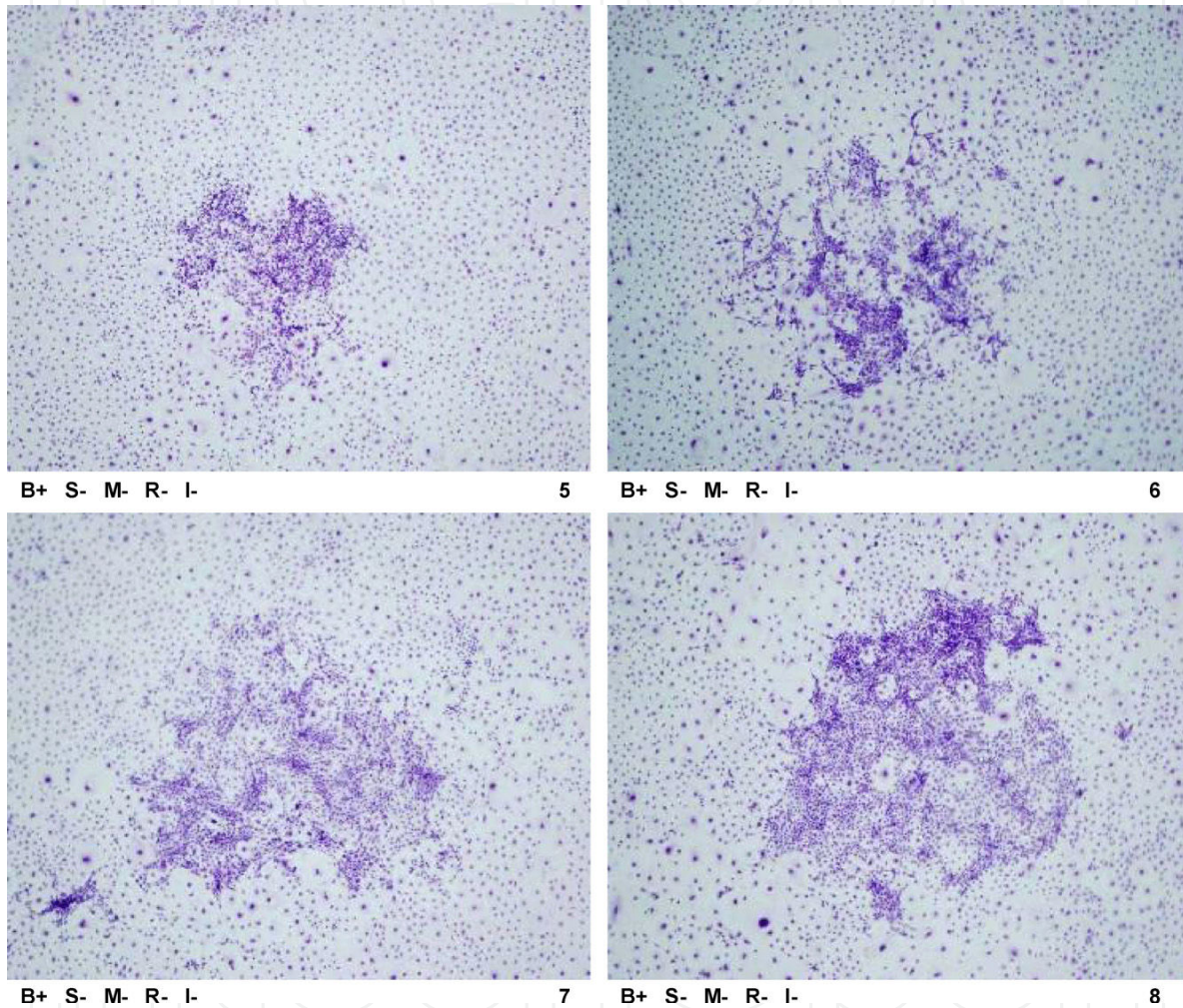
Figure 2. Type I foci: small, non invasive BALB/c 3T3 cells with weak basophilic staining. Under each picture in the catalogue, the characteristics are described as basophilic (B), spindle-shaped (S), multilayer (M), random orientation (R), invasive (I) and were evaluated as absent (−), weak (+/−) present (+), or strong (++). Magnification ×50 [62]
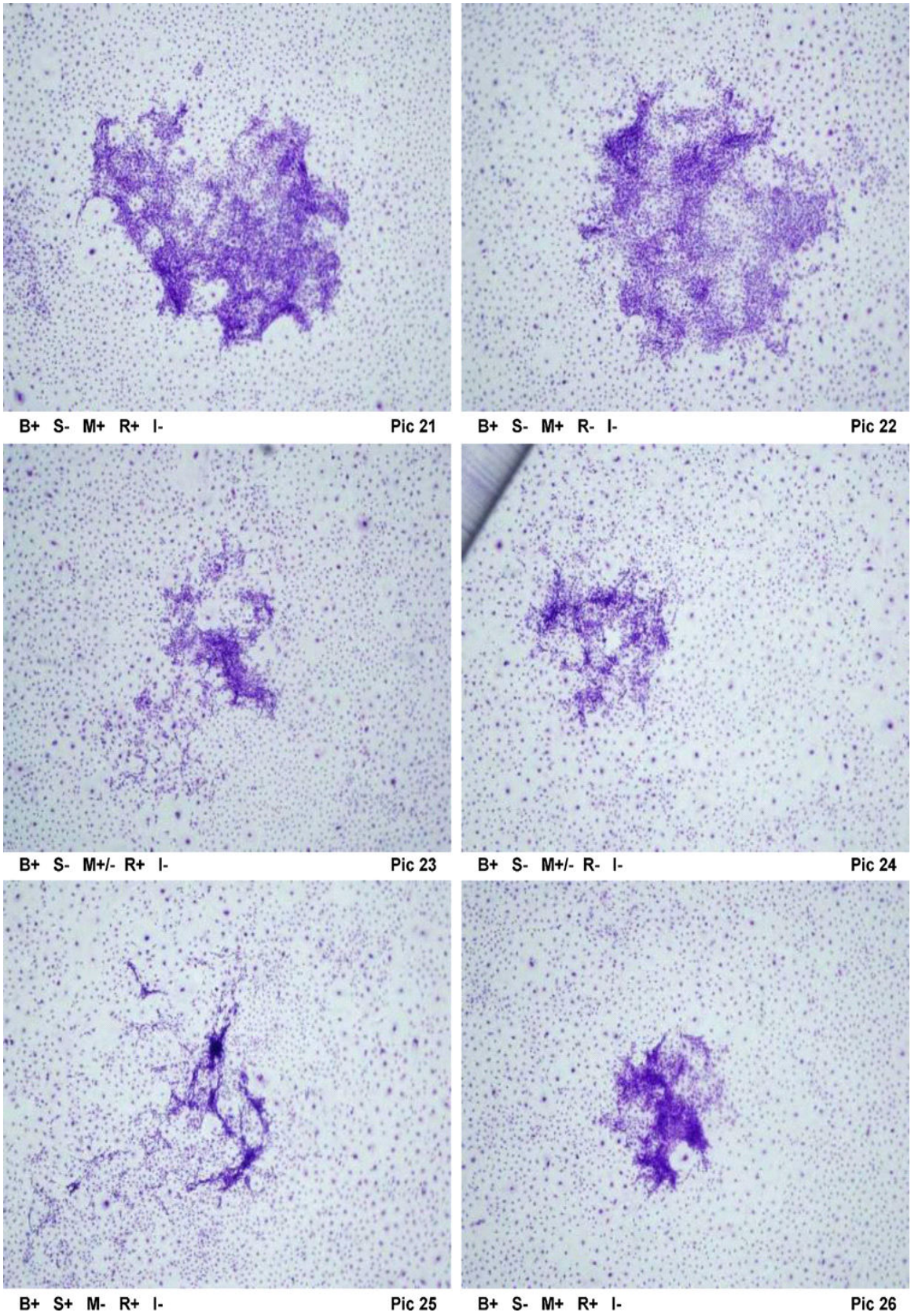
B+ S- M+ R+ I-   Pic 21

B+ S- M+ R- I-   Pic 22

B+ S- M+/- R+ I-   Pic 23

B+ S- M+/- R- I-   Pic 24

B+ S+ M- R+ I-   Pic 25

B+ S- M+ R+ I-   Pic 26

Figure 3. Type II foci: densely packed multi-layered cells, some cells pile up and are criss-crossed. Magnification ×50 [62]

B++ S- M+ R++ I-   Pic 51

B++ S++ M++ R+ I+   Pic 52

B++ S+ M+ R++ I+   Pic 53

B++ S++ M+ R++ I+   Pic 54

B++ S++ M++ R++ I++   Pic 55

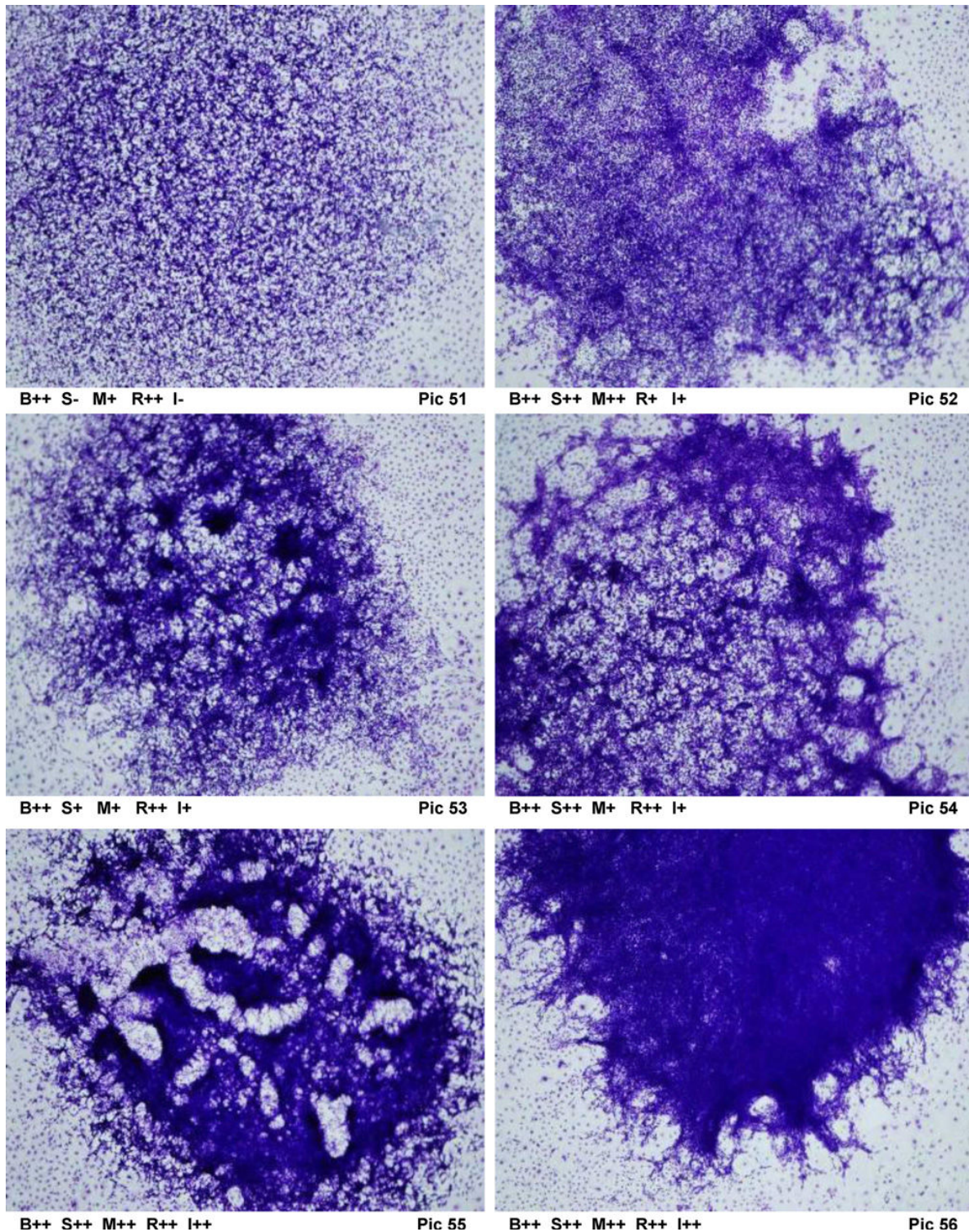B++ S++ M++ R++ I++   Pic 56

Figure 4. Type III foci: Malignantly transformed colony of morphologically different spindle-shaped cells. Cells are densely multi-layered and criss-crossed. Cells are randomly oriented and grow invasively at foci edge. Magnification ×50 [62]

3.3. Herbal toxicokinetics

Herbal toxicokinetics deals with the prediction of toxicity due to pharmacokinetic disposition of an herb, or purified xenobiotics derived from it, due to genetics or from potential herb-drug interactions [63] Testing usually begins with assays using human liver microsomal Cytochrome P450 isoforms to identify early enough, metabolites which are known to cause toxicological modulation at any level of cellular organization. Modulation of Cytochrome P450 has great significance as this largely affects drug biotransformation to active or inactive forms. For a drug that is dependent on these enzymes for inactivation via conjugation to chemical polar groups prior to elimination, any herb that induces these enzymes would lead to rapid inactivation and clearance of such a drug. Converesly, a herbal medicine that inhibits enzyme activity will lead to high concentrations of a drug whose inactivation relies on the inhibited enzyme. From findings in a recent survey [64], potential adverse drug herb interactions were observed in 40 % of patients receiving conventional therapy and taking a herbal product. Clinically significant drug-herb interactions may occur when an herb interacts with metabolism of a co-administered drug and either reduces its efficacy due to decreased formation of an active metabolite or increases its toxicity due to reduced metabolic elimination. The latter type of interaction potentially predisposes human consumers to adverse reactions or toxic drug effects, especially if the drug has a narrow therapeutic range. This is important because, approximately 73 % of all known drugs are metabolized hepatically by mixed function oxidation reactions, catalyzed by Cytochrome P450 enzymes [65]. Of all its isoforms, CYP3A4, CYP2C9, CYP2C19, CYP1A2 and CYP2D6 are implicated in over 80 % of oxidative drug reactions and are highly subject to inhibition owing to their broad specificity for structurally diverse substrates [66]. Some herbs, notably St. John's Wort (*Hypericum perforatum*), ginkgo (*Ginkgo biloba*), ginseng (*Panax ginseng*), kava (*Piper methysticum*) and garlic (*Allium sativum*) reportedly show significant interaction with some co-administered drugs by modulation of Cytochrome P450 [67]. In order to predict clinically significant effects that can occur when a herbal product inhibits or induces these enzymes, *in vitro* metabolic data can be used to correlate metabolic disposition of a test substance *in vivo* [68].

From the early 1990s onwards, new techniques for generating as much information as possible from one experiment were developed including DNA sequencing, microarrays to study gene expression, protein and metabolite profiling [69]. Further structure-activity relationship of metabolites or pure compounds can be extrapolated from computer-based models and simulation studies. Thereafter, pattern databases of tissue/organ response to drugs which allows for the parallel sequencing of all the relevant genes, measurement of genome transcription, protein expression and quantitation of metabolites produced by direct or indirect actions of the expressed protein. A final screening category for the compound or metabolite utilizes an integrative system biology approach; comprising databases of metabolic pathways, genes, regulatory networks and protein interactions [69].

Despite the high efficiency of these techniques, no single approach is sufficient to predict toxicokinetics *in silico* and harnessing the different assays will be effective in predicting metabolic fate of the test molecule in humans.

3.4. Toxicogenomic screening tools

Herbal toxicogenomics is a collective term that refers to the combination of toxicology with different '–omics' tools that measure the potential toxic outcomes of interactions of the herbal extract or compounds at sub molecular (epigenomics, transcriptomics), molecular (proteomics), cellular, tissue and organ (metabonomics) levels [70]. It is aimed at elucidating molecular mechanisms involved in the expression of toxicity, and to derive molecular patterns (i.e. molecular biomarkers) that predict toxicity or the individual susceptibility to it.

There are three major aspects within this field as outlined below:

DNA microarrays: These are carried out using specially designed microarrays. They usually provide the most information, providing not only clear prediction of cellular response to chemical toxicants, but also mechanisms through which such toxicity is elicited [71]. For an herbal mixture with a diversity of chemical entities, the data obtained cannot usually be extrapolated to that of data libraries of existing chemical compounds.

Proteomics: This high throughput screening tool is applied in protein identification. It is a sequential process of peptide separation and profiling, followed by mass spectrometry and NMR detection. Based on the assumption that a chemically related group of xenobiotics exhibit specific patterns of protein expression, only purified phytochemicals with known chemical structures can have their protein expression profiles correlated to existing databases of those of xenobiotics. The use of proteomics has been considered more advantageous than microarrays which assess gene expression, because they measure proteins which are closer to toxicology endpoints, as not all genes are translated to proteins and expressed proteins are liable to structural changes post-translation [72].

Metabonomics: This is an aspect of toxicity evaluation, performed through the large scale analysis of metabolic profiles of metabolic enzymes and metabolite composition resulting from the action of chemical stressors. This can be a very efficient approach as it can be applied in *in vitro* metabolic profiling, in animal toxicity tests for promising lead selection and in humans during clinical stages of safety testing for the development of biomarkers of safety [73].

3.5. High throughput next generation sequencing

Molecular studies have witnessed rapid developments since DNA was first sequenced in 1997 [74] to the creation of large volumes of DNA sequences at unprecedented speed; also referred to as next generation sequencing (NGS). Apart from its application in personalized medicine, it has also been applied in the creation of large genetic databases of plants, which can serve in the identification of potentially toxic plants, or those that may contain allergens. For example if functional gene transcriptomes present in Aristolochia species are found present in another specie under investigation, it is likely that such a specie may contain aristolochic acid. NGS technology has already been applied in unravelling the genome of Gingko [75] and holds potential for biomarking toxicity in the 21st century.

3.6. Animal tests

The whole animal is usually presumed to be closely correlated to human toxicity as the system incorporates pharmacokinetic (absorption, distribution, metabolism) disposition of the test substance when administered by a route similar to its intended use. It also takes into consideration, other physiological events in an organism that influence toxicity. While cell-based assays measure is predictive of potential toxicity, the whole animal experiment measures the critical toxicity of a test substance, which are the signs of toxicity that manifest as a result of a gradual increase in the dose of the test substance.

Certain drawbacks to animal testing however do exist; the costs of the animals to be used can be prohibitive and subtle differences within species can affect the type of effects that are observed and they are usually more tedious to arrange, in terms of duration of experiments.

4. General tests

Standard guidelines for the conduct of animal toxicity tests have been harmonized by the Organization for Economic Co-operation and Development [76] as part of continuous efforts to internationally harmonize test guidelines.

Before conducting safety study of an herb or its product in animals, some major factors that need to be considered are:

Preparation of test substane: Herbal products can be prepared into different dosage forms like capsules, tablets, ointments, creams and pastes. For correct administration of a pre-defined dose of the product, the product should be quantitatively standardized and administered based on its intended use in humans.

Animal welfare considerations: Guidance on the use of clinical signs as humane endpoints for experimental animals used in safety evaluation [77] have been reviewed elsewhere and the reader is advised to look it up. In particular paragraph 62 of the guideline thereof, should always be followed. This paragraph states that "In studies involving repeated dosing, when an animal shows clinical signs that are progressive, leading to further deterioration in condition, an informed decision as to whether or not to humanely kill the animal should be made. The decision should include consideration as to the value of the information to be gained from the continued maintenance of that animal on study relative to its overall condition. If a decision is made to leave the animal on test, the frequency of observations should be increased, as needed. It may also be possible, without adversely affecting the purpose of the test, to temporarily stop dosing if it will relieve the pain or distress, or reduce the test dose."

Animals: Different rodent and non-rodent species are used in animal toxicity tests. In chronic studies, justification is often required for choice of specie or strain of animals used. All animals should be housed in acceptable environmental conditions and adequately catered for in accordance with stipulated guidelines [78].

Regulatory requirements: An independent animal ethics committee usually reviews, approves and supervises animal experiments and ensures that the experiment is well justified and in agreement with provisions for animal welfare. These regulations may differ, dependingon different countries, but basic requirements to be met remain unchanged.

4.1. Acute systemic toxicity

This test measures relative toxicological response of an experimental organism to single or brief exposure to a test substance [79]. Test organisms range from simple systems like brine shrimp to other animals like mice, rats, guinea pigs and rabbits. This test is also used to calculate median lethal dose (LD_{50}) of a substance, using various standardised methods including Lorke's and acute toxic class methods [79, 80]. Exposure routes may be by oral gavage, inhalation/mucosal, dermal; or by injection into the bloodstream, abdomen, or the muscles. Following administration of a test product, animals are observed individually at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days in the case of delayed toxicities [79]

4.2. Sub-acute/sub-chronic toxicity

This is repeat-dose study performed to expose any deleterious changes in organ, haematological and biochemical indices that may arise in the course of repeated administration of a test substance, usually ranges from weeks to a few months. The terms 'sub-acute' toxicity and 'sub chronic' toxicity can be differentiated on the basis of exposure, the former having a duration period of one month (28-30 days) and the latter ranging from two to three months (60-90 days). The test product or compound is usually administered daily throughout the test period and at the end of the study, data generated will include general parameters such as daily food consumption and water intake measurements and body weight measurements. Other specific endpoints of toxicity assessed will additionally include serum biochemical parameters (Lipid, protein, urea, creatinine, electrolytes, liver transaminases and phosphatase), enzymatic and non-enzymatic liver oxidative stress indicators (thiobarbituric acid reactive substances, reduced glutathione, catalase) and haematological parameters (white blood cells and differentials, red blood cells, haemoglobin, haematocrit, platelets, lymphocytes). Various organs are examined for gross pathological changes and tissue slices obtained from respective organs are prepared for detailed histological examination.

Results of many sub chronic toxicity tests of various plant extracts showed that the major organs usually affected are the liver and kidneys. Hepatotoxic and nephrotoxic effects are mostly to be expected, as the liver acts as the main detoxifying organ for chemical substances, while the kidney is a principal route of excretion for many chemical substances in their active and/or inactive forms [81].

Liver injury associated with the use of herbal medicine ranges from mild elevation of liver enzymes to fulminant liver failure often requiring a new transplant; and carcinogenesis [63]. Established hepatotoxic phytochemicals include podophyllin, eugenol, neoclerodane diterpenes, among others [83-88].
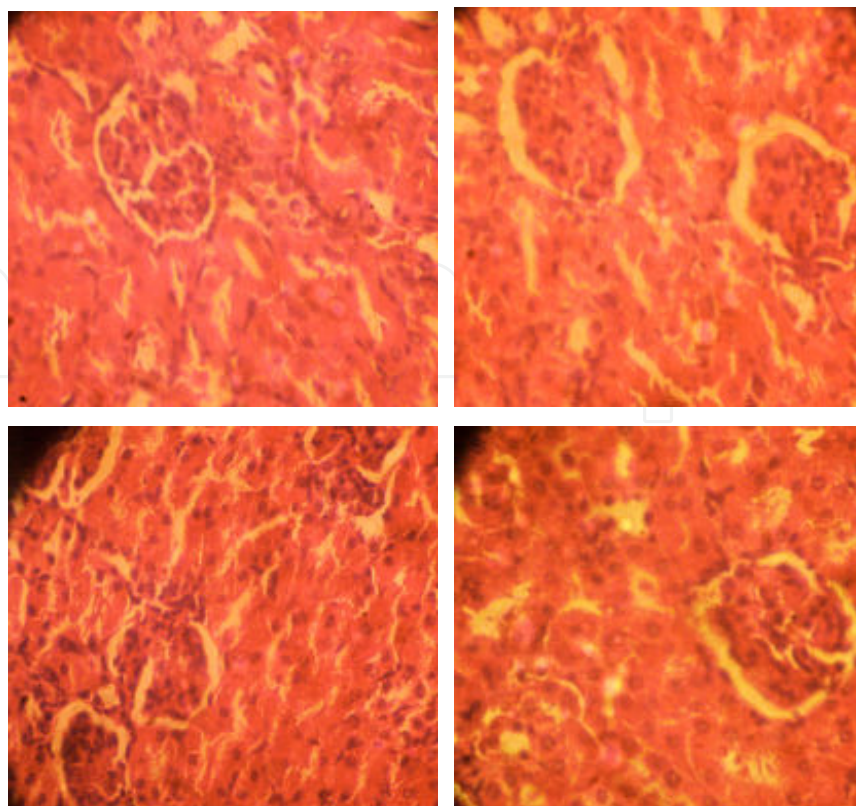
Figure 5. Clockwise from top left: Photomicrographs (×400) of kidney tissue slices from rats treated with (a) aqueous vehicle, (b) 25, (c) 50 and (d) 100 mgkg-1 body weight Hymenocardia acida ethanol leaf extract. Fig. 5a shows normal tubular architecture while Fig. 5b, c and d show alterations ranging from mild cortical oedema to tubular distortions. [82].

5. Chronic toxicity/carcinogenity

Chronic toxicity testing is similar to sub chronic studies except that they are conducted with a larger number of animals to reveal toxicity which may arise during exposure to a substance for a 24 months or for an entire lifespan. Oral, dermal or inhalation are the main routes used here, depending on the intended use in humans. In these long-term studies, mutagenic/carcinogenic propensities of test substances and likely organs where they may accumulate are revealed. End points of toxicity which are studied include dose limits of toxicity, that is, the lowest dose at which no toxicity occurs or no observed adverse effect level (NOAEL), mortality, food consumotion, water intake, hematology and clinical biochemistry measurement, organ gross necropsy ang histopathology. Further informmation on study design and execution can be found in OECD draft guidance document on the design and conduct of chronic toxicity and carcinogenicity studies [89].

5.1. Specialized tests

These are tests suited to reveal specific toxicities, such as reproductive toxicity, developmental toxicity, eye and skin irritancy test (Draize test), neurotoxicity and Genotoxicity.

Ocular/Skin irritancy test:

Named after a US food and Drug Scientist, John Draize, this test was developed in the mid-nineteenth century. Eye and skin irritancy tests involve the topical application of the test substance; usually in rabbit cornea or skin. Irritancy is reversible in nature and distinguished from corrosion which is irreversible. This test has become unpopular due in part to the percieved cruelty to the rabbit its very subjective scoring system, leading to poor reproducibility and high variablility between laboratories [90]. A recently developed short term exposure test using Statens Seruminstitut Rabbit Cornea (SIRC) cells has been demonstrated to be a potential alternative for eye irritancy test in rabbits [91].

Neurotoxicity:

Neurological effects such as convulsions may arise followed acute systemic exposure to some phytomedicines; while cerebrovascular accident, encephalopathy and psychosis can become evident in sub acute, sub chronic and chronic tests for toxicity. It is important to note that the presence of high levels of metals in the herbal medicine can contribute to neurotoxicity [92]. Microbial biosorptive removal using granulated *Cladosporum cladosporioides* and chelation with dithizone have been shown to be effective in removing heavy metal contaminants from herbal extracts [93, 94].

Genotoxicity:

Genotoxicity is a special area in toxicities, as it is often the most difficult to detect. It may be defined as a chemically induced mutation or alteration of the structure and/or segregation of genetic material.Recently, a guidance document on the assessment of genotoxcicity of herbal preparations has been drafted by the European Medicines Agency [95]. The first stage utilizes the Ames test with *S. typhimurium*, although some potent genotoxins like Taxol (*Taxus brevifolia*) and vincristine (*Catharanthus roseaus*) are not reliably identified at this stage and some products rich in flavonoids like quercetin may give false positives. More reliable tests like the mouse micronucleus test and mouse lymphoma assay (MLA) can be used more definitively [95].

Reproductive/developmental toxicity studies:

These studies were developed after it was discovered thousands of offspring of women who used the new drug thalidomide to treat morning sickness were born with serious birth defects [96]. It was later proposed that the drug acts by decreasing transcription efficiency of the genes responsible for angiogenesis in the developing limb bud of the foetus, resulting in truncation of the limb [97]. In designing these tests, a large number of animals are used, which are dosed repeatedly with escalating doses of the herbal test substance before mating, during gestation and after delivery up to the entire lifetime of the new offspring to detect effects of the herb on reproductive performance and/or developing offspring. Toxicity endpoints include spontaneous abortion, premature delivery, and birth defects.

In addition to the use of rodents, research in reproductive and developmental toxicity of traditional Chinese medicine incorporates other animal models like zebrafish and roundworm models and stem cell cultures [98].

5.2. Clinical testing: Clinical/safety trials

After sufficient preliminary investigation showing the safety of an herbal product in pre-clinical studies, further studies can then be initiated in human participants. These type of studies are called clinical trials (CTs) and are carried out in four phases, I – IV [99].

Phase I: These are CTs that are specially designed with a minimum number of human participants that voluntarily consent to partake in assessing the impact of use of the herbal product on vital physiological indices. It is the usually the first stage of testing in healthy humans to determine the safety and maximum tolerable doses of the investigational substance before any further human testing may be carried out. It is however acceptable that for certain herbs with long history of use, this phase may be unecessary [99]

Phase II: Studies carried on a limited number of participants to determine clinical efficacy, also labelled as feasibility studies. In this study, doses that are observed to be relatively safe are used, participants are also monitored for the occurence of adverse effects [99].

Phase III: In this phase, a larger number of participants is used in different centres and the study is designed as a randomized, double-blind, controlled CT. It is a validatory study for clinical efficacy of the herbal product, usually compared with a standard intervention [99].

Phase IV/post-marketing surveilance: Monitoring for rare side effects which may have been unnoticed during Phases I – III but may occur after the product has been introduced to the market [100].

There are critical issues which must be considered to provide justification for the clinical trial of herbal products and guidelines to this effect have been provided by the World Health Organization [99]. These areas of consideration are listed below:

Chemistry-manufacturing-control: Unlike conventional medicines, herbal medicines are frequently monoherbal or polyherbal with wide chemical composition. While it is not required for an active compound to be isolated as it is accepted that the action of the compounds in the product may be synergistic, a means of standardisation has to be used for the product that would be representative of the final product. If the active principle is known, it can serve as the marker for the product. If unknown, a chemical marker of sufficient quantity or a chemical fingerprint of the entire product can be used, within specified limits. Preparation of the herbal medicine intended for administration in a clinical trial also has to be carried out in accordane with WHO guidelines on good manufacturing practices for herbal medicines [101].

Provision of information on the herbal substance and the herbal product is also an important requirement. This includes a description of the source of the plant and its processing, storage conditions and shelf life. Information regarding the product including excepients, dosage form, analytical parameters for active compound or chemical markers, storage conditions over the lenght of the trial and specifications that would be assessed before clinical trial material is released will also need to be furnished.

Non-clinical considerations: This constitutes a supportive background upon which a clinical investigation is based. In general, data on efficacy, toxicity and pharmacokinetics which have been demonstrated or obtained from appropriate literature sources including journal publications and reference pharmacopoiea. A systematic review of earlier trials of the same herb or a related one can be done where possible in order to identify gaps that can be bridged in the proposed trial.

Clinical considerations: At all stages of the trial, ethical standards and quality requirements have to be met. For a phase 1 safety study, the adverse effects related to increasing doses of the test product are observed in human participants recruited within the limits of inclusion based on gender, weight, age and health status. An outline of the basic safety parameters that are monitored are shown in Table 2. The standard intervention is usually the product itself. The study may be randomized, blinded, double blinded or placebo-controlled to minimize bias.

Ethical considerations: All CT protocols require approval by regional ethical board before such trials can be executed. All research that involves human participation, including clinical trials must apply fundamental thical principles and must adhere to standards of good clinical practice [102]. Informed consent of all participants or gaurdians of minor participants must be obtained. It is required that each participant is well informed of any concerns regarding the trial herbal product especially with respect to rarely understood interactions, or known undesirable effects. Risks to participants must be minimized and as such, experienced ethical investigators including clinicians who can promptly identify and treat observed adverse events in participants need to be involved as CT investigators.

Organ/system	Safety parameter
Neurological:	lack of neurologic symptoms
Musculoskeletal:	lack of arthritis or myalgias, normal values of CPK
Skin:	clinical evidence of lack of allergic reactions
Gastrointestinal:	clinical evidence of tolerability
Liver:	normal values of SGOT or SGPT, alkaline phosphatase, total bilirubin,
Kidney:	normal values of BUN or creatinine
Endocrine system and metabolism:	normal values of albumin or total protein, uric acid, glucose, cholesterol, amylase or lipase, sodium/potassium, calcium
Cardiovascular:	normal EKG and blood pressure
Haematopoietic:	normal values of complete blood count
Additionally:	more intensive investigation of any organ system likely to be affected by the product

Table 2. Basic physiological parameters monitored in phase 1 clinical trial [99]

6. Conclusions and perspectives

Summarily, the processes involved in the toxicological evaluation of complex herbal extracts/mixtures and chemically characterized isolated compounds are schematically represented below. It is noteworthy that currently, only chemically characterized phyto compounds are useful candidates for QSAR studies and high throughput toxicogenomic assays, as compound data libraries exists for data comparison.
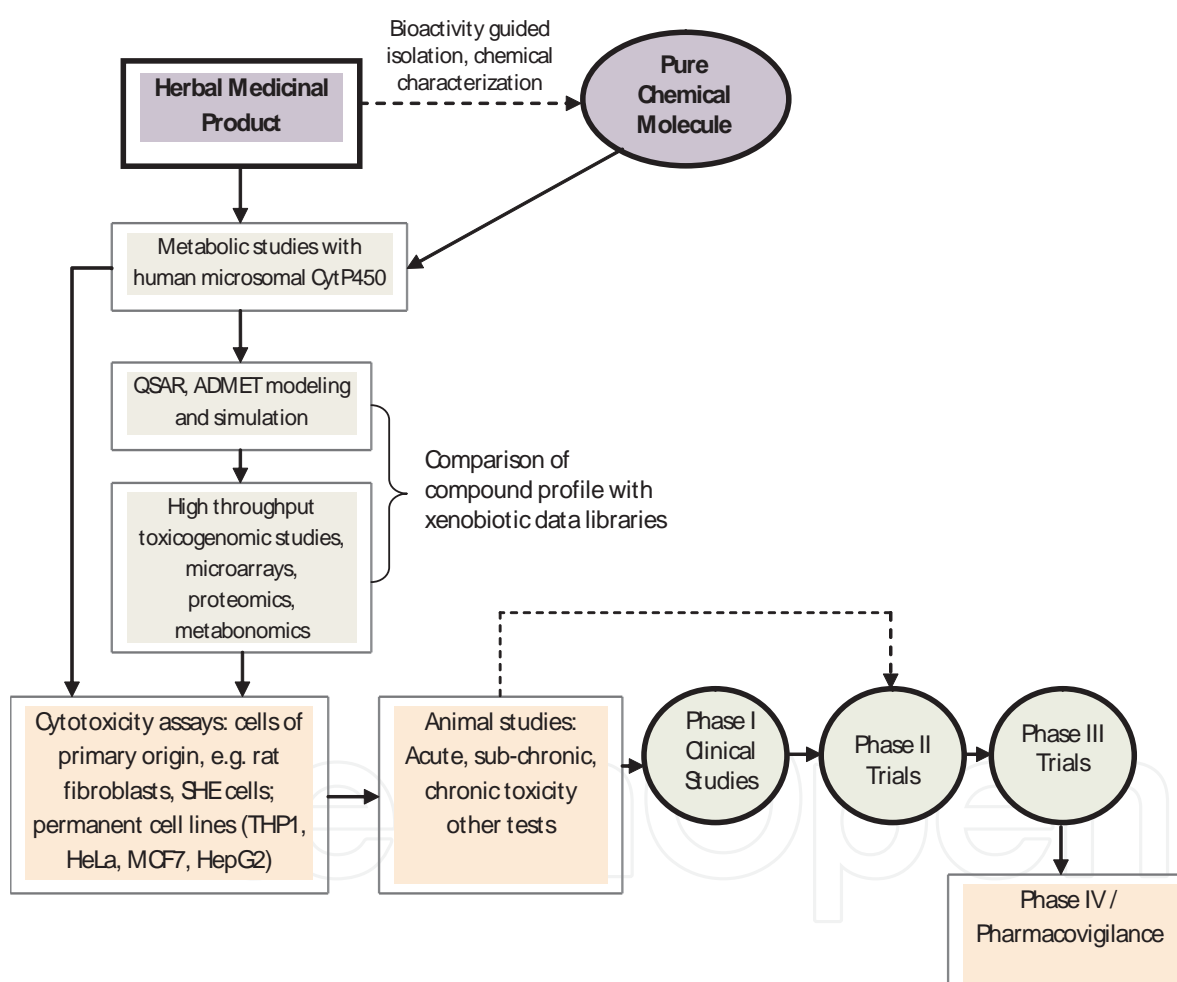

Figure 6. Schematic processes involved in evaluating and establishing the toxicity of medicinal herbs. The broken arrow indicates that for some herbal medicines, phase 1 clinical trials may not always be necessary.

Toxicity testing of herbal medicines in the 21st century tends to begin in a reductionist manner and proceeds through holistic tests to reach clinical conclusions. The challenge however,

remains the identification of unique approaches in testing and developing regulations regarding safety of herbal products. Although some drawbacks to animal testing exists; such as the large number of animals used, financial implications and poor validation which affects correlation to humans, animal testing is still relevant as it is still impossible to predict long term carcinogenicity, embryotoxicity and reproductive toxicity using alternative non-animal tests alone.

A major issue in toxicity testing is "Animal welfare". The use of animals in research gave rise to the adoption of the critical 3 R's to consider before conducting animal-based toxicity testing of herbals. This calls for a fundamental paradigm in regulatory toxicology; there is a need to reduce the number of animals, refine the tests methods used in order to minimize pain and suffering of experimental animals, and replace animal tests with validated alternatives employing human cells where possible. Some instances of efforts in this regard are the development of a transcriptomics based *in vitro* screening method to predict embryotoxicity using the embryonic stem cell test. Additionally, the number of rats used for LD_{50} tests can be significantly reduced by the adoption of *in vitro* cell-based assays and chemicals shown to be harmful to cultured cells are excluded from any further LD_{50} tests and animal tests. It is no longer news that, cellular models of toxicity are more rapid and can easily be adapted to high throughput screening.

Next generation sequencing technology and toxicogenomics are strong predictive tools but databases of genetic biomarkers of toxicity of herbal medicines need to be enriched. It will be worthwhile to develop data libraries upon which prediction of the safety herbal extracts can be done to fully exploit these screening tools. As pointed out earlier, this can be achieved by creating genomic signatures of identified phytochemicals which can serve as data library for herbals.

Standardization of an herbal product in terms of parts per million limits of heavy metals will also eliminate product contamination and its associated toxicity. Chemical standardization of medicinal herbs with High Pressure Liquid Chromatography (HPLC) alone or hyphenated with Mass Spectroscopy (HPLC-MS) or Nuclear Mass Resonance Spectroscopy (HPLC-NMR) would also ensure chemical uniformity and detect chemical adulterants in herbal products.

More so, the integration of recent biotechnological innovations like computer-aided modeling and simulation studies, bioinformatics, high throughput screens, toxicokinetic and toxicogenomic tools in a systems toxicology approach with other necessary tests in experimental animals and appropriately designed clinical observation studies will undoubtedly bring about significant advances in predicting and determining herbal medicine safety.

Acknowledgements

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