Review Article

Recent Advancement in *in-vivo* and *in-vitro* Toxicity Studies for Ayurvedic Formulation

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ABSTRACT

Aim: Ayurvedic formulation consists of natural substance. Many of Ayurvedic formulation consist of heavy metals and some species are poisonous in nature. So it is necessary to determine the toxicity of Ayurvedic formulation. **Background:** Toxicology is a science that involves the study of the adverse effect of the substance on living organism. The toxicity of the substance can be observed by: a) *in vivo* (using the whole animal), b) *in vitro* (Testing on isolated cell or tissue). *In vivo* toxicity study involves acute toxicity, sub-acute toxicity, sub-chronic toxicity and chronic toxicity studies. *In vitro* toxicity testing of substance involves model such as model for cytotoxicity, specific toxicity, genotoxicity and toxicokinetic. The challenges regarding *in vivo* and *in vitro* toxicity study and recent development in the toxicity studies are discussed briefly. **Conclusion:** This review mainly focus on the various methods and model used for *in vivo* and *in vitro* toxicity testing of substance and provides information about the toxicity study which will be useful for the researchers who are working in the field of toxicology as well encourage researchers to work on various areas of research for the development and enhancement in acceptance of Ayurveda.

Key words: Ayurvedic formulation, *in vivo* toxicity, *in vitro* toxicity, Specific toxicity, Toxicokinetic, Toxicology.

INTRODUCTION

Ayurvedic medicine is an ancient system of health care that is native to the Indian subcontinent. Ayurveda is known as "mother of healing".[1] Ayurvedic formulations consist of natural substance which are usually having a wide therapeutic range and effectiveness in large number of disease.[2] Ayurvedic formulations are cheaper and also have less side effect as compared to allopathic drugs. There is also a misconception about the Ayurvedic formulation that they are always safe. Charaka samhita itself has described that ayurvedic medicine have adverse effect. It is known that the presence of heavy metals in pharmaceutical is not allowed, to avoid toxicity. But the concept of Rasa Shastra is practised in large number in which metals are added to form Rasausadhies (Herbo-bio-mineral-metallic preparation). Approximately 6000 medicine in the 'Ayurvedic formulary' contain metals like mercury and lead. These metals shows hepatotoxicity, nephrotoxicity, neurotoxicity and hematotoxicity.[3] Researchers have revealed that metal content or some poisonous species used in Ayurvedic formulation report toxicity cases throughout the last decade[4] (Table 1). Contamination and deterioration of Ayurvedic formulation can be prevented by proper storage method which ensures the safety and efficacy of the product. The storage condition should be avoid for Ayurvedic formulation are i) storing in open spaces, ii) using inappropriate package material, iii) storing the material for long period, iv) keeping the material within

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Table 1: Examples of Ayurvedic Formulation Reported Toxicity.

S.NO	Ayurvedic Formulation	Result	Reference
1.	**Rasasindura**	Acute toxicity result showed that Rasasindura did not produce any signs and symptoms of toxicity or mortality up to an oral dose of 2000 mg/kg in Wistar rats. Chronic toxicity results showed that Rasasindura, even at a level as high as therapeutic equivalent dose×10 level, had no significant effect on the hematological parameters. Although the drug produced mild to moderate adverse changes (in kidney, liver, intestine and stomach) at therapeutic equivalent dose×10 dose level. The observed changes were not seen at the lower dose levels as well as in the recovery study. Hence, it is suggested that the Rasasindura, is safe for consumption at the therapeutic dose level.	9
2	**Krshinadi churna**	In this *in vitro* toxicity assay it is observed the formulation of Krshnadi Churna. Shows non-toxic activity against Hela cell line with IC_{50} value of 40.25 µg/ml for hydroethanolic fraction respectively by MTT assay.	55
3	**Arogyavardhini vati**	Arogyavardhini vati at doses of 50, 250 and 500 mg/kg (1, 5 and 10 times of human equivalent dose respectively) administered to rats for 28 days does not have appreciable toxicological effects on brain, liver and kidney.	10
4	**Vasaguduchyadi Kwatha**	Acute toxicity test was evaluated as per OECD 425 guidelines with 5 000 mg/kg as limit test in Wistar strain albino rats. Test formulations were administered to overnight fasted animals and parameters like body weight, behavioral changesand mortality were assessed for 14 days. Hematological and biochemical parameters were assessed on 14th day. The samples of Vasaguduchyadi Kwatha are relatively safe up to the dose of 5 g/kg.	11
5	**Mahanarayana taila**	Ayurvedic formulation was evaluated for safety in Wistar rats by acute and sub chronic (91 days) dermal toxicity studies. The test drug was applied externally and the animals were observed for the physical and clinical synptoms of toxicity in comparision to animals in control group. Skin and internal organs did not reveal structural changes suggestive of toxicity upon gross examination and histopathology investigation. Mahanarayana taila was found to be safe upon single and repeated dermal exposure in wistar rats during the study.	12
6	**Shwasakuthara Rasa**	Evaluation of the acute toxicity and anti-tussive activity of SKR one prepared with Kajjali (SKR1) and another without Kajjali (SKR2) in sulphur dioxide induced cough model in albino mice. The presence of Kajjali in the formulation is safe on acute administration and further enhances anti-tussive activity of the formulation may be due to increasing bioavailability of Ayurvedic formulation.	13
7	**Hartal (Orpiment) and Rasa Manikya (Processed Product of Hartal)**	3 experimental compounds namely IH (impure hartal in crude form), PH (pure Hartal detoxified with fruit juice of Benincasahispida) and RM (prepared by putting PH between mica sheet and heated for 5- 10 min) were used. On the basis of therapeutic dose, drug dose was decided16.25 mg/kg BW. In experimental part, total 24 albino male mice, each weighing 25-30 gm were taken and randomly divided into 4 groups, 6 in each group (control, IH, PH and RM). After 28 days blood was collected by extirpating eyeball for RFT and LFT there after all animals were sacrificed, dissecting kidney, liver, part of intestine and skin out for histopathological study. Thus IH is probably toxic, PH is non-toxic and RM is mildly toxic.	14
8	**Tamra Bhasma**	In this study Tamra Bhasma was administered orally, daily to different groups of albino rats in TD (Tamra Bhasma) and 2 TD (Tamra Bhasma 2 x Therapeutic Doses) doses for 3 months. Tamra Bhasma was found to be relatively safe at these dose levels. There was no mortality	15
9	**Navratna rasa**	The drug was screened for its safety/toxicity studies in acute and chronic models. No mortality and behavioral changes were observed during the course of acute toxicity study. The chronic toxicity study reveals that, the test drug has no serious toxicity potential to most of the important organs in therapeutics doses.	16
10	**Punarnava Mandur**	Study for repeated dose oral toxicity study in Wistar rats for 90 days. Total 48 Wistar rats (24 male and 24 female) were selected based on the body weight and were randomly distributed into four groups followed by administration of Punarnava Mandur at the dose of 0, 90, 450, 900 mg/kg body weight for 90 consecutive days. Hence, the dose level 450 mg/kg of Punarnava Mandur was found as NOAEL (No Observable Adverse Effect Level). However, the NOEL (No Observed Effect Level) could not be established. It was suggested to carry out a toxicity study at possible higher doses so as to establish target organ of toxicity.	17
11	**Swamala**	Swamala at the doses of 0, 3, 6 and 15 g/kg was administered for 90 consecutive days. After 90 days of oral administration Swamala did not show any gross toxicological signs and histopathology also when compared with normal. All animals in Group IV showed significant increase in body weight as compared to that of control group animals. No mortality was observed throughout the period.	18

abnormal heat and moisture.[5] Toxicology is a science that involves the study of the adverse effect of the substance on living organism. Adverse effect depend on two main factor: i) route of exposure (Oral, inhalation, dermal) and ii) dose (Duration and concentration of exposure).[6] The toxicity of the substance can be observed by: a) *in vivo* (Using the whole animal), b) *in vitro* (Testing on isolated cell or tissue) and c) *in silico* (in a computer simulation).[7,8]

In vivo Techniques

The term *in vivo* is derived from Latin word which is defined as the test study that is performed in living organism. Initially, *in vivo* experiments were aimed for the prediction of acute systemic toxicity usually in rodents. Currently, more sophisticated, targeted and multispecies approaches with well-defined experimental protocols are applied to toxicological studies, especially for regulatory testing. The animals that are most commonly used in toxicological testing are rodents and rabbits (Table 2). Cats and dogs are used less frequently in toxicity testing and mostly in preclinical toxicology or phase I pharmacological studies, whereas nonhuman primates are rarely used and mainly to study metabolism of toxic compounds. Pharmacological effects of drug are same in human as in animals due to which non-clinical studies in animal is required before administration to humans. Toxic effect in species will predict adverse effect in human. Therefore, risk assessment can be done by comparison of toxic doses in test species with predicated therapeutic human dose.[19]

Many toxicity methods include the use of laboratory animals. Therefore 3Rs concepts was first describe by William Russell and Rex Burch in the Principle of Humane.[46] The 3Rs concepts are further define as:

Reduction alternative- decrease the number of animals required for a test method.

Refinement alternative- use procedure that minimized or reduce the pain or distress in animals.

Replacement alternative- use of non-animal system instead of animals or use lower species of live animals.

Institute Ethics Committees

Before conducting any toxicity test on animals the study or protocol should be approved by the Institute Animal Ethics committee (IAEC). In India, the committee for the purpose of Control and Supervision of Experiment on Animal (CPCSEA) guideline are to be followed for the maintenances of experimental animals.[47]

Types of Toxicity Studies

There are many different types of toxicity studies carried out for evaluation of toxic effect of therapeutic agent. The traditional methods of determining toxicity of drug or chemical include acute toxicity study, sub-acute toxicity study, sub-chronic toxicity study and chronic toxicity study.[48]

Acute Toxicity Testing

Acute toxicity refers to that adverse effect of a single dose of a substance on a particular species of animal. In acute toxicity testing, the test sample is administered at different dose levels and the effect is observed for 14 days.[49] All mortalities caused by the test sample during the experimental period are recorded. Acute toxicity testing permits the 50% lethal dose (LD_{50}) of the test sample to be determined. The determination of the LD_{50} involves large numbers of animals and the mortality ratio is high because of these drawbacks modified methods were developed.

Table 2: Examples of Animal Models Used in Selective Toxicity Tests.

Species	Toxicity Tests	References number
Rat	Developmental toxicity	20,21,22
	Carcinogenicity	23,24
	Cutaneous toxicity	25
	Genotoxicity	26,27
	Immunotoxicity	28
	Neurotoxicity	29
	Reproductive toxicity	30,31
Mice	Carcinogenicity	23,24
	Skin sensitization	32
	Genotoxicity	26,27
	Immunotoxicity	28, 33
	Neurotoxicity	29
	Reproductive toxicity	31
Guinea pigs	Cutaneous toxicity/skin sensitization	25,32,33
	Developmental neurotoxicity	34
Hamsters	Carcinogenicity	35
	Genotoxicity	36
Rabbit	Developmental toxicity	37,38
	Cutaneous toxicity	25,40
	Reproductive toxicity	37
Hen	Neurotoxicity	39,41
Dog	Carcinogenicity	36
	Cutaneous toxicity	42
	Neurotoxicity	43
Monkey	Developmental toxicity Cutaneous toxicity	44 45

Fixed Dose Procedure

Fixed dose procedure was first proposed by British toxicology society in 1984. In 1992 this test was proposed as an alternative to the conventional LD_{50} test by the organisation for economic co-operation and development under the OECD test guideline 420.[50] The objective is to identify a dose that produces clear signs of toxicity but no mortality. Depending on the result of the first test, further testing is needed or not is to be decided. If mortality occurs, then retesting is done at a lower dose. If no sign of toxicity occur at the initial dose, it requires retest at a higher dose. The result are thus interpreted in relation to animal survive and evident toxicity.[51] In comparison to conventional LD_{50} test this procedure produce similar result while using fewer animals and cause less pain and suffering.[52]

Up-and-Down Procedure

Up-and-down procedure was developed by OECD in 1981 and revised many times. In the up-and down procedure animals are dosed once at a time. If an animal survive the dose then the increased dose is given next time. If animal dies, then the dose is decrease. It is recommended that surviving animal is to be monitored for the delayed death for a total 7 days. Testing in females alone is recommended, based on the observation that females are more sensitive and selective follow-up in male may sometime indicated.[53] As compared to conventional procedure, this method permits a major reduction in the number of animal used.

Sub-Acute Toxicity Testing (Repeated Dose Acute Toxicity Testing)

Repeated dose acute toxicity is carried out for minimum 28 days. The test substance is administered daily at a specific time. Rodent 5-7 weeks of age are preferred with average weight of 20% the standard deviation. Animals are observed for toxicity signs.[48] The interpretation of human safety details is essential in repeated dose toxicity studies.

Sub Chronic Toxicity Testing

Sub chronic toxicity is the study carried out over 90 days and weekly body weight variation, cardiovascular parameter changes are observed. At the end of the experimental animals are scarified and all the tissue are subjected to histopathological analysis.[54]

Chronic Toxicity Testing

Chronic toxicity is the long term toxicity study that last as long as the life span of the test animal usually 1-2 year. Rodent like mice and rat are mostly used. These types of test can be conducted on drugs developed for terminal disease such as AIDS, cancer. Carcinogenicity testing is under chronic toxicity testing. The animals are scarified for gross pathology and histopathology.[48]

In vitro Techniques

The term *in vitro* is derived from Latin phrase which means "the technique of performing a given procedure in an artificial environment outside the living organism". *In vitro* methods are widely utilised for screening purpose. The *in vitro* models are much more useful as they do not require live animals for toxicity testing.[55] The 3Rs states replacement with non-animal model, reduction- of number of animal and refinement- to decrease animal suffering. This is universally accepted on the basis of good laboratory practices.[56] A number of *in vitro* test gain wide acceptance in order to replace *in vivo* cytogenetic with *in vitro* cytogenetic.[57] The need of *in vitro* models for toxicity assessment is due to increase in ethical issues. The prime concern while using animal model for toxicity testing is to avoid animal killing.[58] There are several types of cell cultures (Table 3) available for *in vitro* testing that offer various degrees of complexity and relatedness to the *in vivo* situation. In order of increasing complexity and genetic similarity to the tissue of origin, these include permanent cell lines, primary cultures, stem cells and organotypic cultures.[59]

Methods used for *in vitro* Toxicity Studies

Many different *in vitro* models have been in use over the year, in which cell lines are the best model for toxicity study. *In vitro* toxicity testing of substance involve model

Table 3: Organization of a Tiered System for *in vitro* Toxicity Testing.60

Culture Type	Suitability/Limitations
Mitotic cell lines	Medium to high throughput studies of basal toxicity (e.g., membrane damage, viability, etc.) and cell proliferation. If immortalized, many cell lines are tumor-like. Limited cell–cell interactions and drug metabolism.
Differentiating cell lines	Medium to high throughput screening and Mechanistic studies of developmental toxicity and target cell specific toxicity. Often short-lived. Limited cell–cell interactions and drug metabolism.
Primary cell cultures	Developmental or target cell-specific toxicity. Genetically more similar to target system but generally heterogeneous and short-lived. Can be used as co-culture systems to simulate cell–cell interactions of target tissue but usually have limited drug metabolism.
Organotypic / whole organ cultures	These are tissue slices or cultures organs that can maintain cell interactions and tissue function. Generally unsuitable for medium to high throughput analysis and may exhibit limited drug metabolism.

for cytotoxicity, specific toxicity, genotoxicity and toxicokinetic.

Cytotoxicity Study

Cytotoxicity is the study of being toxic to the cells. The cytotoxicity test use tissue cells *in vitro* observe to measure the cellular response toward a toxic substance.[48] Some of assay to measure cytotoxicity are.

MTT Assay

MTT assay is a colorimetric assay for measuring cellular growth. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay can be used for toxicity study of the substance. The water-soluble yellow dye MTT is a tetrazolium salt that is taken up by the viable cells and reduced into purple colour formazan by the action of mitochondrial succinate dehydrogenase in mitochondria of living cells.[61] An organic solvent, dimethyl sulfoxide is used to dissolve insoluble formazan crystal, a purple coloured product which is measured by spectrophotometer. The amount of formazan produced is directly proportional to the number of viable cells present in the sample.[62]

Protocol[63]

Cells were culture in a 96-well plate at a density of 1×104 cells/well and allowed to adhere for 24 hours at 37°C in a CO_2 incubator.

After 24 hrs of incubation, culture medium was replaced with a fresh medium.

Cells were then treated with various concentrations of the desired compound for 24 hrs at 37°C in a CO_2 incubator.

After 24 hours of incubation, culture medium was replaced with a fresh medium.

Subsequently, 10 µL of MTT working solution (5 mg/mL in phosphate buffer solution) will be added to each well and the plate was incubated for 4 hr at 37°C in a CO_2 incubator.

The medium were then aspirated and the formed formazan crystals were solubilized by adding 50 µL of DMSO per well for 30 min at 37°C in a CO_2 incubator.

Finally, the intensity of the dissolved formazan crystals (Purple color) will quantified using the ELISA plate reader at 540 nm.

Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically Standard Graph was plotted by taking concentration of the drug in X axis and relative cell viability in Y axis.

$$\text{Cell viability (\%)} = \frac{\text{Mean OD}}{\text{Control OD}} \times 100$$

Neutral Red Dye Uptake Assay

The neutral red (NR; 3-amino-7dimethyl-2-methylphenazine hydrochloride) dye uptake is another cytotoxicity assay, which provides quantitative estimation of number of viable cells in a culture. NR is a weak cationic dye that penetrates into the cellular membranes and accumulates intracellular in lysosomes. Viable cells incorporate NR dye into their lysosomes. As the cell surface alters or cell dies, their ability to uptake NR dye decreases. The absorbance is read using a spectrophotometer. Thus the loss of NR uptake inside lysosomes corresponds to loss of cell viability.[64]

Protocol[65]

Cell monolayers grown in 48-well culture plates were incubated for 48 h at 37°C. Then, medium were removed and 500 µL of NR solution (30 µg/mL in MM) will be added to each well.

The plates were incubated once more for 3 h at 37°C to promote the uptake of the dye by cells.

The monolayers were washed with PBS and 500 µL of extraction solution (H_2O: acetic acid: ethanol) (49: 1: 50) was incorporated in each well.

After gently shaking the plates, the absorbance was read on a multiwell spectrophotometer at 540 nm.

The CC_{50} was calculated from concentration-effect curves after nonlinear regression analysis

Lactate Dehydrogenase Assay

Lactate De Hydrogenase (LDH) is an oxidoreductase enzyme found in mostly all living cells (Animals, plants and prokaryotes) that is released into the cytoplasm upon cell lysis.[66] It is also a colorimetric cytotoxicity assay that measures the membrane integrity. The level of LDH is more in damaged cells as compared to normal cells. The LDH activity is measured on the basis of the conversion of lactate to pyruvate. LDH reduces Nicotinamide Adenine Dinucleotide (NAD) to reduced NAD (NADH) and release H+ ions, these ions catalyze reduction reaction of the tetrazolium salt to give the coloured formazan compound, which shows the absorbance at 490–520 nm wavelength. The oxidation of NADH to NAD+ is detected spectrophotometrically which show absorbance at 340 nm. NADH shows more absorbance in comparison to NAD+ at 340 nm. The amount of colour product formed is directly proportional to the activity of LDH in the sample.

Specific Toxicity

In vitro Models for Liver Toxicity

Cell lines are extensively used for assessment of liver toxicity because they display similar genotypic and phenotypic characteristics of normal liver cells with

functional enzymes responsible for phase I and phase II metabolism.[67] Liver cell lines are the best choice for toxicity testing for detection of toxic substance and evaluating their cellular mechanism of toxicity. Liver cell lines HepG2, Hep3B, HBG and HepaRG are commonly immortalized liver derived cell lines used for *in vitro* testing of liver toxicity study.[68]

In vitro Models for Lung Toxicity

In vitro models have been used for testing of lung toxicity studies. Cell line is recognized as a useful *in vitro* model for the assessment of damaging effects and contributed to increase our knowledge about mechanism involved in pulmonary toxicity.[69] The A549 cell line has been widely used in the study of the human lung damage caused by toxic substances.[70]

In vitro Models for Neurotoxicity

Various cell lines are used to study the effect of various toxic substance on neuronal cells such as neuroblastoma cells. Various classes of chemotherapeutic agent causing human neurotoxicants and neuritis were identified.[71] *In vitro* systems are most successfully used to elucidate the mechanism of neurotoxicity and to describe the developmental changes induced by neurotoxicants.[72]

In vitro Models for Immunotoxicity

The immune system plays a major role in maintaining human health, from the toxicological point of view, this system can be targeted from immunotoxic effects of variety of chemicals including the environmental pollutants like polychlorinated bisphenols, chlorinated dibenzo-p-doxins, pesticides and heavy metals, therapeutic drugs and any other foreign substances often called as xenobiotics.[73] Heavy metals are considered to be immunosuppressive and ranked according to their immunosuppressive properties.[74]

Enzyme-linked immune sorbent assay as well as quantification of activated CD^{4+} and CD^{8+} T-cell subset by flow cytometry clearly demonstrates chemical-induced deregulation leading to autoimmune phenomena.[74] Human microglia (SV 40) and monocytic cell line (THP-1) are commonly used for immunotoxicity studies.

Genotoxicity

In vitro test systems are known to determine the possible genotoxic potential of a test compound, which involves different stages of mutations: (1) gene and (2) chromosome.

Comet (Single-Cell Gel Electrophoresis) Assay

It is the assay for the assessment of the DNA damage. Its simplicity, sensitivity, short time duration and economy make it a prime choice in genotoxicity testing. Comet assay is based on the supercoiled duplex DNA strand breakage. The comets are formed from the broken part of negatively charged DNA molecules and become free to move toward the anode when the electric field is applied.[48]

The rate of DNA damage for each sample was calculated using the following formula:

DI (Damage Index) = $n1 + 2n2 + 3n3 + 4n4$,

Where $n1$ are cells included in category 1, $n2$ in category 2, $n3$ in category 3 and $n4$ in greater damage. Bioassays were performed in duplicate and 200 cells were analyzed per treatment: negative control, positive control and cells treated

Gamma-H2AX Assay

The damage of DNA is an important event able to affect cellular functions. Thus, it is essential for cells to maintain DNA integrity and repair such lesions effectively. Among different kinds of DNA lesions, Double Strand Breaks (DSB) are considered to be the most critical type of DNA damage and misrepair can lead to cell death.[75] In response to DSBs, H2AX are rapidly phosphorylated on its serine residue by several kinases of phosphoinositol 3-kinases, especially ataxia telangiectasia mutated and then called γ-H2AX. γ-H2AX induction is one of the earliest events detected in cells following exposure to DNA damaging agents.

Sister Chromatid Exchange Assay

Sister Chromatid Exchange (SCE) is the reciprocal exchange of chromatin between two identical sister chromatids. SCE possibly occurred during DNA synthesis either due to some replication error or due to inhibition of DNA replication.[76] This assay examines the ability of a test chemical to increase the exchange of DNA in duplicating chromosomes between two sister chromatids. This method is able to stain in the presence of 5-bromodeoxyuridine (BrdU) base, which is introduced to the chromatin.[77]

Toxicokinetic Study

Toxicokinetic study is essentially required to relate the dose or chemical concentration and the mode of action of the chemicals and its various metabolites. The basic toxicokinetic parameter is based on *in vitro* and *in silico* studies, which detects the potential of accumulation and the potential of distribution or inhibition of chemicals in the tissues/organs.[78] Toxicokinetic models can be divided into two broad categories depending on the function of time and dose: data-based compartmental models and physiologically based compartmental models.

Challenges and Consideration

The challenge with *in vivo* studies is using large number of animals in research with the advancement in medical technology. Every year, millions of experimental animals are used all over the world. For the experimental procedure either whole animal or its organ and tissue are used

by killing the animal. Many times, animal survive during the experiment and they are euthanized at the end of the experiment to avoid the later pain and distress.[78] The pain, distress and death experiment by the animals during experimental study have been debating issue for the long time. Animals have the right against pain and distress and thus, the use of animals for experiment is unethical and must stop. Therefore, various act and laws have been passed to bring the control over unethical use of animals. Beside this major concern of ethics, few more challenges of animal experiment are requirement of skilled or trained person and time-consuming protocols.[79] There is even always a limitation with extrapolating the *in vitro* data with *in vivo* studies in toxicological situations. The biggest problem with *in vitro* systems is the lack of biotransformation studies.[79] The authenticity of *in vitro* cell lines is still a big issue as there is always a misconception with contamination and often the type of cell lines are mistaken.[80] *In vitro* screening methods should be developed to test various cell lines parallel to different chemicals and biological metabolites. Special consideration should be given to characterize in cell-based assays so as to develop a possible understanding of the reaction in each cell of a particular assay. *In vitro* assay cannot provide true reflection of *in vivo* parameters. Thus, the use of animals for toxicity studies can be complemented by encouraging replacement, refinement and reduction.[81]

Recent Development in Toxicology

Alternative test are used to support the planning and interpretation of whole animal toxicity studies and are not yet used as substitute for toxicity studies using whole animals. Recent advancement that has been made by *in vitro* studies with isolated cell, tissue and organ.

Need for Developing Alternative Test

Economy and Efficiency

In vitro test may provide toxicity information in a cost effective and time-saving manner. Information generated from *in vitro* test systems can be used to increase the efficiency of whole animal studies and decrease the number of animals used in toxicity testing.

Bioinformatics and Computational Toxicology

Bioinformatics and databases of biological information can be used to create "maps" of cellular and physiological pathways and responses. Computational toxicology is a combination of mathematical and computer models to predict the response of any environmental agent and explain the series of events that follow on adverse effect. Bioinformatics and computational toxicology bridge the gap between data interpretation and software development. Its aim is to rapidly generate models for studying the functioning of cell, multicellular system and finally the organism. It can generate virtual test systems for quick screening of toxic chemicals.[82]

Integrated Testing Strategies

The design of testing strategies aims to make use of both existing and newly generated information to increase the quality of human safety assessment. Depending on the toxicological hazard assessed, there is a significant difference in testing strategies. There are various tests that stand alone for different parameters but a systematic combination of several information is often required. ITS can be described as an arrangement of test batteries covering important mechanistic steps and arranged in a hypothesis oriented form, which is of prime importance to make the efficient use of existing data so as to gain a summative understanding of the hazard or risk posed.[82]

Omics Approach

It involves an overall understanding of the molecules that makes up a cell, tissue or organism. They are aimed fundamentally at the detection of genes (Genomics), mRNA (Transcriptomics), proteins (Proteomics) and metabolites (Metabolomics). These new fields are developing rapidly and now investigation is going on to integrate them with traditional testing techniques. These tools, techniques along with science provide a promising future in the advancement of test methods. Omics technology provides all the necessary tools required for understanding of the difference between DNA, RNA, proteins and cellular molecules between different species and members of same species.[82]

CONCLUSION

People's inadequate knowledge and misconception on the safety of ayurvedic formulation may lead to opposite effect. Therefore the need of toxicity study is necessary but animal ethics is an important issue. So, various alternatives to animal use have been suggested which are needed to be implemented in an effective manner and researchers should also expand the number of compound to be tested that are in need of testing for potentially toxicological effect. Thus toxicological data for ayurvedic formulation will lead to the world wide acceptance.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

IAEC: Institute animal ethics committee; **CPCSEA:** Committee for the purpose of control and supervision of experiment on animal; **LD_{50}:** 50% Lethal dose; **OECD:** Organisation for economic co-operation and development; **AIDS:** Acquired immune deficiency syndrome; **MTT:** (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); **NR:** Neutral red; **LDH:** Lactate dehydrogenase; **NAD:** Nicotinamide adenine dinucleotide; **DNA:** Deoxyribonucleic acid; **DSB:** Double strand breaks; SCE: Sister chromatid exchange; **BrdU:** 5-bromodeoxyuridine; **ITS:** Integrated testing strategies; **mRNA:** Messanger Ribonucleic acid; **RNA:** Ribonucleic acid.

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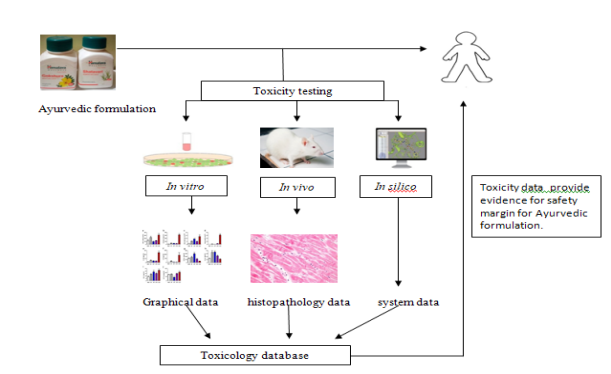
PICTORIAL ABSTRACT



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SUMMARY

Ayurvedic formulations consist of natural substances which are usually having a wide therapeutic range and effectiveness in large number of disease. There is also a misconception about the ayurvedic formulation that they are always safe. Drug experts have estimated that approximately 6000 medicine in the " Ayurvedic formulary" contain at least one metal, mercury and lead are most widely used .The toxicity of the substance can be observed by: a) *in vivo* (using the whole animal), b) *in vitro* (testing on isolated cell or tissue) and c) *in silico* (in a computer simulation). *In vivo* is derived from Latin means "in the living" thus, can be defined as the test is as study that is performed in living organism. Initially, *in vivo* experiments were aimed for the prediction of acute systemic toxicity usually in rodents. Many toxicity methods include the use of laboratory animals. The traditional methods of determining toxicity of drug or chemical include acute toxicity study, sub-acute toxicity study, sub-chronic toxicity study and chronic toxicity study. The term *in vitro* is derived from Latin phrase which means "the technique of performing a given procedure in an artificial environment outside the living organism". *In vitro* methods are widely utilized for screening purpose. The need of *in vitro* models for toxicity assessment is due to increase in ethical issues is the prime concern while using animal model for toxicity testing as it involves in unavoidable killing. The authenticity of *in vitro* cell lines is still a big issue as there is always a misconception with contamination and often the type of cell lines are mistaken. *In vitro* assay cannot provide true reflection of *in vivo* parameters. Thus, the use of animals for toxicity studies can be complemented by encouraging replacement, refinement and reduction. Recent advancement that has been made is Bioinformatics and Computational Toxicology, Integrated Testing Strategies, Omics Approach. Need for developing alternative testis due to Information about human risk Economy and efficiency. It is concluded that the toxicological data for Ayurvedic formulation will lead to the world wide acceptance.

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