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Review Article

Current methodologies in assessing the toxicity of natural products

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Abstract

Herbal ingredients and the combination of several species of plants, account for the different therapeutic and healing effects observed in the "complementary" or "alternative" medicine. The practice of herbal medicine is responsible for extense human knowledge that has developed over time in various cultures, worldwide. Cosmetics or nutracosmetics may cause adverse reactions in the skin, specially when there is a lack of cosmetic vigilance management. It is important to promote, in a relatively permissive regulatory environment, conscious efforts on the part of health professionals, to evaluate the outcomes and validate their pharmacological and cosmetic use. Traditional knowledge about the use of some herbs should be reevaluated and scientific basis should be considered. It is important to conduct a complete scientific research for validating herbal therapeutics and optimizing safety. Scientific toxicological research, using proper models, must continue to prove quality, efficacy, pharmacokinetics and pharmacodynamics interactions with other substances, and safety. Mechanisms of actions, as well as factors affecting toxicity should be studied, revised and taken in account to prevent adverse reactions, when using complementary or alternative medicine.

Introduction

Natural products show, in general, less toxic effects than synthetic products. Phytoingredients from living or dried plants, contain hundreds to thousands of interrelated chemical compounds that may have different biological and therapeutic effects, especially when the whole plant preparation is used. Although relatively rare, adverse reactions to cosmetic containing both traditional synthetic chemicals, as well as natural botanical ingredients, have been documented in the literature.

Yet, many phyto products are adulterated with metals, pesticides and synthetic drugs, mainly in a clandestine way.[1] Caution is required with the so called natural products and over the counter prescriptions, as health food, nutraceuticals, and nutracosmetics, which claim to be natural but include synthetic chemical additives.

Some of these products - which may contain germs, minerals, or metals - may be harmful, particularly if used improperly or without the right direction.[2] For example, some herbs can cause side effects or interact with conventional medicines. Most of the side effects of cosmetics or nutracosmetics, reported in the literature, include skin allergy, irritation, photosensitization, dermatitis, or genotoxicity.[3,4]

Thus in some countries, they are not classified as drugs, and there are no appropriate guidelines or regulatory standards. As for cosmetics, from raw material to finished products, packers and labelers, suppliers of consumables, and distributors, lack GAP (good agricultural practice) and GMP (good manufacturing practice) knowledge, and do not implement any of these guidelines to their operations.

The safety-in-use of cosmetic products has been established in Europe by controlling the substances, their chemical structures, toxicity profiles, and exposure patterns (1223/2009/EC1).

Scientific Committee on Consumer Safety (SCCS) Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation, states that "for the safety evaluation of cosmetic substances, all available scientific data are considered, including the physical and chemical properties of the compounds under investigation, in silico data such as results obtained from QSAR (quantitative structure activity relationship) calculations, chemical categories, grouping, read-across, physiologically-based pharmacokinetics (PBPK) /toxicokinetics (PBTK)

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modelling, *in vitro* experiments and data obtained from animal studies (*in vivo*)". In addition, clinical data, epidemiological studies, information derived from accidents, data from post-marketing surveillance (PMS) and any other human data are taken into consideration.[5]

Whenever evaluating the safety of herbal cosmetic products, each product has to be considered individually on a case to case basis.

Specifications for a Cosmetic Product

It has become crucial to have validated alternative methods (Directive 2003/15/EC2), in particular *in vitro* replacement methods, for the safety evaluation of cosmetic substances and products.[6]

Some of the specifications that are taken into account when considering a cosmetic product are: chemical identity, physical form, molecular weight, characterisation and purity of the chemical including isomer composition, characterisation of the impurities or accompanying contaminants, solubility, partition coefficient (Log Pow); relevant physical and chemical specifications, homogeneity and stability.[6]

For these purposes some authors take in consideration centrifugation assays and temperature variation to assay both homogeneity and stability of cosmetics formulations. High performance liquid chromatography (HPLC) with UV detection is another method chosen to verify chemical stability of cosmetics.

Cell and Tissue Cultures

There are many cell and tissue models available for *in vitro* toxicity testing. A prudent approach is to understand both the strengths and weaknesses of each model. The combination of PBPK models with models that measure changes in target cells, using different test substance concentrations, must take into consideration both toxicokinetics (TK) and toxicodynamics (TD). This combination will be helpful to determine the likelihood of adverse effects from "low-dose" exposure, as well as to assess variation among individuals in specific susceptible groups.[8] TK data are an essential piece of information for inter species extrapolation, route-to-route extrapolation and for mechanistic consideration, visual examination of cell cultures after their exposure to different susbstances, including nanoparticles is very important for *in vitro* toxicity assessment.[9,10]

The use of silico models to describe the cellular system increases our understanding of the (adverse) effects observed in *in vitro* systems, and should also improve the translation of *in vitro* data to the *in vivo* situation.

In Vitro Models

Current European legislation states that it is possible to ensure the safety of finished cosmetic products on the basis of the safety knowledge of the ingredients they contain. Alternative methods which do not involve the use of animals and which are validated at community level by the European Reference Laboratory for Alternatives to Animal Experimentation (EURL-EURLECVAM). The Organization for Economic Cooperation and Development (OECD) recognizes the importance of *in vitro* methods to fully capture the complexity of the toxic response in an intact organism.[8]

In vitro tests offer several advantages, including an apreciable amount of intrinsic toxicity information, controlled testing conditions; a high level of standardization; a reduction in variability between experiments; low cost testing; a small amount of material needed; a limited amount of toxic waste, cells, and human tissues used as well as transgenic cells carrying human genes; and reduced animal testing (reduction of the number of animals, and refinement of the techniques with less animal stress).[11]

Remarkable amount of information can be gathered about intrinsic toxicity and molecular mechanisms in unique type of cells, at appropriate testing conditions. Toxicogenetics at both the transcriptional and translational level is important in the context of mutagenesis and carcinogenesis

With advances in molecular biology and genomics, *in vitro* tests include mutagenicity and genotoxicity test: The Ames test (OECD 471) is the best known and used. The Ames test detects compounds that induce mutations in the genetic material that revert the functional ability to synthesize an amino acid. Mutant strains of *Salmonella typhynurium* (TA97a, TA98, TA100, TA102 and TA1535) and *Eschericha coli* are used which do not synthesize an amino acid. The mutant strains are placed and auxotrophic means for the amino acid which they can not synthesize, the compounds are considered to be mutagenic, if colonies are observed in the media.[12]

In vitro test of DNA damage by *Allium cepa* test in low cost, easily handled, has advantages over other short-term tests, also enables the evaluation of different end points for chromosome aberrations and to detect genotoxicity.[13] This test has also been used for studying environmental toxicity as well as food preservatives.[14]

In vitro test of chromosomal aberration (genetic mutation) in mammalian cells: the cells are exposed to the chemical after a metabolic activation or without such activation. The cells and tissue cultures are analyzed microscopically, after being exposed at different concentrations of colchicine, in order to observe possible chromosomal aberrations.[15]

The mouse lymphoma TK assay (MLA) is designed to determine whether a chemical is capable of inducing mutgenicity in cultured mammalian cells assessing risk prior to *in vivo* testing. The test has the potential to detect the mutations at the thymidine kinase locus caused by base pair changes, frameshift and small deletions (OECD

476). For a more thorough study concerning the possible genotoxic effects of a medicinal plant it is important to include bacterial and mammalian tests, with at least one *in vivo* assay. Also, these tests should be capable of detecting outcomes that include mutation induction, clastogenic and aneugenic effects, and structural chromosome abnormalities.[16,17]

The *in vitro* micronucleus assay (OECD 487), is a cytogenetic test prescribed by the International Conference on Harmonization, used to detect micronuclei in the cytoplasm of interphase cells to identify genotoxic substances that chromosome damaging potential *in vitro*. Micronuclei may originate from acentric chromosome fragments or whole chromosomes that are unable to migrate to the poles during the anaphase stage of cell division. The assay detects the activity of clastogenic and aneugenic test substances in cells that have undergone cell division during or after exposure to the test substance.

In vitro test of phototoxicity of 3t3 neutral red uptake, evaluates phototoxicity by the relative reduction in viability of cells exposed to the chemical in the presence versus absence of UVA/VIS light. It is based on the comparison of the cytotoxic effect of a test substance compounds that are toxic in *in vivo* tests after a sytemic application and distribution to the skin and compounds that are photoirritants, after topical application.

In vitro skin corrosion allows the identification of corrosive and non corrosive chemical substances (solid or liquid) applied to a three-dimensional human skin model, comprising at least a reconstructed epidermis with a functional stratum corneum.[18] It may also provide an indication of the distinction between severe and less severe skin corrosives. This test does not require the use of live animals or animal tissue. Recently, elaborately designed artificial skin models which closely mimic the human skin can be highly valuable and effective tools to replace *in vivo* animal tests for the evaluation of the safety and efficacy in the field of cosmetics.[19]

Currently, internationally accepted test methods for skin corrosion include the traditional animal test (Draize rabbit test)[20] revised in an alternative methodology[21] as well as *in vitro* test methods, including test methods based on reconstructed human epidermis technology (RhE).[22,23] In addition, alternative methods for skin corrosion include the transcutaneous electrial resistance (TER) assay, based on excised animal skin to predict corrosivity potential rather than the degree of corrosive effect.[24]

OECD test for *in vitro* skin irritation, evaluates a chemical when it is applied topically to a 3-dimensional RhE model comprised of non-transformed human-derived epidermal keratinocytes, which have been cultured to form a multilayered, highly differentiated model of the human epidermis. Chemical-induced skin irritation, refered to the production of reversible damage to the skin, is manifested mainly by erythema and oedema, is the result of a cascade of events beginning with penetration of the chemicals through the stratum corneum. Burnett et al[25] reported that amino acid alkyl amides used as surfactants in cosmetics, are safe in the present practice of use, when testing dermal irritation and sensitization. It is important to note that the European Centre for the Validation of Alternative Methods (ECVAM) has validated the use of EpiSkin™ nd EpiDerm™ to replace the usual *in vivo* rabbit Draize in conejos skin irritation test.[26]

Although the majority of different countries and regional regulatory authorities prefer or suggest animal data to assess skin sensitization potential, many are flexible in their consideration and acceptance of non-animal alternative methods.[27]

The compatibility test is performed to check that there is no dangerous effect when applying the cosmetic for the first time on the skin or on some mucosa. Cosmetics are applied to the human body for several purposes, and some may cause adverse reactions. When testing a cosmetic product in a human volunteer, to assess skin and mucous membrane compatibility, ethical practices, as well as complete information of the finished product, have to be considered.[28]

What About Animal Testing?

The commercialization of any cosmetic products containing ingredients tested on animal models was forbidden in 2009.[24] However, beyond the controversy that has brought the use of laboratory animals, the ban for EU cosmetic legislation, it is unlikely that just one *in vitro* test would be sufficient to make a decision point for safety assessment. However, experimental animals should be used, only if considering the 3R's: replacement, reduction and refinement.[29]

A series of alternative well characterized screening models may provide important information as to predict *in vivo* effects with a low incidence of false positive or negative results. Results obtained *in vitro* should be able to show potential toxicity mechanisms at a molecular level, identifying targets, especially when dealing with a new group of molecules.

Much effort is directed to the improvement and validation of other alternative methods and combinations of methods for predicting toxic effects, however, the limitations of *in vitro* tests raise numerous questions, as discussed by Kandárová and Letasiová in 2011.[30]

When performing *in vitro* experiments, the systemic impact is not evaluated. Interactions between tissues and organs are not considered. Chronic effects cannot be tested, and the use of one or two exposure concentrations instead of developing a full concentration response curve does not provide the kind of quantitative information required to extrapolate the *in vitro* effects to a relevant *in vivo* reference value, such as a plasma concentration, where toxicity occur. Also, the exposure concentrations

used *in vitro* have little relevance to the maximum plasma concentrations achieved *in vivo*.[31]

It is important to address, when evaluating safety assessment of herbal products, protein binding, metabolic stability and activation, metabolites, temporal relationships, and compound solubility. Some of the *in vivo* methodology, still considered for cosmetic products safety assessment is presented below.

The patch test is used to study whether a specific substance causes allergic inflammation of a patient's skin. It is recommended for any individual suspected of having allergic contact dermatitis. Patch testing helps identify which substances may be causing a delayed-type allergic reaction, and may identify allergens not identified by blood testing.[32]

The photopatch test is a technique of choice to establish the diagnosis in patients with a photoallergic contact reaction and study skin reactions, such as eruptions. The sun-product-exposure of an indiviual, may cause an allergic skin reaction. The photopatch is an application of the suspect substance, usually in the back, for 48 hours. If no reaction is observed, the zone is exposed to a source of ultraviolet radiation. If a reaction develops in the patch area, the reaction is considered as positive. It is important to conduct epidemiological surveillance.[33]

Murine local lymphoid nodule assay (LLNA) is a model used to study skin sensitization reactions with chemicals. The test measures specifically lymphocyte proliferation in the draining lymph nodes which is a hallmark of a skin sensitization response. This assay has been validated and incorporated worldwide into regulatory guidelines providing reliable hazard identification information and necessary information for effective risk assessment and management, however there are several concerns, like false positive responses, variability and predictivity.[34]

The comedogenicity test, which was traditionally made in rabbit ears, it is currently made in humans. In occlusive conditions in the back of volunteers. Comedones are generated when cells lining the sebaceous duct proliferate and sebum production increases, changing the pattern of keratinization. Methods that measure comedogenicity focus on quantifying keratinocyte «plugs» that can occur from the use of a product. [35]

The dermal corrosion test: TER (transcutaneous electrical resistance), is recommended for testing all types of chemical compounds. It is prepared with skin culture from rats of 28-30 days and the chemical is administered, leaving it to act for more than 24 hours.[36]

The corrosion is manifested as an irreversible damage in the epidermal tissue, also called visible necrosis, after a maximum exposure of 4 hours. According to the OECD Guide, in order to evaluate if the substance under study causes corrosion, three patches are applied sequentially, and removed at 3 minutes, 1 hour and 4 hours, after exposure.

The dermal and ocular irritation test is an i*n vivo* and *in vitro* assessment of dermal irritation and sensitization of cosmetic ingredients and products. It is used to examine the potential irritating and sensitizing effects of substances on the skin and eyes, recent developments propose the use of 3D reconstructed human cornea-like epithelium and epidermis models.[37]

Widespread use of nanomaterials requires studies on the impact on human health. Skin and eyes have the highest risk of exposure to nanomaterials, because deposition to the superficial organs has the potential to be a major route of exposure during the manufacturing, use, and disposal of nanomaterials.

The Draize test is used to measure irritation by observing the damages that cause a substance in the eyes and the skin of animals. Product solutions are applied directly to the eyes of conscious immobilized animals or to the shaved and frayed skin, for the observation of the effects of the substance during 7 days; alternative methods for replacement of eye irritation tests are being perssued.[21,38,39]

Traditional *in vivo* methods involve researching a value in the literature for the no observed adverse effect level (NOAEL), which is the maximum safe daily level of the substance usually derived from animal chronic toxicity studies and dermal absorption and systemic toxicity.[40] If a reliable NOAEL value cannot be found, it may be possible to estimate it from related substances. The margin of safety (MoS) is then calculated from a knowledge of the amount of the substance to which an individual will be exposed each day (including dermal, inhalation and oral exposure).

Final Remarks

There is constant review and innovation about the toxicity porocedures for evaluating side effects and toxicologycal risk in daily used cosmetics, particularly when validation and safety use non-animal models.

The population needs to be aware that herbal products are chemical drugs, and as such, may have pharmacological or cosmetic benefits, but may also present side effects and become a risk under certain conditions during observations. Consumer studies of beauty and cosmetic products provide rich and important information based on consumers' expectation from a product.

There is a need to integrate coordinated efforts between organizations and health care professionals, as a team to promote and incorporate validated alternative toxicological testing methods, by scientifically based research to support regulatory decision-making on risk assessment. An international agreement is necessary to standarize methodology for efficacy testing studies on individuals. There is a need for a "phytovigilance" program to evaluate the potential chronic toxicity of herbal products.

In addition, clinical data, epidemiological studies,

information derived from accidents, data from PMS and any other human data are taken into consideration. Long-term safety aspects of cosmetics has driven special attention, since cosmetic products may be used extensively over a large period of time in some populations.

Competing Interests

None.

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