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A Guidance Manual for the Toxicity Assessment of Traditional Herbal Medicines

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Dedicated to Prof. Dr. Wilhelm Fleischhacker on account of his 85th Birthday.

Herbal remedies have been used for thousands of years in worldwide traditional medicines for their potential health benefits. Although they are generally presumed safe unless a significant risk has been identified in humans, increasing number of case reports notify acute or chronic intoxications resulting from their use. This study aims to produce a scientific guide for the evaluation of traditional herbal medicines (THMs) in terms of their toxicity risks based on the published regulatory documents. For this purpose recommended *in vitro* and *in vivo* toxicity tests on medicinal products for human use issued by the international regulatory bodies are overviewed and they are then adopted to be used for the toxicity assessment of THMs. Accordingly, based on compilation of these issued regulations, the following tests are recommended for the toxicity assessment of THMs; *in vitro* cytotoxicity, genotoxicity, acute and repeated dose toxicity, carcinogenicity, reproductive and developmental toxicity, local tolerance tests, toxicokinetic studies, and additional toxicity tests including safety pharmacology, immunotoxicity and antigenicity, endocrine system toxicity, gastro-intestinal toxicity, renal and hepatotoxicity, and drug interaction studies. This study describes and discusses the applicability of these tests for the risk assessment in THMs.

Keywords: Toxicity assessment, Traditional herbal medicines, Herbal remedy, Herbal medicinal products.

Traditional medicine has served as a unique health provider for human beings for thousands of years. In the contemporary world herbal remedies still play a significant role in the health care delivery to people living particularly in developing countries, where the availability of health facilities and basic medicines are limited. On the one hand, recently, a growing number of patients have come to rely on these kinds of remedies for preventive or palliative care; on the other hand herbal medicinal products (HMPs) are becoming increasingly popular all over the world [1]. Mostly, herbal remedies are presumed safe unless a significant risk has been identified in humans. However, increasing numbers of case reports notify acute or chronic intoxications resulting from their use. These toxic effects vary from mild gastrointestinal symptoms and allergic reactions to renal and/or hepatic toxicity, haematological, cardiovascular, and neurological complications, carcinogenic effects, and death depending on acute or chronic consumption as well as amount of the HMPs or the traditional herbal medicines (THMs) [2-3]. Without specific investigations, only acute and severe adverse effects are likely to be identified. It should be emphasized that the absence of evident toxic effects does not mean totally safe.

According to the report by Chhabra *et al*. [4], approximately 1500 botanicals are sold as dietary supplements or local traditional medicines/herbal formulations in the United States (US) market without any Food and Drug Administration (FDA) premarketing approval. The US National Toxicology program initiated some research on several medicinal herbs and phytochemicals to examine possible carcinogenicity, reproductive toxicity, neurotoxicity, immunotoxicity risks or adverse effects, after administration at high acute doses and chronic low doses. Eventually, the FDA banned comfrey containing products due to their pyrrolizidine alkaloid content on July 6, 2001 [4].

According to European Directive [5] amending European Directive on Medicinal Products for Human Use [6], a **herbal medicinal product** is defined as '*Any medicinal product, exclusively containing as active ingredients one or more herbal substances or one or more herbal preparations, or one or more such herbal substances in combination with one or more such herbal preparations*'. This directive also determines herbal substances and herbal preparations. A **herbal substance** is defined as 'All mainly whole, fragmented or cut plants, plant parts, algae, fungi, lichens in an unprocessed, usually dried form but sometimes fresh. Certain exudates that have not been subjected to a specific treatment are also considered to be a herbal substance. Herbal substances are precisely defined by the plant part used and the botanical name according to the binominal system (genus, species, variety and author)'. A **herbal preparation** is defined as 'Preparations obtained by subjecting herbal substances to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation. These include comminuted or powdered herbal substances, tinctures, extracts, essential oils, expressed juices and processed exudates'.

There is a common belief that "*It is natural; then it is harmless*". However, this conjecture might only be more rational on condition that the plant remedy has been practiced safely in traditional medicines during the centuries. This long standing experience might be the reason for the common belief, not vice versa! Maybe due to this foresight, scientific evidence on the safety and toxicity levels of THMs are insufficient in number and quality [7-9]. To overcome this gap, a growing number of investigations have recently been carried out for the safety and toxicity assessment of THMs [10].

The main aim of this document is to provide a general overview for the researchers who are planning to evaluate the toxicity potential of

traditional herbal remedies based on the regulatory documents published by the international institutions and authorities. The present document focuses on both general toxicity tests for THMs and the corroborative tests in order to seek a possible sign of any toxic effect. It is proposed that safety assessment of a THM should utilize all existing knowledge and should compare the material with adequate comparators under consideration of the history of human use. Such an approach is considered as a prerequisite for identifying potential hazards and to determine the need for further information such as experimental toxicological data [11-12].

Toxicological data assessment

General aspects: In the current scientific rational, even for herbal remedies with a long history in traditional use, safety on human health should be supported with scientific evidence. In general, history on the traditional utilization of the plant material is expected to enlighten for planning the experimental toxicity studies. Besides, some special toxic effects including developmental and reproductive toxicity, genotoxicity and carcinogenicity should be evaluated for THMs since these effects may not be manifested during traditional practice. If there is not sufficient data on traditional herbal preparations or there is a suspicious status about these products, additional pre-clinical testing would be necessary [12-13]. On the other hand, in order to avoid unnecessary use of experimental animals, the EMEA stated that well-presented clinical evidence, epidemiological studies and data as well as post-marketing experiences for HMPs may be considered. This consideration may not be applied for THMs if the available data or data on historical use are not adequate or where there is a safety concern recognized or suspected pre-clinical studies are required.

***Selection of the test models*:** The selection of relevant animal models or other alternative test systems has supreme importance so that scientifically valid information can be derived.

Animal models as well as *ex vivo* and *in vitro* preparations can be used as test systems. Animal species to be used in test systems are mentioned in the related sections. *Ex vivo* and *in vitro* alternative test systems can include, but are not limited to, isolated organs and tissues, cell cultures, cellular fragments, subcellular organelles, receptors, ion channels, transporters and enzymes. It must be remembered that experimental results observed in bacterial and cell culture systems can be different when compared with those in whole animals or human. Cell culture systems may not reflect some protective mechanisms of living intact animals. Cells in culture may be subjected to different toxic endpoints that would rarely occur in the intact animal. In some instances, vice versa, toxicity in intact animal may not be observed in cell culture [14]. Therefore, *in vivo* tests on laboratory animals might be more valuable than *in vitro* studies for toxicity identification or characterization [3]. However, validated alternative test systems are currently suggested to replace some animal tests due to 3R (reduction, refinement and replacement of test animals) initiatives. Moreover, *in vitro* systems can also be used in supportive studies, for example, to reveal the mechanism of effects observed during *in vivo* studies or to investigate the general toxicity profile of the test sample.

On the other hand, since most of the plant constituents are subjected to metabolic processing in the body once orally administered, *in vitro* test conditions would generally not reflect the real toxicity profile of the oral THMs. Therefore, *in vitro* testing should include tests in the presence of S9-mix. For example, in the mid-1970s flavonoids including quercetin were reported to be mutagenic components of plants in the *in vitro Salmonella typhimurium* assay system [15-16]. These results opened a discussion that flavonoids having a widespread distribution in food plants including tea or onion might be carcinogenic. However, later investigations have revealed that a greater ratio of flavonoids is subjected to C-ring fission in the gastro-intestinal system, particularly by the colonic microbiota, to yield small phenolic fragments before systemic absorption. At the present time, quercetin is not classified as a human carcinogen by the National Toxicity Programme in the USA [17]. In fact, worldwide health authorities suggest quercetin containing foods in cancer prophylaxis.

Sample size: Sample size is mentioned in some common official guidelines released by Organization of Economic Co-operation Development (OECD), FDA or European Authorities. Appropriate negative and positive control groups should be included in the experimental design. Official test guidelines mentioned in the subsections of this manuscript include suitable negative and/or positive control substances.

Route of administration (*in vivo*): The ethnomedical information on the traditional use of the plant remedy will provide a valuable guidance for the selection of administration route in a toxicological survey. Most of the THMs are administered orally. Therefore, it is essential to investigate the gastrointestinal toxicity and to find out affected tissues or organs after administration of the test sample by oral gavage or by dietary supplementation to test animals [12]. On the other hand, administration by intraperitoneal injection is sometimes used to investigate toxic potential of test substances, but this type of administration would not reflect the real risks which might be due to lack of gastro-intestinal metabolites of the plant ingredients. With these explanations, the selection of administration route is critical to evaluate safety of any materials.

Dose levels or concentrations of plant extracts: *In vivo* experiments should be designed to define the dose-response relationship, but *in vitro* experiments to establish a concentration-activity correlation. The doses eliciting the toxic effect should be compared with the doses eliciting the primary biological effect in the test species or the proposed therapeutic effect in humans.

***Testing strategy*:** Frequently beneficial effects of a THM may be manifested following long term repeated administration which may increase the toxicity risks. For instance, liver toxicity symptoms may become apparent once the organ is severely compromised, which could be after several weeks of using a THM. Although acute or subacute toxicity signs are often easily recognized and predicted by humans, there have not been any predictable symptoms (developed delayed toxic effects) about the long lasting use of the herbal remedy. Therefore, sub-chronic and chronic toxicity tests are more valuable for the safety assessment of the THMs beside acute and sub-acute toxicity tests.

On the other hand, THMs may influence the pharmacokinetics and/or pharmacodynamics of the pharmacotherapy, eventually resulting in a reduction of efficacy or augmentation of side effects [18]. Therefore it is also reasonable to investigate possible interactions between the THMs and the pharmaceuticals like digoxin, warfarin, antiepileptics and others with a narrow therapeutic index.

Furthermore, cytotoxicity, genotoxicity, mutagenicity, carcinogenicity and reproductive and developmental toxicity studies should be carried out in order to support the safe and sound use of THMs, particularly if there is a suspicion of genotoxicity [19-21]. In addition, immunotoxicity or neurotoxicity investigations may also be considered [3, 22].

Contaminants in THMs: THMs can be contaminated with environmental pollutants in nature or during the processing; *i.e.* heavy metals, microorganisms (mycotoxins, endotoxins, exotoxins), agricultural residues (i.e., pesticides, fertilizers), and industrial wastes, which are not the natural components of plants. Due to the potential toxic effects, these pollutants may interfere with the correct interpretation of the results from toxicological tests on THMs. Therefore THMs should be monitored for such pollutants before submitting to toxicity tests.

- Due to the increasing environmental risks heavy metal contamination has currently become an increasing concern for herbal medicines. Heavy metals like lead and cadmium are known to induce nephrotoxicity and/or genotoxicity and therefore contamination of THMs with such toxic metals would interfere with the correct interpretation of the experimental results. Therefore possible pollutants which might interfere with the results, *i.e.* pots used for preparation of remedy or collection site of the plant material, should be carefully considered.
- Mycotoxins (for example, aflatoxins and ochratoxin) contamination is another external risk factor for both crude and processed plant materials. This is particularly a frequent case for plant materials purchased from herbalists or spice shops due to improper processing or storage conditions. Mycotoxin contamination would also interfere with the results of carcinogenicity and/or hepatotoxicity tests on THMs.
- Contamination with agricultural agents, particularly of pesticides, is also considered among the critical risks for plant materials. Such contaminations are particularly valid for cultivated plants, but wild plants collected from the vicinity of cultivation areas may also have such risks.

Identification of the THMs' toxicity: According to European and other national authorities, preclinical testing and clinical trials may sometimes be disregarded in THMs due to their long term practice in public health provided that satisfactory documentation on efficacy and safety is available [5]. This is particularly effective in registration of traditional herbal products. However, even a long tradition does not exclude the possibility of concerns with regard to the product's safety.

Some components of herbal ingredients, such as aristolochic acid, pyrrolizidine alkaloids, and ephedrine alkaloids, have been associated with the occurrence of nephropathy, hepatic veno-occlusive disease, and heart attack and stroke, respectively [2, 4, 23]. Therefore, if the herbal remedy is known or suspected to contain such potentially toxic components, along with chemical identification, several toxicity tests from acute toxicity to chronic toxicity should be made. Furthermore, depending on the history of use and the information on health benefits of THMs, further toxicological investigations may be required for identification of potential hazards [3].

In framing the toxicity test for herbals, differences in the pharmacological and toxicological characteristics of a whole herb, its crude extract, subextract or the isolated active component should be taken into consideration [3, 24]. Particularly, there may be significant variations in pharmacokinetic [absorption, distribution, metabolism, elimination (ADME)] behaviours of fractionated extracts compared with isolated pure components. On the other hand, it is very difficult to conduct ADME studies with THMs due to their multi-component composition. This difficulty may be overcome for only structurally defined active components in ADME studies, but this solution is only relevant for very few THMs. Such a point should be considered before framing the tests.

Toxicity assessment of HMPs should follow the international guidelines released by the competent authorities i.e., OECD, European Union (EU) Authorities, International Conference on Harmonization (ICH) or World Health Organization (WHO) [3, 12, 13]. However, for toxicity evaluation of plants not in traditional practice or with indeterminate safety, a minimum toxicity test battery should be set similar to that for medicinal products. These are as follows: toxicokinetics, single dose toxicity, repeated-dose toxicity, genotoxicity, carcinogenicity, reproductive and developmental toxicity, local tolerance and other toxicity studies including antigenicity, immunotoxicity, and impurities. For the plants used in traditional medicine a reduced battery of tests, including those for developmental and reproductive toxicity, genotoxicity and carcinogenicity, set by the authoritative institutions are required [6, 13].

Toxicity test battery

In vitro cytotoxicity tests: *In vitro* cytotoxicity tests have a widespread application in toxicity evaluation of a wide range of devices and inorganic and organic materials following international guidelines such as ISO 10993-5:2009 [25]. In these tests, test samples and cultured mammalian cells are co-incubated and responses of mammalian cells are measured by using appropriate biological parameters, as pointed out below. Results of these tests may provide some rapid information on the safe use of THMs in clinical practices, but may not be considered as complete safety evidence. On the other hand, Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) published a report which stated that *in vitro* cytotoxicity assays can be useful as one of the tools (e.g., SAR or bridging from similar compounds or mixtures) in setting a starting dose for the *in vivo* assessment of acute oral toxicity [26]. Accordingly, to predict human toxicity of a test material, two cell lines (one from human and the other from rodent) should be employed.

Inhibitions of cell death, cell growth, cell proliferation or colony formation are essential parameters used for the evaluation of cytotoxic endpoints. The number of cells, amount of protein, release of specific enzymes, release of vital dye, reduction of vital dye or any other measurable parameter may be quantified as an indication of cytotoxicity. In the legislative documents, Neutral Red Uptake (NRU), Colony Formation, MTT and XTT Cytotoxicity Tests are recommended for this purpose [25].

Brine shrimp (Artemia salina) lethality assay: Brine shrimp (*Artemia salina*) lethality assay is used as a simple and rapid acute toxicity test system for the evaluation of the potential toxicity of some herbal preparations [27], but this is not included in the protocols of any international health institution for toxicity testing, due to its questionable value. On the other hand, there are some reports recommending this test for ecotoxicity testing, but it is also not included in any standard ecotoxicity test guideline as a test organism [28]. Major drawbacks are lower sensitivity, standardization and characterization problems of *Artemia* strains, and resistance of the organism to several phytochemicals such as phenolic compounds and minerals [29].

Genotoxicity tests: Because of the direct correlation between genotoxicity and cancer development, assessment of genotoxic activity is usually practiced as the first step in exploring the mode of action [30-31]. Genotoxicity studies are obligatory for any new drug candidate by official authorities [6]. If a new drug candidate molecule or a substance/extract from a THM is found to be mutagenic and genotoxic it is no longer considered as a medicinal product and other toxicity tests are not considered. Genotoxicity test

guidelines for pharmaceuticals are issued by OECD, ICH and European Medicine Agency (EMEA) committees. Genotoxicity testing of THMs is also stated in the Guideline EMEA/HMPC/32116/2005 [13]. However, as THMs are composed of various components, the regular testing procedure for synthetic medicinal products should be customized to THMs [32]. If reliable evidence is not available on genotoxic potential of THMs, the following tests should be performed [26].

Genotoxicity testing can include the tests for evaluation of the presence of DNA adducts and other primary DNA damage (e.g., with assays for strand breaks) in the target organs, or indicators of genetic damage, such as micronucleated erythrocytes in the test animals at the end of the sub-chronic toxicity study [31]. To detect possible genotoxicity endpoints a battery of genotoxicity tests are employed, which include *in vitro* pro- and eukaryotic systems and *in vivo* experimental setups with and without metabolic activation. Through application of these preliminary tests, the majority of genotoxic carcinogens may be identified. *In vivo* test models on rodents are generally designed to observe the effects of test material on the intact living systems based on the administration route, exposure time and pharmacokinetic (absorption, distribution, biotransformation and elimination) profiles [33].

International regulatory authorities such as ICH [34] have proposed a standard test battery for genotoxicity evaluation of substances. In addition, EMEA suggested a stepwise approach to test the genotoxicity in HMPs. These tests are as follows:

(a) bacterial reverse mutation test in different bacterial strains;
(b) a cytogenetic test for chromosomal damage (the *in vitro* metaphase chromosome aberration test or *in vitro* micronucleus test), or an *in vitro* mouse lymphoma thymidine kinase (Tk) gene mutation assay;
(c) *in vivo* test for chromosomal damage using rodent hematopoietic cells either for micronuclei or for chromosomal aberrations in metaphase cells [13, 32].

In addition to these standard tests, other genotoxicity tests such as measurement of DNA adducts, DNA strand breaks, DNA repair or recombination can be further options for testing. Molecular techniques to reveal mechanisms of genotoxicity in the standard battery systems may be useful for risk assessment. Recent tools, such as transgenic animals and genomics technologies may be helpful in this regard. Particularly, rodent transgenic mutation assays, in vivo Comet assay and determination of DNA adducts are considered to be the key tests for genotoxicity assessment [4, 22, 31, 35-38].

Gene mutation test in bacteria (Ames test): *Salmonella typhimurium* (TA1537, TA1535, TA97, TA97a, TA98, TA100, TA 102 strains) and *Escherichia coli* (WP2 uvrA strain) are the most commonly employed bacteria for this test [32, 39]. This is a reverse mutation test; mutant bacteria (histidine dependent) in culture medium return to the wild type strain (histidine independent) with substances with mutagenic potential. Bacteria should be exposed to the test sample both in the presence and absence of an appropriate metabolic activation system (i.e., S9 fraction of rat liver).

Cytogenetic evaluation of chromosomal damage with mammalian cells

a) In vitro mammalian cell gene mutation test [40]: Mutant cells deficient in Hprt enzyme activity in the HPRT test or xprt enzyme activity in the XPRT test are resistant to the cytostatic effects of the purine analogue 6-thioguanine (TG). The Hprt (in the HPRT test) or gpt (in XPRT test) proficient cells are sensitive to TG, which causes the inhibition of cellular metabolism and stops further cell division. Thus, mutant cells are able to proliferate in the presence of TG, whereas normal cells, which contain Hprt (in the HPRT test) or gpt (in XPRT test) enzyme, are not. L5178Y mouse lymphoma cells, the CHO, CHL and V79 lines of Chinese hamster cells, and TK6 human lymphoblastoid cells can be used for the HPRT test, and CHO-derived AS52 cells for the XPRT test.

b) In vitro micronucleus test in mammalian cells: The *in vitro* micronucleus assay detects micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from chromosome fragments lacking a centromere or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division. This test is accredited for determination of chromosome loss or breakage [37, 41, 42]. Cultured mammalian cells from human peripheral blood lymphocytes or rodent cell lines such as CHO, V79, CHL/IU and L5178Y are used in this *in vitro* assay system. Other cell lines may also be used, but they are not validated yet. Selected cell cultures are exposed to the test materials both with and without an external metabolic activation system unless cells with an adequate metabolising capability are used. Recently, an *in vitro* micronucleus test has frequently been practiced in genotoxicity testing due to its simplicity in scoring and wider application in different cell types. In future it might be interesting to consider whether the *in vitro* micronucleus test might represent an interesting alternative to the *in vivo* bone marrow micronucleus test [41, 43].

In vivo test for chromosomal damage using rodent hematopoietic cells

Mammalian bone marrow chromosome aberration test [44]: This test is used for determination of structural chromosome aberrations induced by the test sample on bone marrow cells of animals, usually rodents. Due to its high vascularisation and possession of a population of rapidly regenerating cells, bone marrow is the target tissue in this test. Chromosome mutations and related events result in alterations in oncogenes and tumour suppressor genes leading to cancer in humans and experimental systems.

Animals are exposed to the test sample through an appropriate route and then sacrificed after a certain period. Prior to sacrifice, animals are treated with a metaphase-arresting agent (e.g. colchicine or Colcemid®). Chromosome preparations are then made from the bone marrow cells and stained, then metaphase cells are analysed for chromosome aberrations.

Three dose levels are used, within a range from the maximum to little or no toxicity. Gastric gavage is the generally preferred tool for oral administration, but other routes may also be acceptable, justified by the method of application in traditional medicine. However, intraperitoneal injection is not generally suggested to avoid possible antigenic interactions due to the non-sterile and complex composition of herbal preparations. Immediately after sacrifice, bone marrow is removed and exposed to hypotonic solution and fixed. The mitotic index is determined as a measure of cytotoxicity in at least 1000 cells per animal, both for treated (including positive controls) and untreated animals. The number of cells scored, the number of aberrations per cell, and the percentage of cells with structural chromosome aberration(s) should be evaluated.

Single dose toxicity (acute toxicity)

The single-dose toxicity test must be carried out in accordance with the relevant guidelines published or approved by the international

authorities. Investigation of the acute toxicity is the first step in the toxicological analysis for any drug candidate, including THMs. In the official documents, i.e. ICH Guidance [45], this is defined as '*The toxicity produced by a drug when it is administered in one or more doses during a period not exceeding 24 hours*'. The purpose of acute toxicity tests is to determine a clearly toxic but sublethal dose which would be referred for dose selection in the following repeated dose toxicity tests. The test sample is administered to experimental animals in increasing doses to find the dose causing major life-threatening toxicity. The use of vehicle control groups should also be considered. Test animals are generally rodents and rat is the preferred species. Acute toxicity tests are also used to assign the LD_{50} dose (*the dose which causes the death of half of the test animals*). A sufficient number of dose levels should be applied to determine the approximate lethal dose [3, 7, 22, 46, 47]. According to recent modifications on acute toxicity studies, instead of the classical LD_{50} test procedure, the following three types of acute toxicity tests are more likely to become practiced:

(a) *The fixed dose method* [46]: This method abandons lethality as an endpoint and is designed not to cause death. Pain or distress to the animals is measured and thereby this is a useful refinement for an alternative method.
(b) *The acute toxic class method* [48]: This method does not aim to determine a precise LD_{50}-value, but is applied to determine a range of exposure dosages where lethality is expected to be occurring. The test follows a complex stepwise dosage scheme. An advantage of this method is the significant reduction in the number of experimental animals.
(c) *The up-and-down procedure* [47]: This method allows an estimation of the LD_{50}-value and confidence intervals, and the observation of signs of toxicity. The number of experimental animals used is significantly reduced.

The initial dose for the fixed dose levels can be 5, 50, 300 and 2000 mg/kg, both for the herbal extracts and the isolated substances, as a dose expected to produce evident toxicity, if possible, based on the evidence from *in vivo* and *in vitro* data. In the absence of such information, the starting dose will be 300 mg/kg.

Due to the animal protection rights, the number of rodent species should be restricted to the smallest possible number, for instance three to five rodents per sex for each dose level. In the ICH guidance and OECD guidelines the observation period after single dose administration is 14 days. During this period all mortalities, time of onset and duration of observed clinical signs, and reversibility of toxicity should be recorded. Gross necropsies should be performed on all animals, including those sacrificed moribund, found dead or terminated at the end of the test period.

Initial information on the THMs' toxicity may be obtained by acute toxicity testing. It is assumed that the data obtained from acute toxicity studies is used to provide evidence of the likely risks of acute overdose in humans. Moreover, single oral dose studies are also used to define the extent of toxicity in the absence of other data and may help to determine the possible target organs of toxicity [49-50].

Repeated dose toxicity

The primary objective of repeated dose toxicity testing is to determine the *in vivo* effects of repeated daily exposure to THMs over periods of 28 days or longer. Rodent species are used for these test systems, but rat is preferred. Such studies should reveal any targets for toxicity, ranging from organs or tissues to cells. Any physiological and/or anatomo-pathological changes induced by repeated administration of the test samples are determined. The test should also permit determination of dose-response relationships for any targets of toxicity, thereby allowing the nature and severity of toxic effects to be ascertained. In the framework of HMPs safety assessment, repeated dose toxicity studies are usually considered to be core studies, particularly for new HMPs [3, 22, 50]. However, even for traditional medicines with a long history of safe use, risks for repeated dose exposure should not be disregarded. Licorice is a good example for such risks. Licorice extracts and their principle component, glycyrrhizin, have extensive use in foods (black licorice, chewing gum, herbal teas, soft drinks), tobacco and in traditional medicine (for cough, stomach ailments and constipation). Both products have been approved for use in foods by most international regulatory agencies based on their traditional use. However, experimental studies indicated that glycyrrhizinates inhibit 11ß-hydroxysteroid dehydrogenase, the enzyme responsible for inactivation of cortisol. Consequently, long-term and high concentration consumption of licorice products may produce hypermineralocorticoid-like effects in humans [10]. As a matter of fact, recently a toxicity case report was linked to the consumption of licorice candy cigars [51].

Sub-acute toxicity

These studies provide information for the possible risks in repeated oral exposure and may be useful in designation of dose regime for longer term administrations based on the results of additional studies following OECD and ICH guidelines. The test sample is orally administered daily in graduated doses to groups of experimental animals (rat, mouse) for a period of 28 days [52]. This test also provides information on the dose-response relationship and the determination of the No-Observed Adverse Effect Level (NOAEL) of the test sample.

Dose levels should be adjudicated by considering any available toxicity and (toxico-) kinetic data. The highest dose level inducing toxic symptoms without death or severe suffering should be selected and thereafter a descending sequence of dose levels, preferably at two to four fold intervals, should be applied. A fourth test group may often be recommended employing very large intervals (e.g. more than a factor of 10) between dosages.

During the administration period the test animals are observed daily for any morbidity and mortality. Initially, before the first exposure (to allow for within-subject comparisons), and at least once a week thereafter, detailed clinical observations should be made for all animals. At the end of the test, surviving animals are also euthanized and necropsied.

Changes in body weight and food/water consumption should be noted at least once a week. At the end of the test period, haematological analysis, clinical biochemistry tests, gross necropsy and full histopathology of organs are performed in all test animals. Based on the results obtained, additional tissue analyses may be required.

Sub-chronic toxicity

The test sample is orally administered daily in graduated doses to the groups of experimental animals for a period of 90 days [53]. A similar protocol is applied with the sub-acute toxicity tests for the selection of dose levels, as well as observation and evaluation patterns in the experimental animals [54]. If necessary, ophthalmoscopy, electrocardiograms or other specific toxicological investigations can also be performed [55]. Subchronic toxicity experimentation provides evidence for setting safety criteria for human exposure based on the finding observed, i.e. major toxic

effects, affected organs, and estimation of NOAEL value, as well as in selection of dose levels for chronic studies.

Chronic toxicity

In chronic toxicity tests, the test substance is administered daily to the experimental animal groups in graduated doses, as described previously in the repeated dose toxicity tests. Rodents are preferred in chronic toxicity testing, while non-rodent species may also be required under certain regulatory regimes. Since the administration period has not been harmonised between the worldwide institutions, ICH has recently attempted to conduct harmonisation initiatives within the European Region, Japan and US. Accordingly the ICH guideline [56] determines this period as 6 months for rodents and 9 months for non-rodents, while in the OECD guidelines this period is set up to 12 months with longer or shorter durations. The oral route is the main administration route, but depending on the application way in traditional use, inhalation or dermal route may also be used. The study design may also include one or more interim kills, e.g. at 3 and 6 months. During the administration period animals are observed closely for any morbidity and mortality. Animals which die during the study or are sacrificed at the end of the study are necropsied, as described previously in the subacute toxicity test [57].

Carcinogenicity tests

Carcinogenicity studies are practised to determine the tumorigenic potential in experimental animals and to extrapolate possible risks to humans. If there are some concerns about the carcinogenic potential, e.g. proximity of test extract or compound to those reported to be carcinogenic, or suspicion arises from repeated dose toxicity studies, carcinogenicity studies are recommended. On the other hand, genotoxic compounds are expected to induce cancer formation. Therefore, if a genotoxic effect is determined during the *in vitro* and *in vivo* experimentation, then it is likely to be a carcinogen, so there is no need to carry out the carcinogenicity tests [6].

Carcinogenicity tests are not routinely performed. The duration of the product in clinical use is the main determinant for the carcinogenicity test requirement, which is 3 months in FDA regulations and 6 months in European Commission regulations [13, 58].

According to the EMEA guidance document, if there is no potential risk for carcinogenic effect of a HMP under consideration, carcinogenicity studies are not needed [13]. Even if there were a carcinogenicity suspicion about a traditional or well-established herbal preparation, a carcinogenicity study is not necessarily required. However, depending on the quality of available carcinogenicity data, as well as mutual genotoxic or epi-genetic risks of HMPs, carcinogenicity studies should be considered [13].

For the evaluation of carcinogenicity potential of a product, all relevant information should be reviewed, briefly the identity, chemical structure/or composition, and physico-chemical characteristics; any information on the mode of action; results of any *in vitro* or *in vivo* toxicity tests, including genotoxicity tests; anticipated use(s) and potential for human exposure; available (Q)SAR data, mutagenicity/genotoxicity, carcinogenicity and other toxicological data on taxonomically-related plants; available toxicokinetic data and other repeated exposure studies. Carcinogenicity studies should be carried out following the 28-day and/or 90-day repeated dose toxicity tests; short-term cancer initiation-promotion tests would also provide useful information [59]. Sequential testing of chronic toxicity and carcinogenicity studies instead of separate testing might be more efficacious in determination of the possible risks [60].

The National Institute for Environmental Health Sciences (NIEHS) has proposed a modified method by combining the "*Salmonella* Mutagenicity Assay" and the "Mouse Micronucleus Assay" techniques. This is currently the most effective primary screening test for test materials to identify potential carcinogenicity risk to humans [14, 61].

Reproductive and developmental toxicity tests (teratogenicity/pre-natal developmental toxicity tests)

Possible adverse effect of THMs on male or female reproductive function, embryo-foetal development, and prenatal and postnatal development should be studied by following the internationally issued test methods. However, in the EMEA guidance document, these tests are considered unnecessary unless the HMP is administered during either pregnancy or lactation [13]. On the other hand, any uncertainty on the reproductive risks which appear through assessment of scientific data and/or post-marketing experience reprotox tests may be considered.

The embryotoxic and especially the teratogenic potential of a test material is tested by following the prenatal developmental toxicity text described in either OECD TG 414 [62] or the US-EPA Health Effects Test Guidelines OPPTS 870.3700 [63]. For the evaluation of pre- and postnatal effects and influence on fertility, the one-generation reproduction toxicity study OECD TG 415 [64] and two-generation reproduction toxicity study OECD TG 416 [65] or the US-EPA Health Effects Test Guidelines [66] are pursued. However, these tests, especially the two-generation study, are very cost- and time intensive. Therefore, screening test guidelines OECD 421 Reproduction/ Developmental Toxicity Screening Test (US-EPA equivalence of this test is [67]) and OECD 422 Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (US-EPA equivalence of this test is [68]) have been developed to provide preliminary information about possible risks on reproduction and/or development [69]. Male and female reproductive functionality such as gonadal function, mating behaviour, conception, development of the conceptus and parturition are focused in these screening tests. All the data on all aspects of reproduction and development are not provided by screening tests. However, due to limited number of endpoints and the short duration of the study, this method will not provide sufficient evidence for reproduction/developmental risks. Therefore screening test guidelines for Reproduction/Developmental Toxicity is neither a viable alternative to, nor the replacement for, the above existing guidelines [70].

The screening tests and the one- and two-generation studies provide information on pre-, peri- or postnatal mortality, body weight changes in the offspring, externally visible changes and grossly visible structural malformations. However, skeletal and visceral anomalies are usually detected by the prenatal developmental toxicity test [70].

There is a segmental approach to evaluate the reproductive and developmental toxicity of a test material: studies on the administration of the test material prior to and in the early stages of pregnancy (*Segment I*), during the period of organogenesis (*Segment II*), and during the perinatal and lactation periods (*Segment III)*. Segment II and III testing are normally required only if the HMP or THM is indicated for use during pregnancy or lactation [3].

On the other hand, recently, hormone-like actions of inorganic or organic substances, so called endocrine disruptors, have attracted great concern. Therefore this kind of possible effect of THMs should also be evaluated. Such examinations provide thorough evaluation of the reproductive organ functions including sex hormone levels. Micromorphological investigations of ovaries, testicles and epididymides are always required. In the one-generation study, together with reproductive organs such as uterus, cervix, vagina, seminal vesicles, prostate, coagulating gland, and pituitary gland, in particular cases other potential target organs should also be evaluated by histological means [3, 70].

Embryo/foetal toxicity studies should normally be conducted on two different mammalian species, one of which should be a species other than a rodent. Peri- and postnatal studies should be conducted in at least one species. If the metabolism of a medicinal product in a particular animal species is known to be similar to that in man, it is desirable to include this species in the experimental protocol. On the other hand, it is also required that one of the species employed in testing should be the same as that used in the repeated dose toxicity studies [6].

Local tolerance tests

Local tolerance studies are conducted to determine the tolerability of body parts when exposed to test materials (both crude extracts and active ingredients) [6, 71]. Local tolerance studies should be a part of the general toxicity studies in place of stand-alone studies. Clinical signs and macroscopic and microscopic examination of the application site are evaluated in this study [71].

Selection of animal species, as well as the dose levels, duration, frequency and route of administration are determined based on the available data for the test material in traditional medicine or clinical practice [6].

For medicinal products, including HMPs and THMs applied to the skin or mucosa (e.g. dermal, rectal, vaginal), the sensitising or irritating potential should be evaluated in at least one of the test systems currently available i.e. the Guinea-pig assay or the local lymph node assay for sensitising; animal test or validated alternative recombinant human epidermis test for irritating potential [6]. Current validated *in vitro* tests may be replaced with animal tests.

Toxicokinetic studies

Pharmacokinetic and toxicokinetic data are the important parameters to establish a scientific basis for the pharmacological efficacy and to estimate possible toxicity risks of a THM. Bioavailability studies to assess to what extent and how fast compounds are absorbed after the administration of THMs, clarifying the possible metabolic pathways yielding potentially active new metabolites, and the assessment of elimination routes are included in kinetic studies. These data are important to evaluate the pharmacological and toxicological effects of the test material. In spite of availability of detailed pharmacokinetic and toxicokinetic information on the active chemical pharmaceutical ingredients, such information is quite rare for HMPs and phytochemicals. These studies are a constant challenge due to the complex composition of herbal medicines [24]. Such kinetic data are mainly required for a herbal ingredient with known therapeutic activity or a defined constituent(s) with a specific toxicological profile [13]. With increasing knowledge of putatively active compounds and availability of highly selective and sensitive analytical methodologies, bioavailability and pharmacokinetics data on particular THMs have increasingly become available in the last decade [24].

Additional toxicity tests

Additional toxicity studies are not a part of general toxicity screening procedures, but may be required based on the results of primary findings from the toxicity studies described above or prior to clinical applications. Moreover, information on the biological actions of taxonomically related botanical species may also address the need for these tests [3]. For example, additional studies on immunotoxicity or neurotoxicity may be a focus of attention if the THMs show such biological activities.

The hierarchy of organ systems can be developed according to their importance with respect to life-supporting functions. Vital organs or systems such as pulmonary, cardiovascular, and central nervous systems are considered to be the most important ones to assess in primary toxicity studies.

Safety pharmacology studies

At this point, pre-clinical safety pharmacology studies are helpful to understand the effects of THMs on vital organ functions. ICH guidelines recognize the safety pharmacology core battery test system for medicinal products. Core battery tests determine the effects of medicinal products on the central nervous, cardiovascular, and respiratory systems [72, 73]. This guideline can be applied to THMs. In addition to the core battery, follow-up studies for safety pharmacology core battery and supplemental safety pharmacology studies are determined in this guideline. The pre-clinical safety pharmacology test systems and measured parameters described in the official guidelines are compiled and an adapted list for THMs is shown in Table 1 [72-74].

Immunotoxicity and antigenicity, endocrine system toxicity tests [22], hepatic function tests as an indicator for hepatotoxicity [9, 75], and drug interaction studies [24] are other additional toxicity tests which can be carried out under specific conditions [12].

Immunotoxicity and antigenicity tests

All new pharmaceutical products for human use should be evaluated for their potential effects on immune parameters (suppressive or stimulating) following the standard toxicity studies and additional immunotoxicity tests [76]. Eventually such type of tests should also be applied to HMPs not in traditional use, but may also be considered for THMs. Particularly if there are any pharmacological data on the use of THMs relevant to anti-inflammatory activity, immunotoxicity studies may have priority. These include:

(a) Haematological changes such as leukocytopenia/ leukocytosis, granulocytopenia/ granulocytosis, and lymphopenia/ lymphocytosis,
(b) Alterations in organ weight and/or histology of the organs involved in the immune system (e.g., changes in thymus, spleen, lymph nodes, and/or bone marrow),
(c) Changes in serum globulins.

Additional immunotoxicity tests may also be required based on the reported traditional use and results of the standard immunotoxicity tests, such as T-cell dependent antibody response (TDAR), immunophenotyping, natural killer cell activity assays, and host resistance studies [76].

Endocrine system toxicity tests: Possible effect of HMPs on endocrine system, such as effects on the pituitary thyroid axis may

Table 1: Pre-clinical safety pharmacology test methods and related parameters arranged for THMs.

Core battery	Measured parameters
Central nervous system Irwin's functional observation battery (FOB)	Motor activity, behavioural changes, coordination, sensory/motor reflex responses, body temperature.
Cardiovascular system QT interval (telemetry) hERG* Isolated Purkinje fibres (Langendorf isolated hearts) (Proarrythmia models)	Blood pressure, heart rate, electrocardiogram, left-ventricular pressure, **TRIaD, hERG IC_{50}
Respiratory systems Plethysmography	Respiratory rate, tidal volume
Follow-up studies for core battery	
Central nervous system	Behavioural pharmacology, learning and memory, ligand-specific binding, neurochemistry, visual, auditory and/or electrophysiology examinations
Cardiovascular system	Cardiac output, ventricular contractility, vascular resistance, the effects of endogenous and/or exogenous substances on the cardiovascular responses
Respiratory system	Airway resistance, compliance, pulmonary arterial pressure, blood gases, blood pH
Supplemental safety pharmacology studies	
Renal/urinary system	Urinary volume, specific gravity, osmolality, pH, fluid/electrolyte balance, proteins, cytology, and blood chemistry determinations such as blood urea nitrogen, creatinine and plasma proteins
Autonomic nervous system	Binding to receptors relevant for the autonomic nervous system, functional responses to agonists or antagonists *in vivo* or *in vitro*, direct stimulation of autonomic nerves and measurement of cardiovascular responses, baroreflex testing, and heart rate variability
Gastrointestinal system	Gastric secretion, gastrointestinal injury potential, bile secretion, transit time *in vivo*, ileal contraction *in vitro*, gastric pH measurement, pooling
Other organ systems	Dependency potential, effects on skeletal muscle, immune and endocrine functions

*hERG: human ether-a-go-go, **TRIaD:triangulation: reverse use dependence and instability

be evaluated by measuring the changes in blood thyroid hormone (T3, T4) and TSH levels according to the updated 28-day repeated dose toxicity test [52]. The following histopathologic tissue analysis may also provide valuable information for endocrine-related effects: ovaries, testes, uterus, cervix, vagina, epididymides, seminal vesicles with coagulation gland, dorsolateral and ventral prostate, pituitary, male and female mammary gland and thyroid.

Hormonal effects can be investigated by using receptor binding studies (*in vitro*) or by using specific studies such as the rodent uterotrophic assay (*in vivo*) [77].

Gastro-intestinal toxicity evaluation: In addition to the parameters given in Table 1 for gastro-intestinal toxicity tests, morphological analysis of stomach mucosa and determination of ulcer score in the stomach of experimental animals may be evaluated as preliminary evidence of gastro-toxicity for THMs. Ulcer score is particularly important for the safety assessment of herbal extracts or isolated components with anti-inflammatory activity due to the ulcerogenicity risk induced by most of the available cyclooxygenase inhibitors on the market [78].

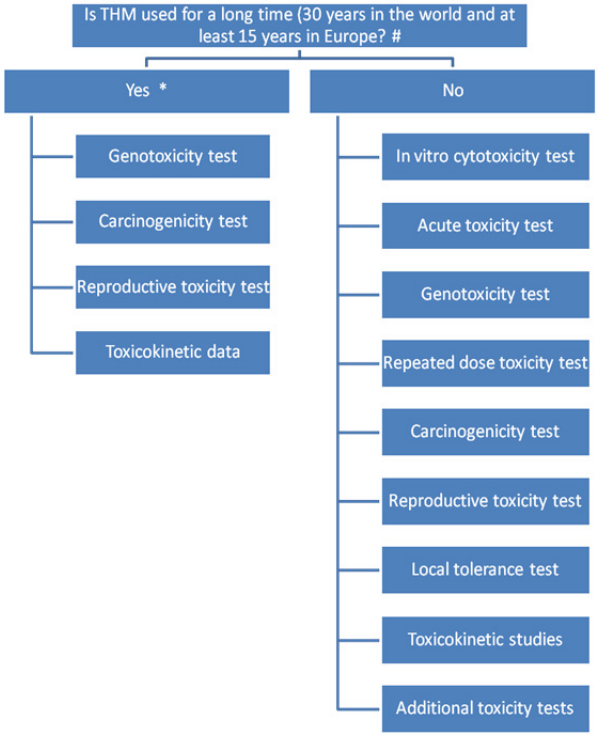
Renal and hepatic functions tests: There are frequent reports on nephrotoxicity (aristolochic acid) and/or hepatotoxicity (pyrrazolidine alkaloids) cases induced by THMs and/or their active ingredients [79]. Therefore evaluation of their renal and hepatic safety should be considered. In addition to the renal toxicity tests described in Table 1, inulin clearance [80], and various urinary enzyme activities such as γ-glutamyl transferase (GGT) and *N*-acetyl-β-D-glucosaminidase (NAG) may also be performed for nephrotoxicity evaluation [81]. Elevations in serum enzyme levels [alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP)], as well as total and conjugated bilirubin levels, are considered to be an indication of hepatocellular injury [82]. Compared with normal liver reference values, elevations up to three-fold in ALT, two-fold in ALP or more than two-fold in total bilirubin (TBL) levels are considered to be an indication of possible liver injury [82].

Drug interaction studies: Biotransformation enzymes and membrane transporter proteins are the essential factors responsible from the pharmacokinetic interactions among the pharmacotherapy, HMPs, and food ingredients in the body. Cytochrome P450 (CYP) isoenzymes (CYP isomers such as CYP2C9, 2D6, 2E1, 3A4, and 1A2) and P-glycoprotein (P-gp) are the major source of such interactions. Many components in the THMs may affect the pharmacokinetic and/or pharmacodynamic characteristics of prescribed medicines. Due to potential drug interactions, *in vitro* screening tests and clinical studies may be requested to identify such interactions, in particular for those plant ingredients with a narrow therapeutic index, i.e. alkaloids [24, 30]. Although drug interaction studies may not be a common part of a general toxicity test battery, these studies can be included to reveal possible risks in particular patient groups. For example, THMs such as garlic or ginkgo may increase clotting times and therefore have an additive effect to antiplatelet drugs such as aspirin or warfarin and increase the risk of life-threatening bleeding [83].

As another example, St John's Wort has a long history in traditional medicine as a safe herbal remedy against many diseases including ulcers, depression, and for wound healing. In the toxicological reference texts, the most commonly reported risk for St John's Wort is its possible phototoxic effects on humans. However this adverse effect has never been documented with clinical case reports so far. On the other hand, more recent documents reported adverse effect risks, in fact life-threatening drug interactions, due to induction of p-glycoprotein and cytochrome P450 3A4. This is particularly important after organ transplantation due to the increased clearance of immune suppressants, which can lead to organ rejection. Also other drug interactions with pharmacotherapy including cardiac glycosides, antidepressants, and antiplatelet drugs have been reported [84, 85]. Therefore, all possible interactions with all THMs should preferably be evaluated in the light of these findings.

***Conclusions and recommendations*:** Traditional use of an herbal remedy is auxiliary in evaluating the possible systemic toxicity risks and also to determine the relevant toxicity studies. However, if there is no adequate evidence on its safety, toxicity tests should cover all of the related characteristics stated in the medicinal product directives [6], or guidelines issued by international health authorities such as EMA, OECD and ICH.

Generally, in common regulatory documents [13], if an herbal medicine has been used traditionally for long periods, it is not required to put into practice a general toxicity screening pattern except for reproductive toxicity, genotoxicity, carcinogenicity and toxicokinetic data [13]. If a THM is found to be mutagenic and genotoxic in these tests, it should not be considered further as a medicinal product and the other toxicity tests are discontinued. The long historical use of THMs will usually pre-empt the need to carry out acute toxicity tests. However, even THMs in long traditional use may have high risks of organ toxicity. For example, plants containing unsaturated pyrrolizidine alkaloids have been proven to be associated with the development of specific liver damage, namely hepatic veno-occlusive disease [86]. Therefore oral use of THMs containing pyrrolizidine alkaloids has been restricted by the



* These tests are not compulsory for THMs, however conditions where these tests are required are described in the text; # However, documentation of long use does not exclude concerns about product safety [5].

Figure 1: A framework for pre-clinical toxicity testing of traditional herbal medicines (THMs) based on the European regulations for medicinal products [5, 6].

health authorities worldwide. Among these *Tussilago farfara* L. (coltsfoot) from Asteraceae and *Symphytum officinale* L. (comfrey) from Boraginaceae, which were used traditionally even for children for hundreds of years, have been withdrawn from therapeutic application [87].

Possible interactions between THMs and prescribed medicines are other important concerns when used concomitantly, which should not be ignored. Therefore physicians and pharmacists should become vigilant about possible herb-drug interactions and should report them to the competent authority. Beside special interaction studies, interaction data reported from clinical use may help to establish an interaction database and correspondingly future serious interactions can be anticipated and thus avoided.

It is not possible to give a simple checklist of toxicological tests and investigations which will be appropriate for establishing the safety of all the THMs in human use. A decision tree approach was previously suggested as an aid in guiding the safety evaluation process for HMPs and dietary supplements [3], but the authors also emphasized that an exact toxicity test battery for this purpose could not be given and a case-by-case evaluation was recommended.

On the other hand, during toxicological studies contamination risks in THMs emanated from environmental toxins (i.e., heavy metals), microorganisms (mycotoxins, endotoxins, exotoxins), agricultural contaminants (pesticides, fumigants), and other toxins i.e. industrial wastes etc. should not be ignored. Such kinds of contamination may interfere with the correct interpretation of the results of toxicological tests on THMs.

Another important point in toxicological risk assessment is the type of extract to be investigated in tests. If an aqueous extract is practiced in traditional receipt, then the toxicity tests should be carried out on this extract. Any toxicity determined with the alcoholic or any other solvent extract would not reflect the real risks for traditional receipt. However, parallel experimentation with both aqueous and alcoholic or other solvent extracts may help more comprehensive evaluation.

As a result, this guidance is not an attempt to re-define the safety evaluation guidelines issued by national/international authorities or organizations, but to provide a compilation of information on how the THMs are evaluated for their toxicity potentials. Based on the EU regulations on human medicinal products and HMPs [5, 6], a framework which was derived and modified from these directives has been constructed for the safety evaluation of THMs (Figure 1). It is essential to mention here that the prevention of unnecessary animal use for safety evaluations is of vital importance due to ethical concerns. Pre-clinical studies should only be performed if there are gaps in human data or concerns around possible toxicity risk. Gathering all the published information, clinical experiences, historical use data, information about the closely related THMs, and structure-activity relationship may render a judgement for further toxicity testing.

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Abbreviations: (ADME) Absorption, distribution, metabolism, elimination; (ALT) Alanine aminotransferase; (ALP) Alkaline phosphatase; (AST) Aspartate aminotransferase; (EMEA or EMA) European Medicine Agency; (EU) European Union; (FDA) Food and Drug Administration; (GGT) γ-glutamyl transferase; (HMPs): Herbal medicinal products; (HPRT) Hypoxanthine-guanine phosphoribosyl transferase; (ICH) International Conference on Harmonization; (NAG) *N*-Acetyl-β-D-glucosaminidase; (OECD) Organization of Economic Co-operation Development; (THMs) Traditional herbal medicines; (TFT) Trifluorothymidine; (TK) Thymidine kinase; (XPRT) Xanthineguanine phosphoribosyl transferase.

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