Food and Chemical Toxicology 197 (2025) 115277

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Review

A proposed screening strategy for evaluating the genotoxicity potential of botanicals and botanical extracts

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ARTICLE INFO

Handling Editor: Dr. Bryan Delaney

Keywords:
Botanical safety
Genetic toxicity
DNA damage
Testing strategy
In vitro assays
Herbal medicines
Dietary supplements

ABSTRACT

Botanicals have long been used to promote health and treat diseases, but the safety of many currently marketed botanicals has not been adequately evaluated. Given the chemical complexity of botanicals, which often contain numerous unknown constituents, and their widespread use, comprehensive toxicity assessments are needed. The Botanical Safety Consortium was established to address this challenge. This international group of experts in toxicology, chemistry, bioinformatics, and pharmacognosy is developing a toolkit of assays to generate reliable toxicological profiles for botanicals. Genotoxicity assessment is especially critical, because, unlike other toxicities, genotoxicity is not adequately identified by adverse event and history-of-use reports, and genotoxicity is directly linked to health consequences such as cancer and birth defects. The Consortium's Genotoxicity Technical Working Group is exploring a genotoxicity testing strategy based on the use of *in silico* modeling and the bacterial reverse mutation and *in vitro* micronucleus assays and including several options for additional tests to further characterize genotoxicity and mode of action when indicated. The effectiveness of this testing strategy is being evaluated using 13 well-characterized botanicals with existing toxicological data as case studies. A brief overview of each of these 13 botanicals is provided. The final strategy for developing comprehensive genotoxicity profiles of botanicals will incorporate published genotoxicity data, chemical composition information, *in silico* and *in vitro* test data, and human exposure data, reducing the need for animal testing.

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https://doi.org/10.1016/j.fct.2025.115277
Received 19 September 2024; Received in revised form 14 January 2025; Accepted 21 January 2025
Available online 23 January 2025

1. Introduction and background

1.1. Botanical use and safety evaluations

Botanicals are widely used throughout the world in dietary supplements, herbal medicines, and consumer products. An estimated 20% of adults in the United States use botanical supplements (Bailey et al., 2011; Clarke et al., 2015). Estimated U.S. retail sales of herbal supplements were more than 12 billion dollars in 2021 with an annual growth of 8.9% from 2011 to 2021 (Smith, 2022). The U.S. Food and Drug Administration (FDA) estimated in 2019 that more than 50,000 botanical supplement products were on the market (MacGregor, 2019) In other parts of the world the use numbers may be even higher (e.g., at least 70% of the population of the developing world directly rely on traditional medicine for their primary health care (Astutik et al., 2019). Thus, intentional human exposure to botanicals is extensive.

The existing framework for the safety evaluation of botanical supplements operates under the general assumption that these products are safe (DSHEA, 1994).

This assumption is usually based on historical usage of the botanicals and is typically anecdotal. Although adverse event reporting (e.g., ephedra) can highlight an immediate hazard, history of use is generally ineffective at identifying delayed toxicities such as cancer, genetic damage, effects in susceptible sub-populations, or interactions with other biologically active chemicals such as pharmaceuticals (Mitchell et al., 2022). Hence, safety determinations require more formal scientific methods.

To complicate matters, types of botanical-based products on the market vary widely, ranging from entire plant materials (such as aloe vera) to single, purified chemicals (such as the medicinal drug digoxin), to water or solvent extracts of specific parts of the plants (e.g., roots, leaves). Traditional toxicity testing for regulatory approval has focused primarily on single chemicals (e.g., pharmaceuticals, food additives, pesticides) that have stringent regulatory requirements and agreed-upon testing protocols to verify safety (ICH, 2011, 2014; OECD, 2016, 2020, 2023). However, botanical products and their extracts are complex mixtures of dozens or hundreds of chemicals of varying structures and chemical and biological activities. This complexity and variability of botanical products and their extracts makes evaluating their potential toxicity challenging.

The chemical compositions of botanical products are influenced by a range of factors including the part of plant used, geographic location, local climate and cultivation conditions, harvest schedule, plant variety, the solvents used for extraction, and the steps, if any, taken for extraction and purification (Rider et al., 2018). As a consequence, different batches of the extract from the same or different sources could have different proportions of their chemical components (Belwal et al., 2018; Kriker, 2013; Ryan et al., 2019; Vidhya et al., 2014). Substances that might be found to be biologically active when tested in their pure states may be present in diluted concentrations, at or below the sensitivity of the test. Thus, information on chemical constituents is critical to data interpretation.

Although traditional toxicology tests often rely on rodent responses, regulatory bodies internationally have been advocating for a shift from animal testing toward non-animal predictive methodologies (Cattaneo et al., 2023; Reddy et al., 2023). This shift in testing philosophy underscores the pressing need for innovative *in vitro* approaches to evaluate the toxicity of botanical products.

In 2019, to address the need for an internationally accepted standardized approach to assure the safety of botanical products, the FDA, the U.S. National Institute of Environmental Health Sciences (NIEHS), and the Health and Environmental Sciences Institute (HESI) formed a public/private partnership – the Botanical Safety Consortium (BSC). The goal of the BSC is to bring together international experts in a variety of disciplines to develop a framework to facilitate the robust evaluation of the safety of dietary and medicinal botanical substances. Within the BSC there are currently technical working groups focused on the areas of genotoxicity, hepatotoxicity, cardiotoxicity, neurotoxicity, developmental and reproductive toxicity, dermal toxicity, ADME (absorption, distribution, metabolism, and excretion), *in vitro* to *in vivo* extrapolation (IVIVE), botanical-drug interactions, chemical analysis, data analysis, and pharmacognosy. We present herein the objectives and progress to date of the Genotoxicity Technical Working Group (GTWG).

1.2. The importance of testing for genotoxicity in characterizing the safety of botanicals

Genotoxicity is an umbrella term for toxic effects that modify the structure or function of the genetic material or the process of cellular inheritance. Genotoxicity includes the induction of gene mutations (permanent heritable changes in the genetic material) as well as structural (chromosomal) DNA changes and adverse effects on processes critical to maintaining the regulatory fidelity of the genome (e.g., spindle apparatus, DNA polymerases, DNA repair systems, topoisomerases). Testing for mutagenic (an umbrella term for gene and chromosome mutations, along with aneugenic effects (i.e., imbalances in chromosome number) focuses on identification of heritable alterations, both genic and chromosomal, in somatic or germ cells. Such effects are a key consideration in consumer safety evaluation and regulatory decision-making.

Genetic damage can lead to a variety of adverse health impacts, including cancer and heritable genetic damage, depending on the type of damage induced, as well as the life stage and cell type that is affected in the exposed individual (Heflich et al., 2020; Phillips and Arlt, 2009). In germ cells, genotoxicity can lead to birth defects or adult-onset diseases, for example. In somatic cells, genetic damage may lead to cancer, cardiovascular disease, neurological defects, or other health effects. In this manuscript, we discuss *in silico* and *in vitro* tools that could be used to characterize the genotoxicity of botanicals. We also briefly describe the data-rich botanicals that were selected as case studies to explore the suitability of the proposed tools and the rational for choosing. The work represented herein is not meant to be a comprehensive assessment or listing of genotoxic botanicals, but rather an examination of methods that may allow for the rapid assessment of genotoxicity potential of a chemically complex botanical product. Reviews of genotoxic botanicals have been published previously (Celik, 2012; Prinsloo et al., 2019; Zhou et al., 2013).

2. Assays for evaluating genotoxicity

Because genotoxicity is induced through various mechanisms, a battery of assays is needed to screen for the full range of potential damages. There are various established regulatory guidelines and strategies for assessing different classes of compounds (e.g., ICH S2(R1) for active pharmaceutical ingredients (ICH, 2011) or ICH M7 for impurities in pharmaceuticals (ICH, 2014). Although there are many assays available for genotoxicity testing, the GTWG strategy leans heavily on commonly used, well-accepted assays or tools, accepted by regulatory agencies, that were originally designed to test individual, well-characterized chemicals (Fig. 1). Determining the suitability of these assays for testing chemically complex and largely uncharacterized botanical products is a main goal.

For initial assessment of genotoxicity using established *in vitro* assays, we selected a two-test battery consisting of the bacterial reverse mutation test (Ames test) (OECD TG 487) and the *in vitro* mammalian cell micronucleus (MN) test (OECD TG 474). This assay combination has been recommended as an efficient approach to capture most genotoxicity endpoints with high predictivity for *in vivo* genotoxicity (Kirkland et al., 2011; Pfuhler et al., 2007). Subsequently, we had the opportunity to conduct ToxTracker® assays (Hendriks et al., 2024) on each of the 13

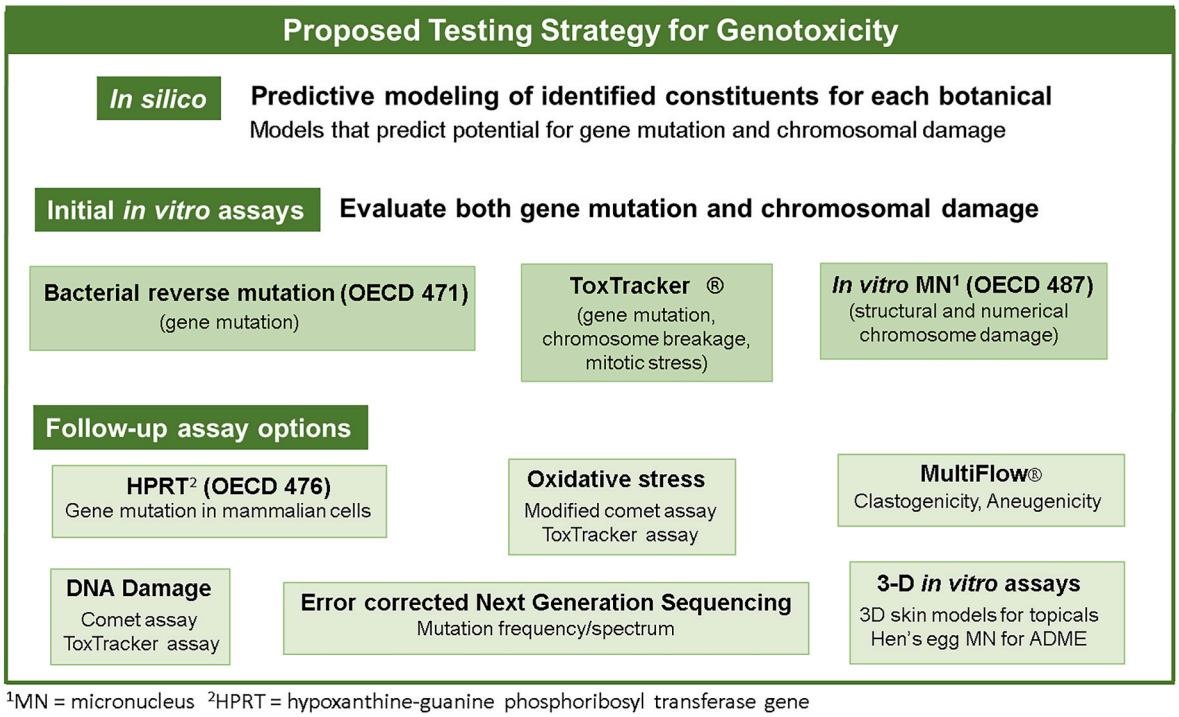

Fig. 1. Proposed testing strategy for genotoxicity.

botanical case studies, allowing us to gain mechanistic insights into observed responses in the Ames and MN tests, and to further corroborate both positive and negative results seen in either of these assays. The data from all three assays should prove helpful in guiding the selection of any additional follow-up tests.

2.1. The bacterial reverse mutation (Ames) test

Mutation is considered a necessary step in the development of cancer, either as the initiator of the cancer development process and/or as an intermediate step. For this reason, mutagenicity tests, such as the

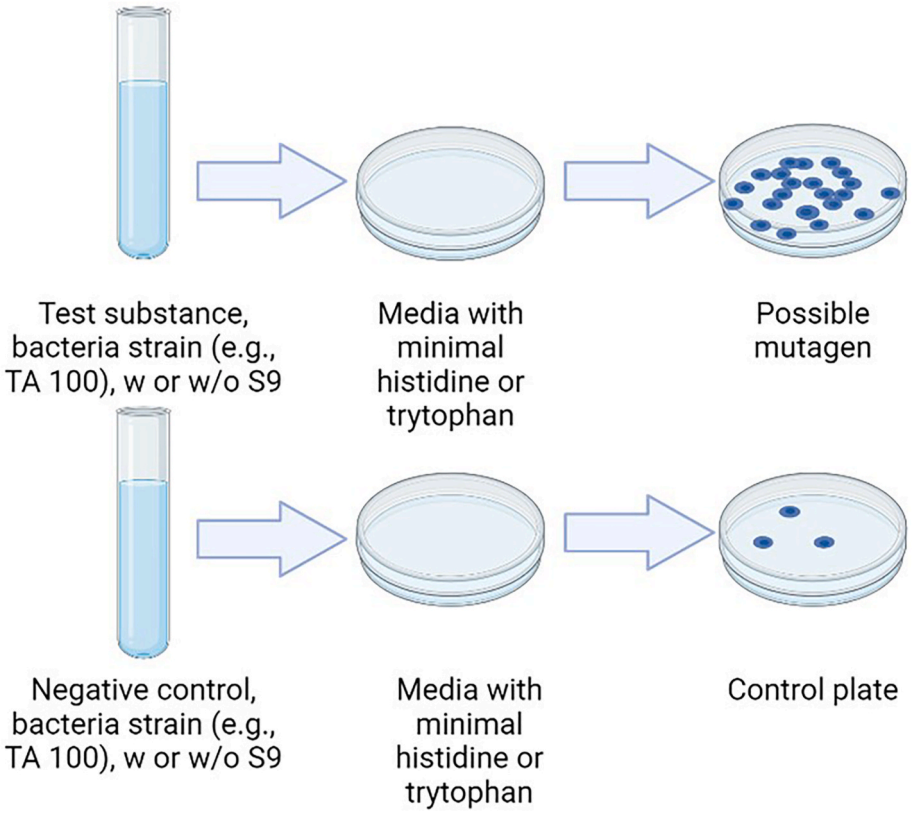

Fig. 2. Overview of the Ames test.

Ames test, are used to screen for potential carcinogens in addition to human germ cell mutagens. The Ames test for mutagenicity (bacterial reverse mutation), developed in the early 1970s, remains a relatively rapid and simple procedure to identify mutagenic chemicals and defined mixtures using multiple strains of *Salmonella typhimurium* and/or *Escherichia coli* bacteria (Ames et al., 1975; Cross and DeMarini, 2023; OECD, 2020; Williams et al., 2019; Zeiger, 2019) (Fig. 2). Each of the five bacterial tester strains typically used in the OECD-compliant assay has a different mutation in one of the genes needed for synthesis of a required amino acid (histidine in *S. typhimurium* or tryptophan in *E. coli*) that prevents the cells from growing and forming colonies on agar plates that are deficient in histidine or tryptophan. Incubation of these specialized bacterial strains with mutagenic chemicals results in reversion of the original incapacitating mutation, allowing the bacterial cell to regain the ability to manufacture the required amino acid. Reverted bacterial cells can then grow and form colonies on agar that lacks the required amino acid. In the Ames test, the bacterial strains are exposed to the test substance with and without a mammalian metabolic activation system derived from rodent (usually from rats induced with phenobarbital or Aroclor 1254) liver (designated "S9"). Because many chemicals require metabolism for their biological activity that the bacteria cannot provide, the use of S9 simulates the metabolism that is expected to occur in test animals and humans.

Although the test measures mutations in bacterial genes, a positive (mutagenic) response has ≥70% sensitivity for rodent carcinogenicity depending on the chemical classes tested (Zeiger, 1998). The test is required by regulatory authorities worldwide as an initial screen for chemical mutagens and carcinogens. The test requires only standard laboratory equipment and supplies. This increases its portability and allows it to be performed by laboratories with minimal facilities. Detailed information on the molecular basis of the test and a description of its various procedural modifications can be found in Mortelmans and Zeiger (Mortelmans and Zeiger, 2000) and OECD Test Guideline 471 (OECD, 2020).

A concern that is unique to the bacterial reverse mutation test is that an elevated presence of the free amino acids histidine or tryptophan in the plant extract being tested could lead, in theory, to false positive responses by allowing un-mutagenized bacterial tester strain cells to grow into colonies resembling those formed by mutagenized bacterial cells. This concern may be addressed in several ways, e.g., by examining the bacterial lawns that form on the agar plates during the first few cell divisions while histidine or tryptophan are normally still present, by noting the size of the colonies, or by chemical analysis of the extract prior to testing. Another concern when using *in vitro* methods to test for genotoxicity is the presence of constituents, (e.g., glycosides) that require metabolism by intestinal enzymes rather than by the enzymes found in the typical induced rat liver S9 preparations used to mimic mammalian metabolism in these cell-based systems. Such concerns are surmountable, but they underscore that the complex nature of botanical products and extracts must be considered in conducting *in vitro* assays and interpreting the results.

2.2. The in vitro micronucleus (MN) assay

Genetic changes resulting from environmental exposures are not limited to gene mutations. Another type of damage that may be induced is chromosomal damage, in the form of structural changes (clastogenicity) due to double-strand DNA breaks, or numerical changes (aneugenicity) due to loss of whole chromosomes through disruption of processes controlling chromosome segregation during cell division. Acentric fragments resulting from breakage and nonmigrating chromosomes resulting from disruption of the mitotic spindle apparatus are not incorporated into either of the two daughter nuclei formed at the end of mitosis. Instead, these lagging pieces of chromatin form a micronucleus (MN) in the cytoplasm of one of the two daughter cells in the subsequent interphase (Fig. 3). Thus, a rapidly dividing cell population is required for the MN assay. Micronuclei are similar in appearance to the main nucleus of a cell except for size. They are clearly visible with a light microscope or easily detected and enumerated using flow cytometry, which eliminates scorer bias and allows for thousands of cells to be evaluated for presence of MN, thereby increasing the sensitivity of the assay (Bryce et al., 2007).

A detailed description of the *in vitro* MN assay and protocol recommendations are published in the OECD Test Guideline 487 (OECD, 2023). There are also numerous publications describing the principle of the assay, protocol variations, methods of data collection, and statistical approaches suitable for data analysis (e.g., (Avlasevich et al., 2021; Bryce et al., 2008; Bryce et al., 2013; Bryce et al., 2007; Doherty, 2012; Sobol et al., 2012). As with the Ames test, the *in vitro* MN test is conducted with and without induced rat liver S9, as some clastogens are known to require metabolic transformation to induce chromosome breakage.

It should be noted that structural chromosome aberrations can be assessed directly in stained metaphase cell slide preparations (OECD, 2016). However, evaluation of chromosome aberrations requires highly trained personnel, is time consuming, the assay is less reliable than the MN assay in detecting aneugenic damage, and is more subject to experimental artifacts (Corvi et al., 2008).

2.3. The ToxTracker® assay

The ToxTracker assay will be used to further characterize the genotoxicity of each of the 13 botanical case studies, providing confirmation of the Ames and *in vitro* MN test results in a complementary assay and also supplying mode-of-action insight, for example, by assessing DNA damage and oxidative stress induction (Hendriks et al., 2024). The

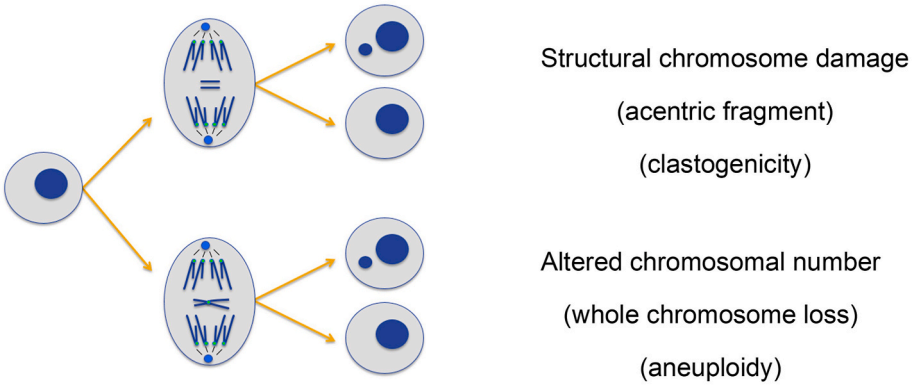

Fig. 3. Formation of micronuclei during mitosis. Shown are structural chromosome fragments (above) and a whole chromosome that is not incorporated into the main nucleus (below).

ToxTracker assay has recently completed a successful international interlaboratory validation (Hendriks et al., 2024) and has been accepted into the OECD test guideline development program. The assay uses six genetically modified mouse stem cell reporter cell lines. Each of these cell lines is designed to express a fluorescent reporter protein that is under the control of a promoter specific to a particular stress response pathway (e.g., DNA damage, DNA double-strand breaks, oxidative stress, protein damage) (Fig. 4). When a compound triggers a stress response in the cell, the corresponding reporter protein is expressed, providing a visible indication of the activation of that pathway.

In a typical testing scheme for a botanical, a laboratory might choose to conduct either a bacterial reverse mutation assay and an *in vitro* MN assay initially to assess the two broad categories of genotoxicity, or, as an alternative, conduct a ToxTracker assay as an initial step. Then, depending on the output, the ToxTracker assay might be followed by either a mutation or a chromosome damage assay. Guidance on the approach will be offered once all our data have been analyzed and interpreted. Several well-characterized and informative genotoxicity assays are available to assess in greater detail mutation and/or chromosome damage effects seen in the initial screening assays (Fig. 1).

2.4. *In silico predictive modeling*

For botanicals, we plan to evaluate the individual constituents that were identified and quantified in the botanical case studies. These computational tools utilize various algorithms and predictive models to estimate the likelihood of genotoxic effects. They typically incorporate large genotoxicity databases and employ structure-activity relationship (SAR) analysis to identify patterns of responses and predict test outcomes.

Advantages of *in silico* tools for genotoxicity assessment include their ability to rapidly screen large numbers of compounds with minimal cost and effort. Furthermore, *in silico* methods offer the ability to explore the potential mechanisms of genotoxicity and predict outcomes for novel compounds with limited experimental data. On the other hand, disadvantages of *in silico* tools for genotoxicity assessment include their reliance on the quality and completeness of the underlying databases. Inaccuracies or gaps in the reference databases can lead to incorrect predictions. Additionally, these tools may not be applicable to novel compounds with unique structures or mechanisms of action that are not well-represented in the existing data – likely the scenario faced in the context of phytochemicals. There is also the challenge of interpreting the results, as *in silico* predictions may not always correlate directly with *in vivo* outcomes (or even *in vitro* outcomes). Finally, regulatory acceptance of *in silico* methods varies, and they are usually used in conjunction with other testing approaches rather than as stand-alone tools.

Initially, we are exploring available tools such as Derek Nexus or models available from Leadscope (now InStem). Our focus will be on predictive models for bacterial mutation and for induction of MN. The individual constituents from each of our 13 selected botanical case studies will be evaluated. How best to integrate the *in silico* test results from the individual constituents and the results from *in vitro* tests of the whole extract will be determined in collaboration with our chemistry and our bioinformatics working group partners. Finally, the value of the *in silico* predictions as supplements to the *in vitro* test results will be determined.

2.5. *Follow-up mechanistic studies*

Fig. 1 shows several optional follow-up assays that may be used to obtain additional mode of action information, clarification of results, or genotoxicity data from more complex 3-D test systems to aid in estimation of human risk from exposure to a particular botanical extract. Decisions on the use of any of the suggested follow-up assays will be made on a case-by-case basis, considering the interpretation of the data obtained from the initial mutation and chromosomal damage assays.

For example, if deemed necessary, a modified comet assay that evaluates DNA damage induced by oxidative stress may be run to further explore this pathway to genotoxicity (Muruzabal et al., 2021). This comet assay modification enables indirect measurement of oxidized DNA bases through the use of base excision repair enzymes (e.g., formamidopyrimidine DNA glycosylase or endonuclease III). These enzymes recognize and cleave DNA at the sites of such modified bases, leading to an increased occurrence of strand breaks (Cordelli et al., 2021).

Moving to more complex test systems (e.g., the 3-D test options listed in Fig. 1) could provide valuable additional insight into the genotoxicity potential of a botanical that is administered topically (3-D human skin MN assay), for instance, or assess clastogenic potential of a botanical in a non-animal system with ADME capability (Hen's egg MN test or HET-

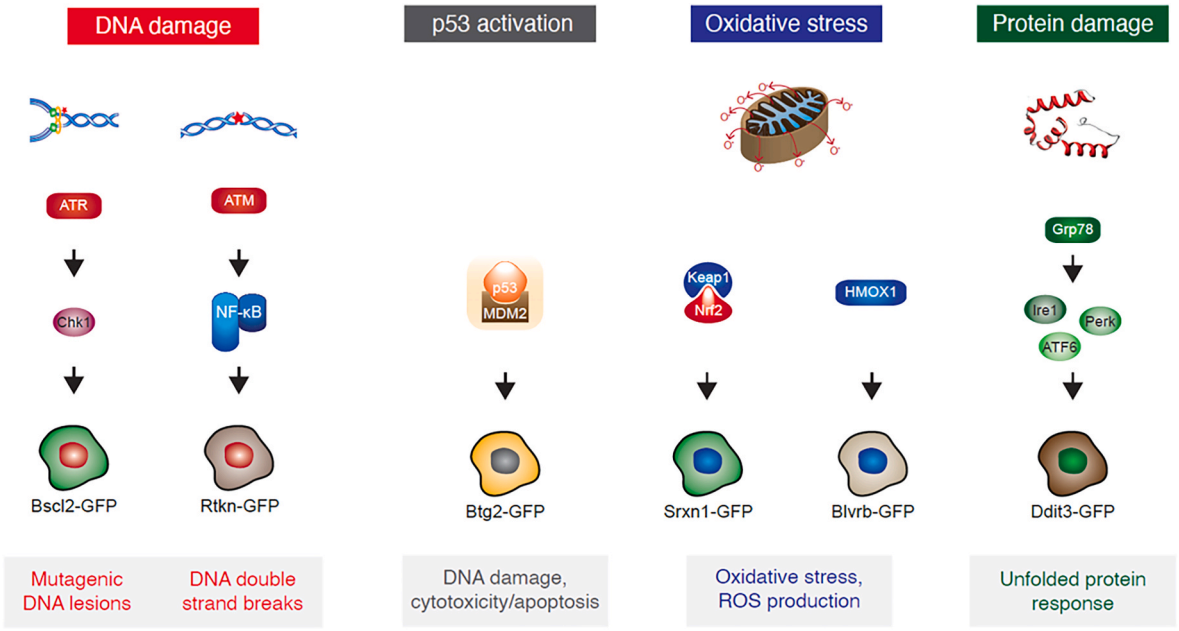

Fig. 4. Schematic representation of the ToxTracker® assay readouts from 6 reporter cell lines. Reprinted from Toxys.com, with permission.

MN).

Looking beyond hazard identification, which is what the assays shown in Fig. 1 provide, there may be a need to estimate the human risk posed in the context of anticipated exposures to a botanical. Approaches exist to aid in this task, including *IVIVE* techniques, but there may be cases that call for *in vivo* genotoxicity data to enable informed risk estimates. It should be emphasized, however, that conducting *in vivo* genotoxicity assessments and recommending risk management measures are currently beyond the scope of the BSC.

3. Botanical case studies for evaluation of proposed testing strategies

To evaluate the acceptability of the proposed safety evaluation framework, each working group in the Consortium that focused on a particular type of toxicity (e.g., neurotoxicity, hepatotoxicity, cardiotoxicity, genotoxicity) was asked to identify and select a set of well-studied botanicals that had been found to either cause, or not cause, the toxicity of interest. Literature reviews were conducted but these were not true systematic reviews. Although many well-studied botanicals were identified, not all had available information on chemical characterization; those botanicals with information related to constituent quantification and chemical characterization were prioritized for use.

The BSC selected 13 botanicals from this list to serve as case studies (Table 1). Seven of the 13 botanicals were nominated by the GTWG, and these are shown in bold in Table 1. The 13 selected botanicals were sourced from reputable suppliers with certificates of analyses, and various analytical techniques were used to characterize constituents (CEBS, 2024; Waidyanatha et al., 2024). Below we provide a brief overview of the data available for each selected botanical, with a primary focus on genotoxicity. A concise summary of the genotoxicity information for each botanical is presented in Table 2.

3.1. Aristolochia fangchi

Aristolochia fangchi (*guang fang ji*) is a flowering plant, native to Vietnam and southern portions of China. It is a member of the Aristolochiaceae family of plants. Aristolochia species are widely cultivated and have been used extensively in traditional Chinese medicine. This botanical was selected as a key reference botanical by the GTWG because the aristolochic acids (AAs) that occur in certain Aristolochia species are well documented causes of botanical-induced genotoxic and carcinogenic toxicities in humans. Many products currently on the market contain AAs and are used for a variety of ailments including arthritis, gout, and inflammation (NTP, 2021). However, AAs are highly toxic, and have been shown to induce severe kidney toxicity, DNA damage, and cancer, especially urothelial cancer, upon exposure to relatively low dose levels for short periods of time both in humans and in animal models (Grollman, 2013; NTP, 2021). Aristolochic acids are potent mutagens in bacterial and mammalian cell mutagenicity assays, and in laboratory animals and humans, but are weak inducers of micronuclei *in vivo* (Bhalli et al., 2013). Aristolochic acid-induced tumors in animals and humans carry the unique DNA adduct and mutational signatures associated with exposure to the bioactivated AAs (Bhalli et al., 2013; Grollman, 2013; Grollman et al., 2007; Hoang et al., 2013; Zhang et al., 2004). The adduct and mutational signatures provide strong evidence for the genetic mechanism of AA-induced cancers and allow monitoring for exposure to these agents in human populations (Boot et al., 2020). The Internation Agency for Research on Cancer (IARC) has classified plants containing AA as carcinogenic to humans (Group 1)(IARC, 2012).

In conclusion, available data indicate that *Aristolochia fangchi* is a potent mutagen and human carcinogen. It was nominated by the GTWG as a positive case study.

3.2. Ashwagandha

Ashwagandha (the Sanskrit name for *Withania somnifera* (L.) Dunal) is an evergreen shrub that is a member of the *Solanaceae*, or nightshade, family. Ashwagandha is also known as Indian winter cherry or Indian ginseng. It is native to India, the Middle East, the Mediterranean area, and parts of Africa. Ashwagandha root is reported to improve sleep, and reduce stress and anxiety (ODS, 2023). This botanical is also used for treatment of a wide variety of additional ailments (Mukherjee et al., 2021; Paul et al., 2021). A few cases of liver injury have been reported in

Table 2
Summary of the genotoxicity profiles for the 13 botanical case studies.[a].

Standardized common name	Genotoxicity summary
Aristolochia fangchi	Genotoxic and carcinogenic in humans
Ashwagandha	Unknown genotoxicity potential
Asian ginseng	Not genotoxic and not carcinogenic in animals
Blue cohosh	Unknown genotoxicity potential
Comfrey	Genotoxic and carcinogenic in animals
Ephedra	Unknown genotoxicity potential
Green Tea	Mixed evidence for genotoxicity; not carcinogenic in animals
Goldenseal	Not genotoxic, but carcinogenic in animals
Kava	Not genotoxic, but carcinogenic in animals
Kratom	Unknown genotoxicity potential
Milk thistle	Inconsistent genotoxicity data; not carcinogenic in animals
Usnea	Some evidence of genotoxicity
Yohimbe	Unknown genotoxicity potential

[a] Botanicals shown in bold were nominated by the GTWG.

Table 1
List of source botanicals for extracts used to date by the Botanical Safety Consortium, including their standardized common and scientific names, Distributed Structure-Searchable Toxicity (DSSTox) substance identifier (DTXSID), and the part(s) of the plant used to derive the extract. Botanicals selected by the genotoxicity working group based on existing data are shown in bold.

Standardized Common Name	Scientific Name	DTXSID	Plant part(s)	Description of the extract[a]
Aristolochia fangchi	***Aristolochia fangchi* Y.C. Wu ex L.D. Chou & S.M. Hwang**	**DTXSID201349132**	**Root**	**95% ethanol extract**
Ashwagandha	*Withania somnifera* (L.) Dunal	DTXSID201042372	Root	Commercial dry extract
Asian ginseng	***Panax ginseng* C.A. Mey.**	**DTXSID1023780**	**Root**	**Commercial dry extract**
Blue cohosh	*Caulophyllum thalictroides* (L.) Michx.	DTXSID401042859	Root & Rhizome	95% ethanol extract
Comfrey	***Symphytum officinale* L.**	**DTXSID20274226**	**Root**	**95% ethanol extract**
Ephedra	*Ephedra sinica* Stapf	DTXSID801018482	Aerial Parts	95% ethanol extract
Green Tea	***Camellia sinensis* (L.) Kuntz**	**DTXSID0031398**	**Leaf**	**Commercial dry extract**
Goldenseal	***Hydrastis canadensis* L.**	**DTXSID40274228**	**Root & Rhizome**	**95% ethanol extract**
Kava	***Piper methysticum* G. Forst.**	**DTXSID901018742**	**Root & Rhizome**	**95% ethanol extract**
Kratom	*Mitragyna speciosa* (Korth.) Havil.	DTXSID001334842	Leaf	95% ethanol extract
Milk thistle	***Silybum marianum* (L.) Gaertn.**	**DTXSID8031657**	**Seed**	**Commercial dry extract**
Usnea	*Usnea* spp.	DTXSID701349537	Whole Lichen	95% ethanol extract
Yohimbe	*Pausinystalia johimbe* (K. Schum.) Pierre ex Beille	DTXSID4032291	Bark	95% ethanol extract

[a] Detailed descriptions found in Waidyanatha et al. (2024).

people taking ashwagandha supplements, (Björnsson et al., 2020; Inagaki et al., 2017; Ireland et al., 2021; LiverTox, 2012a; Weber and Gerbes, 2021), but no evidence of hepatotoxicity has been reported in clinical trials (LiverTox, 2012a).

Chemical analysis of ashwagandha root extracts have identified withanolides (steroidal lactones) (Devkar et al., 2015), a large number of alkaloids, including withasomnine, a pyrazole alkaloid (Schröter et al., 1966) and several sitoinosides (Bhattacharya et al., 1987; Scartezzini and Speroni, 2000).

No OECD guideline-compliant genetic toxicity or carcinogenicity studies of ashwagandha extracts or its main constituents have been reported in the literature. A few studies suggest that certain constituents may cause DNA damage or interfere with cellular processes that maintain genomic stability. A withanolide present in ashwagandha root extract, withanone, formed adducts with both calf thymus DNA and glutathione *in vitro*, suggesting that glutathione may influence withanone-DNA adduct formation (Siddiqui et al., 2021). Another withanolide, withaferin A, which is present in the leaves of ashwagandha but not the roots, may interfere with the mitotic spindle assembly checkpoint in colorectal cancer cells (Das et al., 2014; Scartezzini and Speroni, 2000).

Ashwagandha's genotoxicity potential is unknown.

3.3. Asian ginseng

Asian ginseng is native to the mountains of China, Korea, and far eastern Russia. Ginseng root extract (*Panax ginseng*) is used to purportedly aid in helping conditions including lowering blood sugar in patients with Type 2 diabetes and mitigating cold and flu symptoms. Identified constituents include ginsenosides, glycans, and flavonoids.

Extensive toxicological studies have been conducted with Asian ginseng root extract. No evidence of carcinogenicity was seen in 2-year studies in rats and mice (NTP, 2011). Furthermore, no evidence of genotoxicity has been reported in tests of various ginseng extracts and preparations (Chang et al., 1986; Morimoto et al., 1982; NTP, 2011). In addition, a hydrolyzed extract of ginseng leaf that contained some of the same ginsenoside constituents as the root extract gave negative results in both an Ames test and an *in vitro* chromosomal aberration test (Kim et al., 2014).

Several studies reported that ginseng reduced the genotoxic activity of known genotoxicants (i.e., were anti-mutagenic) (e.g., (Ohtsuka et al., 1995; Panwar et al., 2005; Rhee et al., 1991; Zhang et al., 2008).

In conclusion, available data indicate that ginseng is neither carcinogenic nor genotoxic. It was nominated by the GTWG as a true negative case study.

3.4. Blue cohosh

Blue cohosh (*Caulophyllum thalicroides*) root supplements are used primarily for induction of labor or alleviation of various menstrual symptoms (Ali and Khan, 2008; NICHD, 2006). This perennial plant is widely distributed throughout the eastern US and Canada, as well as portions of the Midwest.

Some of the known constituents of blue cohosh include saponins (glycosides) as well as quinolizidine alkaloids (e.g., anagyrine, baptifoline, *N*-methylcytisine), aporphine (e.g., magnoflorine, taspine, boldine), norlupanine (e.g., sparteine, cytisine, lupanine), and piperidine (e.g., thalictroidine, caulophyllumines A and B) (Ali and Khan, 2008; Matsuo et al., 2009; Rader and Pawar, 2013); in humans, blue cohosh has been associated with a number of adverse effects, namely severe gastrointestinal and cardiovascular toxicity, along with teratogenicity and embryotoxicity (Dugoua et al., 2008; Rader and Pawar, 2013). Cases of perinatal stroke, acute myocardial infarction, congestive heart failure, multiple organ injury, and neonatal shock were reported in infants born to mothers who consumed the supplement (Dugoua et al., 2008; Rader and Pawar, 2013). These effects are mainly attributed to alkaloids, in particular *N*-methylcytisine, which exhibited teratogenic activity in rat embryo culture (Kennelly et al., 1999) and nicotinic toxicity in humans (Rao and Hoffman, 2002; Schep et al., 2009). In addition, titerpene glycosides likely contribute to the oxytocic and vasoconstrictive effects of blue cohosh (Rader and Pawar, 2013).

Several studies investigated mechanisms underlying the toxic effects of blue cohosh. In one study, a methanol extract of blue cohosh was shown to impair mitochondrial function by disrupting membrane integrity of human breast tumor T47D and hepatoma Hep3B cells (Datta et al., 2014). In another, interference with GATA2-endothelin1 (GATA2-EDN1) signaling was associated with teratogenic effects in Japanese medaka (Wu et al., 2010). Triterpene glycoside constituents of blue cohosh extract were cytotoxic to promyeloblastic HL-60 cells (Matsuo et al., 2009), and the alkaloid constituents caulophynie A and taspine were cytotoxic to alveolar epithelial A549 cells and rodent embryonic cells, respectively (Kennelly et al., 1999; Wei et al., 2022).

Blue cohosh was nominated as a positive case study for documented developmental toxicity. There are no available genotoxicity studies with blue cohosh or its constituents, and therefore, its genotoxicity is unknown.

3.5. Comfrey

Comfrey *(Symphytum officinale* L.) is a large perennial shrub native to parts of Europe and Asia. It is present in herbal teas and many topical supplements recommended for treatment of inflammatory conditions. It contains pyrrolizidine alkaloids (PAs), which are of particular concern due to their documented hepatotoxicity, genotoxicity, and carcinogenicity (EFSA, 2011, 2017). Pyrrolizidine alkaloids with a 1,2-unsaturated necine base are especially toxic. They can be metabolically activated by cytochrome P450s to form pyrrole intermediates that react with cell proteins and DNA, forming pyrrole adducts that cause hepatotoxicity, genotoxicity, and carcinogenicity (Chan et al., 2003; Fu et al., 2004, 2010; Yang et al., 2016). The European Food Safety Authority (EFSA) evaluated the risks for human health related to PAs in, e.g., herbal infusions and food supplements containing comfrey, concluding that there is a concern for adverse health effects in people who frequently consume PA-containing teas, herbal infusions, and food supplements (EFSA, 2017). This conclusion is supported by human case studies reporting toxicity following the consumption of herbal preparations from pyrrolizidine producing plants including comfrey (EFSA, 2011; Ridker and Mcdermott, 1989). Thus, PA-containing botanicals including comfrey may present a hazard not only for genotoxicity and carcinogenicity, but also for hepatotoxicity that may occur after short term exposure.

Data on genotoxicity of comfrey preparations are limited and consist of studies reporting detection of pyrrole DNA adducts following *in vitro* and *in vivo* exposure to PA-containing botanical preparations including comfrey root extract and comfrey compound oil (Chen et al., 2010). Dietary comfrey induced mutations in the Big Blue transgenic rat assay, with a relatively high frequency in liver (Mei et al., 2005, 2010; Mei and Chen, 2007) similar to that reported for individual PAs; G:C to T:A transversions and tandem base substitutions were the major mutations detected, supporting that PAs in comfrey are responsible for its carcinogenicity. While data on comfrey extracts are sparse, several recent studies have confirmed the genotoxicity of individual PAs in comfrey extract (Allemang et al., 2018; Li et al., 2020; Schrenk et al., 2020).

Comfrey was nominated by the GTWG as a true positive case study based on the demonstrated genotoxicity and carcinogenicity of its PA constituents. Furthermore, the results for comfrey extract in genotoxicity tests will aid in understanding whether these assays are sufficiently sensitive for detecting genotoxicity of a mixture of individual PA constituents, each of which is present in low levels in this extract.

3.6. Ephedra

Ephedra *(Ephedra sinica)* is a perennial plant native to China and its dried herbaceous stem, called *ma huang* (麻黄)in Chinese, has been historically used to treat ailments such as asthma, colds, fever, and headaches, and is believed to aid in weight loss (NCCIH, 2020). Key constituents of interest include alkaloids like ephedrine, pseudoephedrine, and N-methylephedrine. The US FDA prohibited the sale of dietary supplements containing ephedrine alkaloids in 2004 due to severe cardiovascular and neurological side effects, including heart attacks, seizures, strokes, and death (FDA, 2004). Despite its minor short-term weight loss benefits, the significant health risks were concluded to outweigh the benefits.

In 2013, an EFSA panel reviewed the genotoxicity data for ephedra and concluded that the quality of the available mutagenicity and chromosomal damage studies did not permit a definitive conclusion to be drawn on the genotoxicity of ephedra, although all results were negative (EFSA, 2013a). One of the key constituents, ephedrine sulphate, was also non-mutagenic in an OECD-compliant Ames test (NTP, 1986).

Ephedra was chosen as a case study due to its documented cardiotoxicity and neurotoxicity. No comprehensive, high-quality studies on its genetic toxicity exist and therefore, the genotoxicity of this botanical is unknown.

3.7. Green tea

Green tea, from the Theaceae family, is consumed globally, especially in Asia (IARC, 1991). Its chemical composition varies by factors like geography and growing conditions. The dried leaves contain significant polyphenols (30–35%), proteins, carbohydrates, and other substances, with catechins like epigallocatechin gallate (EGCG) showing diverse biological activities (Bedrood et al., 2018; NTP, 2016). Although marketed as beneficial for health, few claims are clinically verified. For the BSC, a decaffeinated, concentrated green tea extract was selected as a case study based on evidence of hepatotoxicity and botanical-drug interactions.

Extensive studies have focused on green tea's anti-mutagenic and anti-carcinogenic properties, generally confirming these benefits. However, studies on its toxicity and potential genotoxicity are limited and inconsistent. For example, low concentrations of green tea solutions decreased DNA damage in human lymphocytes, while higher concentrations increased DNA damage and oxidative stress in various cell types, including human laryngeal carcinoma and leukemic cells (Durgo et al., 2011; Elbling et al., 2005; Ho et al., 2013). Studies by the NTP showed positive results with green tea extract in the Ames test with S9, but no induction of MN in mice (NTP, 2016).

Experiments with green tea catechins showed dose-related cytotoxicity and chromosomal aberrations in Chinese hamster lung cells and DNA damage in human lymphocytes. However, a standardized catechin mixture, Polyphenon E, was not mutagenic in the Ames test or in Big Blue mice, and did not induce micronuclei in Swiss-Webster mice (Chang et al., 2003). A 2-year NTP cancer bioassay in rats and mice showed no evidence of carcinogenic activity (NTP, 2016). The IARC classifies tea, including green tea, as a Group 3 substance, indicating it is not classifiable regarding human carcinogenicity (IARC, 1991).

Green tea extract was nominated by the GTWG due to mixed evidence of genotoxicity and no evidence of carcinogenicity.

3.8. Goldenseal

Goldenseal (*Hydrastis canadensis*) is a perennial herbaceous plant that is native to the eastern regions of the United States and southeastern parts of Canada. It has a long history of use for a wide range of medicinal applications. Traditionally, it is believed to aid various health issues such as skin conditions, gastrointestinal problems, urinary tract infections, and other potential applications. More modern uses include taking its root as a dietary supplement, often in combination with other plants like echinacea. Some of its known constituents include the alkaloids hydrastine, berberine, and canadine.

The NTP tested goldenseal root powder in short-term toxicity studies, long-term carcinogenicity bioassays in rats and mice, ADME studies (in human cell lines), and genotoxicity assays (NTP, 2010). Goldenseal was negative in the *in vivo* peripheral blood micronucleus test in male and female mice following 3 months of exposure via dosed feed, and it was also negative in the Ames test. Additionally, berberine, an alkaloid constituent in goldenseal root powder, was negative in the Ames test and an acute *in vivo* bone marrow micronucleus test in male and female mice. However, in the 2-year feeding study, male and female rats exposed to goldenseal showed significant increases in hepatocellular adenomas compared with concurrent control animals. Treated male mice showed increases in liver hepatoblastomas and hepatocellular adenomas while no evidence of carcinogenicity was observed in female mice. Exposure concentrations in the carcinogenicity test overlapped with estimated human exposure ranges. These results highlight species and sex differences in responses to goldenseal exposure and suggest a potentially non-genotoxic mechanism of tumorigenesis for goldenseal root powder. However, it should be noted that the goldenseal root powder constituent berberine is a topoisomerase II inhibitor, and this class of chemicals can induce chromosomal aberrations (NTP, 2010). IARC has classified goldenseal as possibly carcinogenic to humans (Group 2B) (IARC, 2016).

This botanical was nominated by the GTWG as a case study that has clear carcinogenic activity but no reported genotoxicity.

3.9. Kava

Kava (*Piper methysticum*) is a plant native to the South Pacific islands and is deeply rooted in the region's cultural and social practices, used as a ceremonial and social beverage, particularly in countries like Fiji, Tonga, and Vanuatu (WHO, 1999). The beverage made from the root of the kava plant is known for its calming and relaxing effects, which has increased its use in other parts of the world, including Europe and the United States (Singh, 1992). The kavalactone constituents in kava are believed to be responsible for its anxiolytic and sedative properties (Sarris et al., 2011).

Interest in kava is increasingly focused on potential medicinal properties, especially in managing anxiety and stress (Ooi et al., 2018; Savage et al., 2015). However, in the early 2000s, reports of hepatotoxicity among kava users led to regulatory actions and warnings in several countries (Schmidt et al., 2022; Steenkamp et al., 2023; Teschke et al., 2014), although years of traditional kava consumption in the South Pacific have not shown similar effects (Soares et al., 2022). Research into the safety of kava continues.

Ames tests with kava extract showed no mutagenic activity (NTP, 2012). Similarly, no mutagenic activity was observed in mouse lymphoma cells exposed to kavalactones or kava extracts (Whittaker et al., 2008). However, a specific n-butanol fraction of kava leaves was reported to exhibit mutagenic activity in some tests (EMA, 2017). No increases in the frequencies of micronucleated erythrocytes were observed in blood samples of male and female mice dosed by gavage for 3 months (NTP, 2012).

In rodent carcinogenicity studies, oral administration of kava extract led to increased incidences of hepatoblastoma and hepatocellular carcinoma in male mice (NTP, 2012). In male rats, exposure to kava extract was associated with marginal increases in the incidence of testis interstitial cell adenoma and renal pelvis transitional cell hyperplasia. IARC classified kava extract as possibly carcinogenic to humans (Group 2B) primarily through mechanisms unrelated to direct DNA damage (IARC, 2015).

Kava was nominated by the GTWG as a case study due to its demonstrated carcinogenicity in mice in the absence of demonstrated genotoxicity.

3.10. Kratom

Kratom (*Mitragyna speciosa)*, is a tropical evergreen tree from Southeast Asia whose leaves are used traditionally to treat ailments like pain, diarrhea, and fever, and for their psychoactive effects (Brown et al., 2017). Kratom (also known as "biak") was introduced into the US following the Vietnam War through increased migration from Asian communities. Over the past decades, kratom use has risen, often as an alternative to opioids for pain management and withdrawal symptoms (CDC, 2023; Garcia-Romeu et al., 2020). Its leaves contain mitragynine and 7-hydroxymitragynine, indole alkaloids that induce stimulatory effects when chewed and sedative effects as tea, depending on the dose (Brown et al., 2017; Prozialeck et al., 2012). Both alkaloids are partial agonists at the μ-opioid receptor (WHO, 2021 #106).

With over 10 million kratom users in the US, its legal and health status is contentious (CDC, 2023) because the FDA has not approved kratom for medical use, citing safety concerns including seizures (FDA, 2023), and the WHO warns of its abuse potential (WHO, 2021 #106). Several states have regulated or banned kratom use due to its reported health risks, including neuropsychological and cardiovascular effects, and rare cases of death and liver injury (LiverTox, 2012b; Post et al., 2019). Kratom research has focused mainly on its opioid-like effects, and over 50 alkaloid constituents of kratom have been identified (Flores-Bocanegra et al., 2020). Although a single report of negative results for both kratom extract and the alkaloid constituent mitragynine in a L5178Y TK $^{\pm}$ mouse lymphoma gene mutation assay was identified (Saidin et al., 2008), no comprehensive studies on its genetic toxicity exist.

Kratom was nominated as a positive case study for neurological effects. Its genotoxicity is unknown.

3.11. Milk thistle

Milk thistle (*Silybum marianum*) is native to western Asia, southern Europe, and northern Africa, although it can now be found worldwide. It is an annual or biennial plant that is considered to be an aggressive invasive species in North America. Its leaves and fruits have been used for hundreds of years for diverse ailments, especially those related to the liver. Clinical benefits deriving from preparations from milk thistle fruits were reported by physicians in the US as early as the late nineteenth century (EMA, 2018). Monographs of the German Commission E describe the use of milk thistle for treatment of toxin-induced liver damage, supportive treatment in patients with chronic inflammatory liver conditions, and hepatic cirrhosis (Blumenthal et al., 2000). The beneficial effects reported for milk thistle preparations are generally attributed its bioflavonoid constituents, including silymarin.

There are a considerable number of studies on the genotoxicity and carcinogenicity of milk thistle extract and its components (NTP, 2011; (EMA, 2018). In the NTP 2-year rodent bioassay, milk thistle extract did not demonstrate evidence of carcinogenicity and only insignificant chronic toxicity (Dunnick and Nyska, 2013). Evidence on the genotoxicity of milk thistle is inconsistent across studies. Some extracts were mutagenic in the Ames assay in the presence of S9, while others were negative with and without S9 (NTP, 2011). No increases in micronucleated erythrocytes were observed in peripheral blood of male or female mice following a 3-month exposure to milk thistle via dosed feed (NTP, 2011). Mixed results for DNA damage assessed by the comet assay were seen with the constituent silymarin in mammalian cells (Duthie and Collins, 1997; Anderson et al., 1997). These genotoxicity studies were highly variable as to extract, test system, and study type, thereby not permitting a clear conclusion regarding the hazard posed and suggesting that additional testing to clarify the variable test results would be helpful in understanding the potential genotoxicity risk of milk thistle consumption to humans (EMA, 2018).

Milk thistle was nominated by the GTWG as a noncarcinogen case study with undefined genotoxicity potential.

3.12. Usnea lichens

Usnea is a genus of lichens that grow anchored on bark or twigs; they are found worldwide. A comprehensive review of the chemistry, botany, history of use, and toxicity of *Usnea* lichens was published by Guo et al. (2008). Usnic acid has long been used as an antimicrobial in traditional medicine, while in recent years, it has been marketed as a dietary supplement for weight loss (Guo et al., 2008). *Usnea* lichen preparations may contain both (+)- and (−)-usnic acid (Chen et al., 2024). Consumption of high doses of preparations containing (+)-usnic acid has been associated with acute liver injuries and liver failure in consumers (Favreau et al., 2002; Gao et al., 2016; Neff et al., 2004). These reports of liver toxicity prompted the FDA in 2001 to send a letter to the manufacturer of one specific product, strongly recommending that the product be withdrawn from the market (CFSAN, 2001b). As reports of liver injuries continued, the FDA issued a warning to consumers in 2011 (CFSAN, 2001a). Despite these warnings, extracts of *Usnea* lichen species remain marketed for use as herbal antimicrobials (NTP, 2022b). To further investigate the risk of acute liver injury posed by usnea extracts, the FDA's National Center for Toxicological Research (NCTR) initiated short term studies in male and female rats and mice (NTP, 2022). Results of these studies confirmed the hepatotoxicity of usnea extracts (NTP, 2022a, 2022b).

Information for evaluating the potential genotoxicity of usnea lichens is limited. Constituents and secondary metabolites of usnea species have been examined in a few *in vitro* assays. Usnic acid and two other lichen constituents, physodic and physodalic acid, were tested in the Ames assay. Physodalic acid was clearly mutagenic, with and without S9 (Shibamoto and Wei, 1984). In contrast, usnic and physodic acids were not mutagenic, with or without S9 (Shibamoto and Wei, 1984). In addition, (+/−)-usnic acid also showed no evidence of mutagenicity in the Ames test in studies conducted by the NTP (NTP, 2022). In an *in vitro* micronucleus assay in cultured human lymphocytes, neither (+)- nor (−)-usnic acid induced significant increases in micronuclei (Koparal et al., 2006). This single *in vitro* study contrasts with the results reported from two *in vivo* studies described below.

Male Swiss albino mice, treated with a single oral dose of (+)-usnic acid, showed increased frequencies of micronucleated erythrocytes in bone marrow samples collected 24 and 48 h after treatment (Al-Bekairi et al., 1991). In a 14-day toxicity study (NTP, 2022), blood samples from male and female B6C3F1 mice exposed to *Usnea* lichens containing (+/−)-usnic acid via dosed feed showed significantly increased frequencies of micronucleated immature erythrocytes.

Usnea lichens was nominated as a case study due to confirmed hepatotoxicity. Its genotoxicity requires additional study.

3.13. Yohimbe

Yohimbe (*Pausinystalia johimbe*) bark preparations are used as an aphrodisiac and to enhance sexual performance. Yohimbine, the main active constituent, acts by blocking alpha-2 adrenergic receptors. Other uses for yohimbe include performance enhancement for athletes and as a general wellness tonic. Yohimbine has been associated with neurotoxic symptoms, including anxiety, tremors, lack of coordination, dissociative reactions, retrograde amnesia, and seizures upon ingestion, but no information on mutagenic or carcinogenic activity is available.

In 2013, EFSA stated that they found no *in vitro* data on the genotoxicity of yohimbe and that no conclusion could be drawn from the one available *in vivo* study, for which several limitations were identified (EFSA, 2013b). Another constituent in yohimbe bark preparations, the alkaloid raubasine (an alpha 1-adrenergic receptor blocker), was reported to be negative in a bacterial mutation test and no relevant data for other known alkaloid constituents were available (von Poser et al., 1990). Yohimbine is structurally similar to the known carcinogen reserpine and was therefore nominated to the NTP for genotoxicity testing. Results of an Ames test, with and without S9, were negative

(CEBS, accessed 04-21-2024).

A literature search for information on genotoxicity or carcinogenicity of yohimbe and some of its main constituents (yohimbine, raubasine, corynanthine) published in the last 10 years since the EFSA report did not yield relevant publications. A few recent papers suggested, however, that yohimbine might be an interesting substance to study for possible anti-carcinogenic effects (Jabir et al., 2022, 2023; Paciaroni et al., 2020). A recent evaluation of cytochrome P450 inducing capacity of botanicals has identified yohimbe as active (Haron et al., 2023). Whether this occurs *in vivo*, leading to altered metabolic processing of xenobiotics, remains to be determined.

Yohimbe was nominated as a case study due to its suspected neuro- and cardiotoxicity. Its genotoxicity is unknown.

4. Conclusions and next steps

Botanical products are widely used globally for medicinal purposes, necessitating the evaluation of their toxicity, including genotoxic effects, to assure consumer safety. The Botanical Safety Consortium is exploring the use of standard guideline *in vitro* assays and New Approach Methodologies (NAMs) for effectively screening botanical products. This strategy manuscript outlines the assays and botanicals chosen for assessing genotoxic potential. The immediate steps involve testing these botanicals for genotoxicity, analyzing the data generated, comparing the results to those reported in the literature, and determining whether the proposed assays can be included in a comprehensive toolkit of suitable and informative assays for genotoxicity screening of these complex mixtures. A diverse team of cross-sector experts has selected various assays to determine their applicability to complex botanical mixtures, using 13 well-documented botanicals as case studies. This strategy manuscript is envisioned as the first in a series of papers reporting on the outcome of the BSC's efforts to develop a toolkit of *in vitro* genotoxicity assays suitable for testing botanicals. Future papers in the proposed series will report the results of all the genotoxicity tests performed on the 13 botanicals (Table 1) selected as case studies and, using the data generated, determine the suitability of the selected assays for testing botanicals. Subsequently, the *in vitro* test data will be combined with the results of *in silico* modeling of constituents, published data, and human use data, to determine exposure hazard in humans, and ultimately, for determining human risk.

CRediT authorship contribution statement

Kristine L. Witt: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration. **Jan van Benthem:** Writing – review & editing, Writing – original draft. **Tetyana Kobets:** Writing – review & editing, Writing – original draft. **Guosheng Chen:** Writing – review & editing, Writing – original draft. **Olaf Kelber:** Writing – review & editing, Writing – original draft. **Julie Krzykwa:** Writing – review & editing, Writing – original draft. **James T. MacGregor:** Writing – review & editing, Writing – original draft, Conceptualization. **Nan Mei:** Writing – review & editing, Writing – original draft. **Constance A. Mitchell:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Conceptualization. **Ivonne Rietjens:** Writing – review & editing, Writing – original draft. **Zehra Sarigol-Kilic:** Writing – review & editing, Writing – original draft. **Stephanie L. Smith-Roe:** Writing – review & editing, Writing – original draft, Visualization. **Helga Stopper:** Writing – review & editing, Writing – original draft. **Yax Thakkar:** Writing – review & editing, Writing – original draft. **Errol Zeiger:** Writing – review & editing, Writing – original draft. **Stefan Pfuhler:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization.

Declaration of competing interest

This article reflects the views of the authors and does not necessarily reflect those of the U.S. Food and Drug Administration (FDA) or the U.S. National Institutes of Health (NIH). The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. The following authors are employed in the personal care industries (at the time of article submission): OK, ZSK, SP.

Acknowledgement

This work was supported in part by the Health and Environmental Sciences Institute's (HESI) Botanical Safety Consortium. We acknowledge the committee members for their support and helpful feedback of this document. This work is supported by U.S. Food and Drug Administration (FDA); Office of Dietary Supplement Programs and Department of Health and Human Services (HHS); National Institute of Environmental Health Sciences (NIEHS); Division of Translational Toxicology, Office of Liaison, Policy, and Review; and Health and Environmental Sciences Institute (HESI) via Department of the Interior (DOI) Federal Consulting Group (FCG) under Blanket Purchase Agreement Order 140D0421F0068. This work is also supported in part by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences, Intramural Research project ZIA ES103316-04.

Special thanks are due to Drs. Raymond Tice and David DeMarini for critical review of this manuscript and helpful comments.

Data availability

No data was used for the research described in the article.

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