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Review

Finding the bad actor: Challenges in identifying toxic constituents in botanical dietary supplements

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ABSTRACT

Botanical-derived dietary supplements have widespread use in the general population. The complex and variable nature of botanical ingredients and reports of adverse responses have led to concern for negative human health impacts following consumption of these products. Toxicity testing of the vast number of available products, formulations, and combinations is not feasible due to the time and resource intensive nature of comprehensive testing. Methods are needed to assess the safety of a large number of products via more efficient frameworks. Identification of toxicologically-active constituents is one approach being used, with many advantages toward product regulation. Bioassay-guided fractionation (BGF) is the leading approach used to identify biologically-active constituents. Most BGF studies with botanicals focus on identifying pharmacologically-active constituents for drug discovery or botanical efficacy research. Here, we explore BGF in a toxicological context, drawing from both efficacy and poisonous plant research. Limitations of BGF, including loss of mixture activity and bias toward abundant constituents, and recent advancements in the field (e.g., biochemometrics) are discussed from a toxicological perspective. Identification of active constituents will allow better monitoring of market products for known toxicologically-active constituents, as well as surveying human exposure, two important steps to ensuring the safety of botanical dietary supplements.

1. Why try to identify active constituents?

Humans for millennia have used botanicals to augment diets or affect metabolism or behavior, either as preventatives or correctives for disease, or for specific ceremonies. The ancient discovery process was through trial-and-error and passed down through oral legend or texts such as Papyrus Ebers (Egypt, *circa* 1550 B.C.). Today the use of herbals or botanicals are most common worldwide in the alternative and traditional medicine markets. Botanical ingredients are used in Traditional Chinese Medicine and Ayurveda, as well as in complementary and integrative medicine practice, commonly in the form of botanical dietary supplements. Sources vary in the reported use of dietary supplements, often with little differentiation between botanical and other dietary supplements (Dickinson et al., 2014; Wu et al., 2011). Data collected as part of the 2012 National Health Information Survey indicate that approximately 18% of the general population in the United States use non-vitamin/non-mineral dietary supplements (Clarke et al., 2015).

The terminology and regulatory frameworks that pertain to botanical products differ from country-to-country and have been recently reviewed elsewhere (Low et al., 2017). The regulatory situation in the United States is briefly outlined to provide context for the scientific and globally-relevant discussion of identifying toxic constituents to follow. In 1994, the U.S. Food and Drug Administration (U.S. FDA) passed the Dietary Supplement Health and Education Act (DSHEA). According to DSHEA, there is no requirement for manufacturers to assess the safety of new products prior to market release for botanical dietary ingredients with adequate market history. However, DSHEA requires manufacturers of dietary supplements to notify the U.S. FDA prior to

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marketing a New Dietary Ingredient (NDI), or any ingredient not demonstrably in the marketplace before 1994. This notification does not necessarily require the manufacturer to demonstrate safety of the NDI, and novel combinations of ingredients in use prior to 1994 do not need to be tested for safety. Concerns over the quality and safety of botanical dietary supplements have driven chemists and toxicologists to develop best practices of toxicity testing for these complex mixtures. Unfortunately, the vast number of products on the market and variability between products, precludes testing all available botanical dietary supplements. Shipkowski et al. (2018) detail the scientific and regulatory challenges in assessing the safety of botanical dietary supplements.

Recent efforts at the National Toxicology Program (NTP) have focused on research to inform the assessment of risk associated with botanical dietary supplements. The NTP has studied the toxicity of several botanical dietary supplements in rodents, including green tea (*Camellia sinensis*) extract (NTP, 2016), *Ginkgo biloba* extract (NTP, 2013), kava kava (*Piper methysticum*) extract (NTP, 2012), ginseng (*Panax* species (NTP, 2011);) and goldenseal (*Hydrastis canadensis*) root powder (NTP, 2010). One major challenge following the completion of these studies is understanding how the composition of the botanical material selected for toxicity testing compares to other available products.

Botanical dietary supplements are complex entities. Trying to understand the biological activity of these complex mixtures can be viewed as a continuum between two extremes: (i) as whole mixtures, with the integrated activity of many constituents contributing to observed activity/toxicity, or (ii) as a complex matrix containing mostly inert material with one or more active constituents driving activity/toxicity. The whole mixtures view requires methods to determine whether or not toxicity data from one mixture (*e.g.* botanical supplement formulation) can be predictive of another mixture – in effect, are two mixtures sufficiently similar (*a.k.a.* phytoequivalent). Alternatively, to identify the active constituent(s), methods are required to determine which component(s) of a botanical mixture are responsible for observed toxicity or biological activity. The term 'active constituent' (*a.k.a.* bioactive) may refer to either efficacious or toxic components. The majority of literature on identifying active constituents from botanicals has been rooted in efficacy research or drug discovery (Findeis et al., 2012; Olivon et al., 2017; Sharma and Gupta, 2015), or finding functional chemicals (e.g., pesticides) for commercial application (Madhu et al., 2010). In contrast, the goal of this review is to highlight the methods of best practice and challenges in identifying botanical constituents associated with toxicity. While methods for identifying active constituents should have relevance for toxicologically- or pharmacologically-active compounds, examples from poisonous plant research are deliberately included to leverage existing knowledge and inform the discussion on toxic constituents.

Active constituent identification and sufficient similarity are both methods being developed and applied to toxicity testing and safety assessment of botanical dietary supplements. Determining the sufficient similarity of botanicals is discussed in a companion paper by Catlin et al. (2018). Sufficient similarity analysis can be a useful tool for understanding the relevance of results from a tested botanical sample to related products in the marketplace. However, a whole-mixtures approach to assessing botanical toxicity does not attempt to identify the specific components responsible for biological activity, rather, it provides an assessment of the activity *en masse*. Regulatory decision making, product authentication and safety assessment are made easier when the active constituents in botanical supplements have been identified.

Examples of regulatory action against botanical dietary supplements, based on a single component, include purported weight loss products Hydroxycut® and OxyELITE Pro®. Reports of toxicity in humans have led to the recall of several formulations of Hydroxycut® since the early 2000s. Prior to 2004, many Hydroxycut® products contained *Ephedra* species. These products were removed and reformulated due to reports of toxicity and the ban of ephedra alkaloids by the U.S. Food and Drug Administration (U.S. FDA) (Soni et al., 2004). Subsequent formulations of Hydroxycut® were linked to cases of human hepatotoxicity (Dara et al., 2008; Fong et al., 2010; Lobb, 2009) and in May of 2009, the U.S. FDA issued a warning for the public to avoid Hydroxycut® products (Avigan et al., 2016; Stickel and Shouval, 2015). While the 2009 U.S. FDA warning did not specifically identify the ingredient (s) responsible for the reported hepatotoxicity incidents, other sources suspected the toxicant to be hydroxycitric acid, a component of *Garcinia cambogia* and *Hibiscus subdariffa* (Lobb, 2009; Lunsford et al., 2016). Following the 2009 warning, a new Hydroxycut® formulation, without hydroxycitric acid, was released and has since been linked to hepatotoxicity in humans (Adike et al., 2017; Araujo and Worman, 2015). It is important to note that in addition to the rapid succession of reformulations, there are also numerous variations available in the marketplace at any one time (e.g., Hydroxycut comes in HD, Elite, Pro, Hardcore, Black, etc. varieties), which can further complicate safety evaluation.

A similar string of reformulations occurred with OxyELITE Pro® products. In 2012, the U.S. FDA issued warning letters to the manufacturers of products that contained 1,3-dimethylamylamine (DMAA), a component of *Geranium* species extract, leading to removal of those products from the market (Avigan et al., 2016). Consumption of products containing DMAA, including OxyELITE Pro®, have been associated with cardiac deaths (Eliason et al., 2012). A different formulation of OxyELITE Pro® was then associated with an outbreak of non-viral hepatitis in Hawaii in 2013, with the suspected active constituent being aegeline, a toxicant from the bael tree (*Aegele marmelos*). These products were eventually recalled (Avigan et al., 2016). Interestingly, recent efforts to assess the safety of a new formulation of OxyELITE Pro found that not all of the ingredients listed on the label were present, but that the product instead appeared to be a mixture of synthetic alkaloids (caffeine, aegeline, higenamine, yohimbine, and coclaurine), along with trace amounts of tannins (Miousse et al., 2017). Miousse et al. (2017) observed hepatotoxicity in mice exposed to the new formulation of OxyELITE Pro for 4 weeks or 13 weeks.

Testing for adulterants in botanical dietary supplements can also help regulatory decision-making. In 2015 a new formulation of OxyELITE Pro® was released that was found to contain fluoxetine, a pharmaceutical antidepressant (Avigan et al., 2016). Botanical supplements with purported weight loss or exercise enhancement effects are common targets for adulteration and have been found to contain ephedra alkaloid derivatives, amphetamines, or androgenic steroids (Baume et al., 2006; Geyer et al., 2008). Multiple lots of the supplement Craze®, made by Driven Sports Inc., were reportedly adulterated with a methamphetamine derivative (Cohen et al., 2014). Other common adulterants of botanical exercise supplements include phosphodiesterase type-5 inhibitors (Singh et al., 2009), drugs used for erectile dysfunction (*e.g.* sildenafil). Pharmaceutical adulterants in this class of supplements not only pose a health risk to consumers but may also lead to unintentional consumption and accusations of "doping" by professional athletes (Somerville et al., 2005).

In the cases of Hydroxycut® and OxyELITE Pro®, human evidence of toxicity occurred prior to the U.S. FDA warnings/bans or manufacturer recalls of these products. Due to the lack of premarket safety testing requirements, adverse event reporting often serves as the first signal of toxicity for botanical dietary supplements. Linking an adverse event to a specific supplement is made more difficult by the number and variation in botanical supplements, the low frequency of adverse events and potential diversity of individual responses, and the lack of a single reporting system for adverse events associated with dietary supplements (Klontz et al., 2015).

Ideally, botanical dietary supplements that pose a threat to human health should be identified prior to evidence of toxicity in humans. Preclinical toxicity assessments in animals are useful for identifying

potential hazard associated with exposure to botanicals. However, translating findings from animal studies to a human exposure context requires some understanding of the pharmacokinetics of the botanical. As discussed in more detail in Waidyanatha et al. (2018), absorption, distribution, metabolism, and elimination (ADME) studies with botanicals rely heavily on knowledge of toxic constituents to be informative for relating toxicity observed in animal studies to human exposure scenarios. Without knowledge of the toxic constituents, reliance on 'marker' compounds (i.e., compounds used to authenticate botanicals) could provide misleading information.

The ability to identify and screen for active constituents in botanical dietary supplements that are likely to produce toxicity will help protect human health. Identification of the toxic constituent(s) is helpful in ensuring the quality of botanical dietary supplements in the marketplace. In cases where the toxic constituent is an adulterant or a structurally-related class of adulterants, products can undergo screening for compounds in the target class (Ma et al., 2017; Singh et al., 2009). Additionally, regulatory action is facilitated when there is a known toxic constituent, such as in the examples described above with ephedra alkaloids, DMAA, and aegeline. Aside from product quality, identification of active constituents also serves an important role in better understanding the toxicity of botanicals. Known toxic constituents can be evaluated in ADME studies to aid in interpretation of animal studies to a human context. Furthermore, once a toxic constituent is identified, the chemical structure can provide additional information through comparison to chemicals with more extensive toxicity data (e.g., chemical read-across). While there are many advantages to definitively identifying the constituent(s) responsible for observed toxicity, there are both technical and natural challenges in the identification of active constituents in botanical supplements.

2. The default in identifying active constituents: bioassay-guided fractionation

Identification of the active constituent(s) in complex botanical mixtures can be challenging. The most common approach used has been bioassay-guided fractionation (BGF), depicted in Fig. 1 and described in depth elsewhere (Weller, 2012). Briefly, the botanical material of interest is extracted using several diverse solvents. The extracts are subsequently tested in a bioassay that is carefully selected and fit-for-purpose. The extracts that show biological activity can then be fractionated, using a variety of methods (Weller, 2012). Through an iterative process, the fractions are tested for activity in the selected bioassay and may be fractionated further until the active constituent(s) can be identified. Identification is carried out using a combination of spectroscopic characterization, involving techniques such as mass spectrometry with dereplication/structure elucidation (Hubert et al., 2017; Nikolic et al., 2012), nuclear magnetic resonance and x-ray crystallography.

Botanical dietary supplements, containing extracts or raw material (whole plant, roots, aerials flowers, *etc.*), are extracted using multiple diverse solvents. The extracts are tested in a bioassay and active extracts are fractionated. Through an iterative process, the fractions are tested in the bioassay and active fractions may be further fractionated. Active constituents are then identified via other methods.

The use of BGF to identify toxic constituents in an Australian botanical, *Swainsona,* is an early example of how effective this approach can be. Cattle poisonings from several species of *Swainsona* have been recorded in Australia for centuries (Everist, 1981) and the manifestation of this poisoning, referred to as locoism, was reported in the United States in the early 20th century (Mathews, 1932). Prolonged consumption of *Swainsona* causes a lysosomal storage disease and is characterized by wasting and decreased reproductive success (James et al., 1999). Researchers were able to replicate the disease pathology in guinea pigs and mice and then determine the mechanism of action, inhibition of lysosomal α–mannosidase, by pathological evaluation and

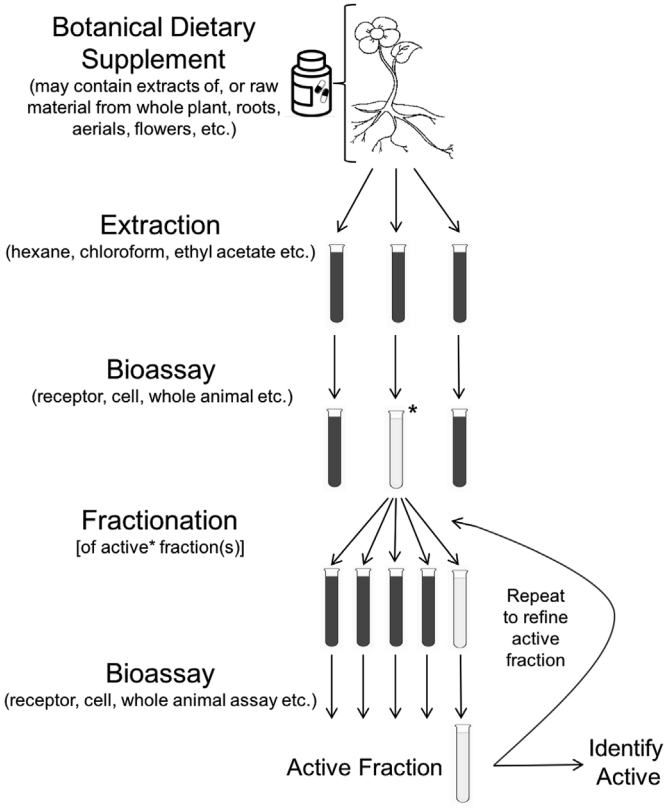

Fig. 1. Bioassay guided fractionation.

enzyme assays (Dorling et al., 1978). When the mechanism of toxicity was elucidated, this botanical became an ideal candidate for the use of BGF to identify the active constituent(s). By knowing the exact mechanism of toxicity, Colegate et al. (1979) were able to select a simple, fast and precise bioassay to determine which *Swainsona* extracts and extract fractions retained the biological activity of interest, α–mannosidase inhibition. Swainsonine was ultimately identified as the α–mannosidase inhibitor responsible for toxicity (Colgate et al., 1979). Subsequently, swainsonine was identified as the active constituent of locoweed (primarily *Oxytropis* and *Astragalus* species), plant species responsible for livestock poisoning in the western United States. The disease presentation resulting from locoweed poisoning was recognized to be similar to *Swainsona*-induced toxicity and swainsonine was suspected and identified. Follow-up research was then able to determine that a swainsonine concentration in plants greater than 0.001% would pose a threat to livestock health and swainsonine analysis may be used as a precautionary measurement in grazing areas (James et al., 1999).

While this example of BGF uses a raw botanical, the same concept may be applied to mixtures of botanicals in dietary supplements. This method can be very powerful but has several limitations and challenges that users must consider, including the reliance on having a known mechanism of action for efficient detection. Additional challenges are introduced by environmental influence and variation of botanical composition that can significantly alter the presence and/or abundance of active components.

2.1. Challenges, pitfalls and limitations

The biggest challenge in implementing BGF to identify active constituents is the selection of an appropriate bioassay. The key to success in bioassay selection for BGF is identifying the mechanism of toxicity. Ideally, to increase fractionation efficiency (*e.g.* higher throughput and lower resource burden), the bioassay selected for BGF should be simple, fast and precise. These methods have been successfully applied for

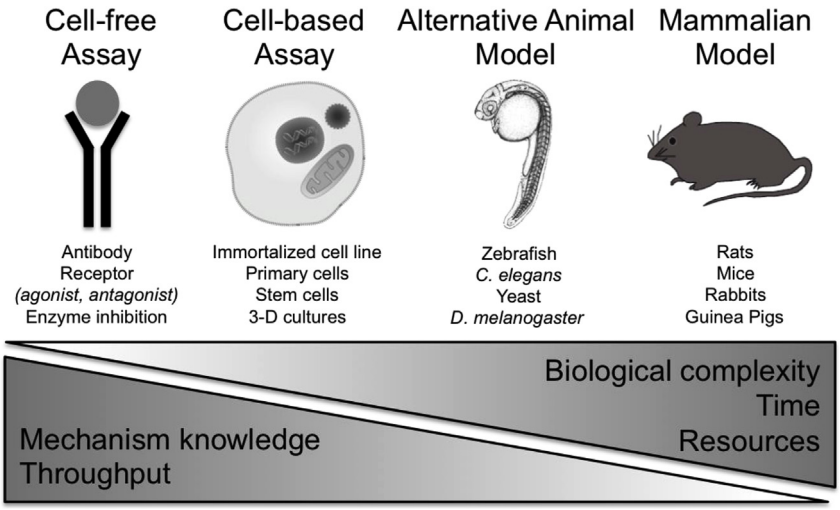

Fig. 2. Bioassay Selection. When the specific mechanism of toxicity is known for a particular botanical, a simple bioassay may be selected that will accurately predict the outcome of a more complex system. When less is known about the mechanism of toxicity a more complex bioassay is required to predict toxicity.

therapeutic applications, with the discovery of several important products including Cox-2 inhibitors and estrogen receptor ligands (Ciesla and Moaddel, 2016). In these cases, the molecular target was known before screening began, allowing use of a simple bioassay. As knowledge about the target mechanism or in our case, mechanism of toxicity decreases, the complexity of the selected bioassay must increase to compensate for unknowns regarding biological activity (Fig. 2). For example, if a botanical dietary supplement is known to act through a specific receptor or other targeted mechanism, as with *Swainsona*, a simple cell-based or cell-free assay can be utilized to determine which constituent(s) in the supplement are responsible for the observed activity (Kellogg et al., 2016; Overk et al., 2005; Powell et al., 2008; Westenburg et al., 2000). When a simplified assay is employed, it is important to consider other limitations, such as the assay's metabolic capacity, chemical solubility and potential partitioning/binding within the assay system.

In stark contrast to the example of *Swainsona,* there are cases in which complex biological systems are required for the selected bioassay to be predictive. This was the case in the identification of isocupressic acid (ICA) as the constituent of *Pinus ponderosa* (ponderosa pine) responsible for spontaneous abortions in cattle (Gardner et al., 1994). Early attempts to identify ponderosa pine active constituent(s) utilized rodents as the bioassay (Allen and Kitts, 1961). Despite the relative complexity, the rodent model could not adequately predict cattle toxicity because perinatal stress in rodents usually results in fetal absorption rather than abortion. Pregnant beef cattle were selected ultimately for the predictive bioassay, and through an iterative process of testing and fractionation of active extracts, ICA was successfully identified as the major abortifacient constituent. Using a resource and time intensive bioassay is not ideal; however, when little mechanistic information is available, this may be unavoidable. With all uses of BGF, but particularly in cases when the mechanism of toxicity is unknown, it is critical to consider the biological relevance of the selected bioassay.

There are several other challenges, relating to extraction and analysis methods, which must be considered in the efficient use of BGF (Kellogg et al., 2016; Sasidharan et al., 2011; Weller, 2012). Unlike testing a whole mixture, BGF takes a reductionist approach to toxicity testing of botanical dietary supplements. While there are many advantages to a reductionist approach (*e.g.* regulatory decision making), one of the biggest pitfalls with this method is the potential to lose antagonistic or synergistic activity when multiple active constituents are present. This has been demonstrated with North American ginseng root (*Panax quinquefolius*) where aqueous extracts were shown to have immune-stimulatory properties while alcoholic extracts had immune-inhibitory properties (Assinewe et al., 2002; Azike et al., 2011; Lui et al., 2012). The presence of residual 'impurities' following purification of a botanical have also been shown to have a profound effect on the biological activity of a product (Qiu et al., 2013). Another potential pitfall that can affect both extraction methods and data interpretation is bias towards abundant compounds. Even when a researcher is aware of this limitation, logistical challenges in isolation/purification of compounds may impact which fractions or components are identifiable. When identifying active constituents, it is critical to consider how various methodologies can affect the type and abundance of components in the extract and how this may impact biological activity and data interpretation.

2.2. Taking the next step- metabolomic and biochemometric approaches

Starting with a BGF scaffold, metabolomic and biochemometric approaches provide complementary advantages compared to BGF alone, by improving the bioassay and analysis methods, respectively. Metabolomic approaches, unlike traditional BGF, do not attempt to identify constituents prior to their interaction with a biological system. Taking a more unsupervised approach, the raw material is applied to a bioassay and the primary and secondary metabolites are the focus of chemical identification. This method avoids some issues identified for BGF (Avula et al., 2016; Prince and Pohnert, 2010) by altering the bioassay exposure paradigm. Specifically, this technique avoids losing the interaction of multiple active constituents. Metabolomic approaches also avoid losing the biological response of low abundance or unstable compounds that might be removed or lost during the fractionation/purification stages of BGF. Similar to BGF, an iterative process of bioassay exposure is required to confirm that identified metabolites retain the biological activity of the whole mixture.

While metabolomic approaches avoid several issues present in traditional BGF, they fail to show correlation between the identified metabolites and biological responses. By using more advanced data analysis techniques, biochemometrics aims to correlate chemometric data with biological responses to help discern which components are most likely responsible for divergent biological responses. Using a BGF approach, generally with a simple bioassay, biological and chemometric data can be processed with a variety of analytical methods, as summarized in Kellogg et al. (2016). The use of S-plots, described in Wiklund et al. (2008), allows visualization of chemometric signals which correlate with divergent biological activity. Another approach, selectivity ratios, weighs the correlation of chemical entities to biological activity by considering the extent to which variance can be

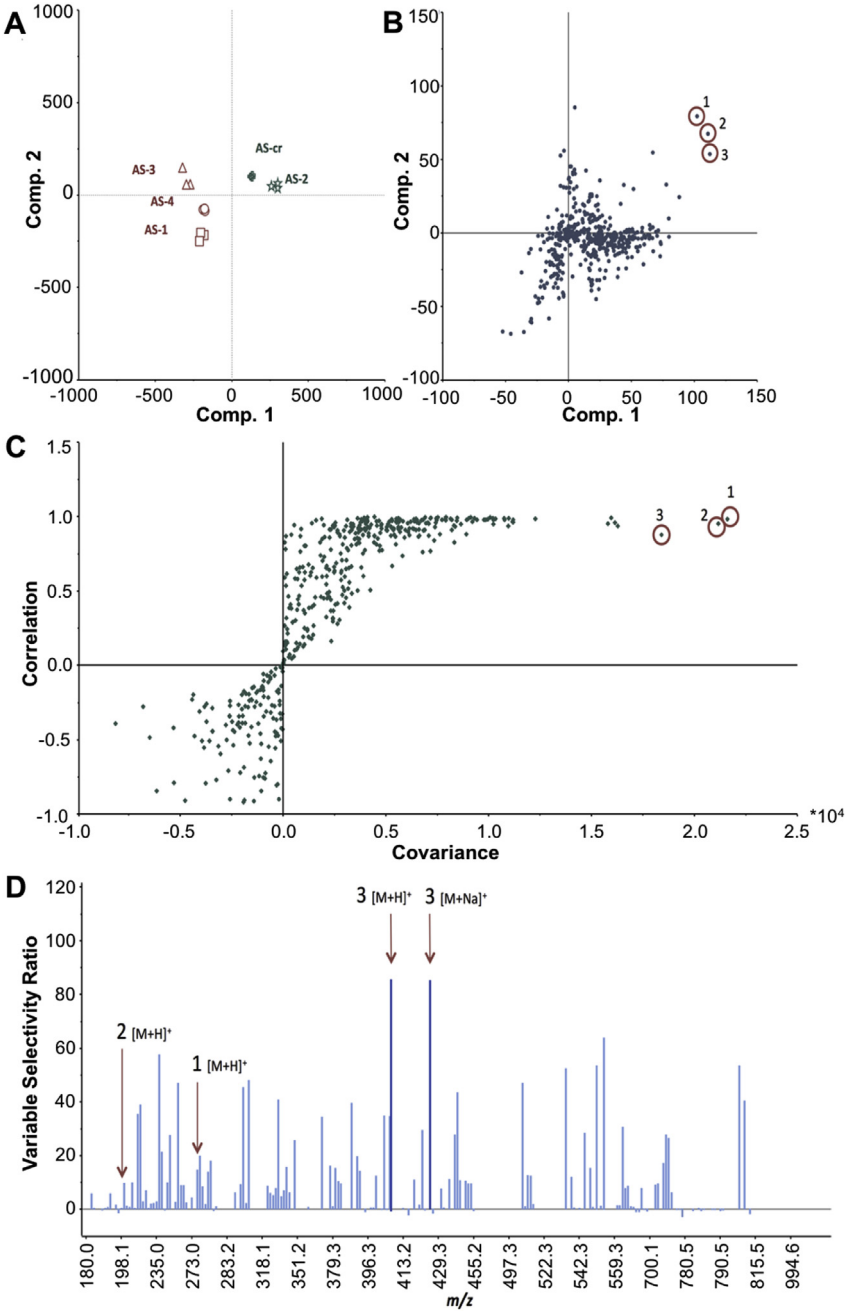

Fig. 3. Biochemometric Analysis of *Alternaria* sp. Mass Spectral Data and Bacterial Growth Inhibition. Reprinted with permission from "Biochemometrics for Natural Products Research: Comparison of Data Analysis Approaches and Application to Identification of Bioactive Compounds", by J.J. Kellogg. 2016. *Journal of Natural Products* 79, 376–386. Copyright 2016 by American Chemical Society and American Society of Pharmacognosy. Reprinted with permission.

explained. The utility of these methods is demonstrated in Kellogg et al. (2016), in which biochemometric methods are used to identify the active constituent(s) responsible for the antimicrobial activity of *Alternaria* sp., an endophytic fungus from the botanical goldenseal (*Hydrastis canadensis*). In Kellogg et al. (2016), the use of more traditional analysis techniques (partial least squares analysis and loading, and S-plots of the PLS analysis) identify three chemical components that correlate with biological activity (Fig. 3A, B, C). However, the selectivity ratio analysis illustrates that only one of the three compounds is strongly associated with the biological activity (Fig. 3D). These advanced data analysis tools work very well with a simple bioassay, but interpretation may be more difficult with more complex biological endpoints.

3. A targeted approach for identifying toxic constituents

The BGF approach discussed above does not require any *a priori* knowledge of potential actives. However, in many cases, there are purported or suspected active constituents for a botanical. In those cases, approaches that correlate biological activity to constituent concentration can be used to build a case for the association between a specific constituent or constituent class and the observed toxicity.

An example of this approach was used by Chen et al. (2013) in studies evaluating the genotoxicity of goldenseal. Goldenseal root powder is a botanical dietary supplement used for promoting immune and digestive health. The isoquinoline alkaloids, hydrastine and berberine, are the purported pharmacologically-active constituents (Abourashed and Khan, 2001). In two-year toxicity and carcinogenicity

studies with goldenseal root powder, liver tumors were observed in mice and rats (Dunnick et al., 2011; NTP, 2010). Follow-up mechanistic studies explored the DNA damaging potential of various goldenseal alkaloids (i.e., berberine, palmatine, hydrastine, canadine, and hydrastinine) and identified topoisomerase II inhibition as the likely mechanism responsible for DNA damage (Chen et al., 2013). Berberine was found to be the most potent of the five alkaloids in a Comet assay to measure DNA damage (Chen et al., 2013).

Chen et al. (2013) measured the berberine content in eight commercial goldenseal products and found a range of 5.3–49.5 mM berberine. They then assessed the DNA damage potential of each of the eight samples in HepG2 cells using measurement of the γ-H2A.X protein as an indicator of double strand breaks. The authors concluded that the berberine content of various goldenseal formulations was correlated to DNA damage (correlation coefficient of 0.98). A future application of this type of approach in clinical studies could be the development of botanical exposure biomarkers for correlation analysis with measured health parameters (e.g., clinical chemistry values indicating liver toxicity).

In the example presented above, the purported pharmacologically-active constituents served as a starting place for identifying the constituent(s) responsible for toxicity. A more agnostic approach for identifying potential toxic constituents is to screen libraries of botanically-derived chemical structures using computational tools to predict toxicity. An example of this *in silico* approach is presented in Glück et al. (2018). In this work, Glück et al. (2018) screen a library of over 600 secondary plant metabolites using a number of different computer models that correlate chemical descriptors to biological activity. The computer models were designed to predict chemicals with genotoxic or carcinogenic potential based on training sets of chemicals with known genotoxicity/carcinogenicity. Through an iterative approach, the researchers identified the optimal combination of models to predict genotoxicity and carcinogenicity. The chemicals within the library were then ranked in terms of their predicted genotoxic and carcinogenic potential, with (−)-asimilobine, aloin (a constituent of aloe vera), annoretine, chrysothrone, coptisine, elymoclavine, and thalicminine predicted with high probability to be genotoxic and the pyrrolizidine alkaloids predicted with high probability to be carcinogenic (Gluck et al., 2018). This type of analysis holds promise for prioritizing botanicals for further evaluation.

4. Nature's contribution to active constituents

While there are many 'man-made' challenges in identifying active constituents that are introduced during processing/investigation, nature also contributes to the complexity of interpretation and reliability of identified active constituents in botanical dietary supplements. Several factors may impact the natural concentration range of active constituents in botanicals, including season (temperature; hours of sunlight), stage of plant growth, precipitation (soil moisture), and geographical region (soil composition and nutrients). Seasonal variation has been shown to influence the proportion of toxic constituents in various botanicals (Allen and Kitts, 1961; Cook et al., 2010; Ralphs et al., 1997). Studies in mice have shown that ponderosa pine (*Pinus ponderosa*) samples collected from September to February are more toxic than those collected in spring and summer months (Allen and Kitts, 1961). In addition, the ICA content in ponderosa pine samples has been quantified in samples from various US western states throughout the year, showing that some regions have low annual variation compared to others and that large differences in ICA content can be observed within a single tree (Cook et al., 2010). Season/plant maturation and growth have been shown to influence the concentration of toxic constituents in four species of larkspur (*Delphinium barbeyi, D. glaucum, D. glaucescens, D. occidentale*) (Ralphs et al., 1997); the concentration of toxic alkaloids was shown to decrease with plant maturation and concentrations were consistently higher in flowers and pods when compared to leaves. While seasonal variation in botanical constituents is understandable, it may be surprising that within a small geographical area, the variation between populations of the same species can be larger than the annual changes within a population. Such differences have been observed for a number of botanicals, including American ginseng (*Panax quinquefolius*), broom snake weed (*Gutierrezia sarothrae*), and tienchi ginseng (*Panax notoginseng)* (Dong et al., 2003; Lim et al., 2005; Schlag and McIntosh, 2006). Environmental plant stressors, such as drought or excess water, have been shown to influence the accumulation of other compounds (*e.g.* nitrates and hydrogen cyanide) (UNEP, 2016) and may complicate the impact of identified actives in botanicals.

In addition to geographic and seasonal variations, the part of the plant that is used or how it is processed to produce a supplement can also have an impact on potential toxicity. A well demonstrated example of this is aloe vera. Various forms of aloe vera (i.e., gel, juice, dried exudate) have been used medicinally for a number of ailments (Maan et al., 2018). Generally, the whole leaf and gel have been used topically to promote wound healing; aloe preparations including the outer and middle (latex) layers of the leaf (sometimes referred to as 'non-decolorized' (Boudreau, 2013)) have been taken orally for constipation; while aloe preparations without the latex layer of the leaf (i.e., 'decolorized') have been used to bolster gastrointestinal health (Maan et al., 2018). The distinction between aloe vera preparations is very important because the latex layer of the aloe leaf is known to contain anthraquinones (a.k.a., hydroxyanthracene derivatives; e.g., aloe-emodin, emodin, aloin A and aloin B) (Maan et al., 2018), which are of concern based on potential harmful effects of the anthraquinone class in humans (EFSA ANS Panel, 2018).

Toxicity and carcinogenicity studies with a whole-leaf extract of *Aloe barbadensis* Miller in rats found clear evidence of colon cancer (Boudreau et al., 2013). A subsequent 90-day study by Shao et al. (2013) in rats, found that a decolorized aloe preparation with low anthraquinone content (total aloin A and B < 0.1 ppm) did not induce any of the histopathological alterations observed with the high anthraquinone aloe used in the Boudreau et al. (2013) 90-day studies. The aloe vera example also illustrates the complexity of botanical substances; follow-up work to these initial studies was conducted to elucidate additional toxic constituents that were previously masked by a known toxin. Shao et al. (2013) concluded that anthraquinones were implicated as the toxic constituents in aloe preparations, but they noted that it could not be ruled out that anthraquinones were serving as a marker for an unidentified toxic constituent that co-occurred with the anthraquinone class and was also removed during the decolorization process. Finally, Boudreau et al. (2017) assessed a pure mixture of aloin A and B in a 90-day toxicity study in rats to show that these constituents could induce a similar profile of pathological effects as the high anthraquinone aloe preparation. This study provided convincing support that aloin A and B are driving the observed carcinogenicity of whole-leaf, high anthraquinone aloe, and ruled out the possibility that they are inactive marker constituents (Boudreau et al., 2017).

5. Active constituents identified, what next?

Once an active constituent has been identified for a particular botanical genus or species, additional tools become available for screening and identification of novel active constituents. Structure activity relationship (SAR) analysis may lead to the identification of active moieties that are related to observed biological effects, allowing prioritization for bioassay screening. The use of SAR in the field of toxicology has been described elsewhere (McKinney et al., 2000). This approach was used to investigate *Aristolochia* species, a botanical previously used in supplements for a variety of ailments and associated with nephrotoxicity (Balachandran et al., 2005). SAR analysis was able to identify the structural aspects of aristolochic acid analogues responsible for the observed toxicities.

In the future, the development of a free and open database composed of identified active constituents, families of active constituents, active moieties and common adulterants will help streamline product monitoring and the testing of new products. Identifying potentially dangerous products with ease may allow regulatory agencies to make decisions about these products before humans are exposed. However, in the absence of human toxicity data, agencies must be able to extrapolate toxicities observed in model systems to human exposure. A database of known active constituents can be used for the development of physiologically based pharmacokinetic (PBPK) models and human biomonitoring methods, an area of development with its own set of challenges and discussed in depth elsewhere (Waidyanatha et al., 2018).

Although definitive identification of toxic constituents can be challenging and resource intensive, it can have an important impact on protecting public health. The widespread use of botanical dietary supplements and evidence of toxicity in humans following consumption of select products illustrates the need for standard methods for assessing toxicity and safety. The aloe vera example demonstrates the utility of these efforts; Boudreau et al. (2013) demonstrated that 27.8 mg/kg total aloin A and B, less than 3-fold higher than current allowable levels set by the International Aloe Science Council (10 mg/kg aloin), induced pathological changes in rat colon cells. This work provides support for re-evaluating the allowable limit of anthraquinones in aloe products and highlights the need to screen market products for toxic constituents to avoid harmful exposures. Identification of active constituents, via bioassay guided fractionation and complementary methods, offers a viable path forward to improve the safety of the botanicals market. Active constituent identification is critical for product monitoring and the development of human PBPK models, establishing important factors necessary for regulatory and risk assessment activities with botanical supplements.

6. Disclaimer

The opinions expressed in this document do not necessarily reflect the opinions or policies of any U.S. government agency or university. The mention of specific products, tradenames, or manufacturers is intended only for accuracy, and should not be considered as an endorsement, or otherwise, of that product.

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References

Abourashed, E.A., Khan, I.A., 2001. High-performance liquid chromatography determination of hydrastine and berberine in dietary supplements containing goldenseal. J. Pharmacol. Sci. 90, 817–822.
Adike, A., Smith, M.L., Chervenak, A., Vargas, H.E., 2017. Hydroxycut-related vanishing bile duct syndrome. Clin. Gastroenterol. Hepatol. 15, 142–144.
Allen, M.R., Kitts, W.D., 1961. The effect of yellow pine (*Pinus Ponderosa* Laws) needles on the reproductivity of the laboratory female mouse. Can. J. Anim. Sci. 41, 1–8.
Araujo, J.L., Worman, H.J., 2015. Acute liver injury associated with a newer formulation of the herbal weight loss supplement Hydroxycut. BMJ Case Rep. https://doi.org/10.1136/bcr-2015-210303. 2015.
Assinewe, V.A., Amason, J.T., Aubry, A., Mullin, J., Lemaire, I., 2002. Extractable polysaccharides of Panax quinquefolius L. (North American ginseng) root stimulate TNFalpha production by alveolar macrophages. Phytomedicine : international journal of phytotherapy and phytopharmacology 9, 398–404.
Avigan, M.I., Mozersky, R.P., Seeff, L.B., 2016. Scientific and regulatory perspectives in herbal and dietary supplement associated hepatotoxicity in the United States. Int. J. Mol. Sci. 17.
Avula, B., Wang, Y.H., Isaac, G., Yuk, J., Wrona, M., Yu, K., Khan, I.A., 2016. Metabolomics based UPLC-QToF-MS approach for the authentication of various botanicals and dietary supplements. Planta Med. 82, OA13.
Azike, C.G., Charpentier, P.A., Hou, J., Pei, H., King Lui, E.M., 2011. The Yin and Yang actions of North American ginseng root in modulating the immune function of macrophages. Chin. Med. 6, 21.
Balachandran, P., Wei, F., Lin, R.C., Khan, I.A., Pasco, D.S., 2005. Structure activity relationships of aristolochic acid analogues: toxicity in cultured renal epithelial cells. Kidney Int. 67, 1797–1805.
Baume, N., Mahler, N., Kamber, M., Mangin, P., Saugy, M., 2006. Research of stimulants and anabolic steroids in dietary supplements. Scand. J. Med. Sci. Sports 16, 41–48.
Boudreau, M.D., 2013. Nondecolorized qualifier is a misnomer for the aloe vera whole leaf extract test. Material. Toxicol Sci 133 343-343.
Boudreau, M.D., Mellick, P.W., Olson, G.R., Felton, R.P., Thorn, B.T., Beland, F.A., 2013. Clear evidence of carcinogenic activity by a whole-leaf extract of aloe barbadensis miller (aloe vera) in F344/N rats. Toxicol. Sci. 131, 26–39.
Boudreau, M.D., Olson, G.R., Tryndyak, V.P., Bryant, M.S., Felton, R.P., Beland, F.A., 2017. Aloin, a component of the aloe vera plant leaf, induces pathological changes and modulates the composition of microbiota in the large intestines of F344/N male rats. Toxicol. Sci. 158, 302–318.
Catlin, N.R., Collins, B.J., Auerbach, S.S., Ferguson, S.S., Harnly, J.M., Gennings, C., Waidyanatha, S., Rice, G.E., Smith-Roe, S.L., Witt, K.L., Rider, C.V., 2018. How similar is similar enough? A sufficient similarity case study with Ginkgo biloba extract. Food Chem. Toxicol. : an international journal published for the British Industrial Biological Research Association 118, 328–339.
Chen, S., Wan, L.Q., Couch, L., Lin, H.X., Li, Y., Dobrovolsky, V.N., Mei, N., Guo, L., 2013. Mechanism study of goldenseal-associated DNA damage. Toxicol. Lett. 221, 64–72.
Ciesla, L., Moaddel, R., 2016. Comparison of analytical techniques for the identification of bioactive compounds from natural products. Nat. Prod. Rep. 33, 1131–1145.
Clarke, T.C., Black, L.I., Stussman, B.J., Barnes, P.M., Nahin, R.L., 2015. Trends in the use of complementary health approaches among adults: United States, 2002-2012. National Health Statistics Reports 10, 1–16.
Cohen, P.A., Travis, J.C., Venhuis, B.J., 2014. A methamphetamine analog (N,alpha-diethyl-phenylethylamine) identified in a mainstream dietary supplement. Drug Test. Anal. 6, 805–807.
Colgate, S.M., Dorling, P.R., Huxtable, C.R., 1979. A spectroscopic investigation of swainsonine: an α-mannosidase inhibitor isolated from *Swainsona canescens*. Aust. J. Chem. 32, 2257–2264.
Cook, D., Gardner, D.R., Pfister, J.A., Panter, K.E., Stegelmeier, B.L., Lee, S.T., Welch, K.D., Green, B.T., Davis, T.Z., 2010. Differences in ponderosa pine isocupressic acid concentrations across space and time. Rangelands 32, 14–17.
Dara, L., Hewett, J., Lim, J.K., 2008. Hydroxycut hepatotoxicity: a case series and review of liver toxicity from herbal weight loss supplements. World J. Gastroenterol. 14, 6999–7004.
Dickinson, A., Blatman, J., El-Dash, N., Franco, J.C., 2014. Consumer usage and reasons for using dietary supplements: report of a series of surveys. J. Am. Coll. Nutr. 33, 176–182.
Dong, T.T., Cui, X.M., Song, Z.H., Zhao, K.J., Ji, Z.N., Lo, C.K., Tsim, K.W., 2003. Chemical assessment of roots of Panax notoginseng in China: regional and seasonal variations in its active constituents. J. Agric. Food Chem. 51, 4617–4623.
Dorling, P.R., Huxtable, C.R., Vogel, P., 1978. Lysosomal storage in Swainsona spp. toxicosis: an induced mannosidosis. Neuropathol. Appl. Neurobiol. 4, 285–295.
Dunnick, J.K., Singh, B., Nyska, A., Peckham, J., Kissling, G.E., Sanders, J.M., 2011. Investigating the potential for toxicity from long-term use of the herbal products, goldenseal and milk thistle. Toxicol. Pathol. 39, 398–409.
EFSA ANS Panel, 2018. Safety of hydroxyanthracene derivatives for use in food. EFSA Journal 16.
Eliason, M.J., Eichner, A., Cancio, A., Bestervelt, L., Adams, B.D., Deuster, P.A., 2012. Case reports: death of active duty soldiers following ingestion of dietary supplements containing 1,3-dimethylamylamine (DMAA). Mil. Med. 177, 1455–1459.
Everist, S.L., 1981. Posionous Plants of Australia. Angus and Robertson, London.
Findeis, M.A., Schroeder, F., McKee, T.D., Yager, D., Fraering, P.C., Creaser, S.P., Austin, W.F., Clardy, J., Wang, R., Selkoe, D., Eckman, C.B., 2012. Discovery of a novel pharmacological and structural class of gamma secretase modulators derived from the extract of actaea racemosa. ACS Chem. Neurosci. 3, 941–951.
Fong, T.L., Klontz, K.C., Canas-Coto, A., Casper, S.J., Durazo, F.A., Davern 2nd, T.J., Hayashi, P., Lee, W.M., Seeff, L.B., 2010. Hepatotoxicity due to hydroxycut: a case series. Am. J. Gastroenterol. 105, 1561–1566.
Gardner, D.R., Molyneux, R.J., James, L.F., Panter, K.E., Stegelmeier, B.L., 1994. Ponderosa pine needle-induced abortion in beef cattle: identification of isocupressic acid as the principle active compound. J. Agric. Food Chem. 42, 756–761.
Geyer, H., Parr, M.K., Koehler, K., Mareck, U., Schanzer, W., Thevis, M., 2008. Nutritional supplements cross-contaminated and faked with doping substances. J. Mass Spectrom. : JMS 43, 892–902.
Gluck, J., Buhrke, T., Frenzel, F., Braeuning, A., Lampen, A., 2018. In silico genotoxicity and carcinogenicity prediction for food-relevant secondary plant metabolites. Food Chem. Toxicol. 116, 298–306.
Hubert, J., Nuzillard, J.M., Renault, J.H., 2017. Dereplication strategies in natural product research: how many tools and methodologies behind the same concept? Phytochemistry Rev. 16, 55–95.
James, L.F., Panter, K.E., Steglemeier, B.L., Ralphs, M.H., Pfister, J.A., Gardner, D.R., 1999. *Astragalus* and *Oxytropis* posion livestock with different toxins. In: Sterling, T.M., Thompson, D.C. (Eds.), Locoweed Research. New Mexico State University, Las Cruces, NM.

Kellogg, J.J., Todd, D.A., Egan, J.M., Raja, H.A., Oberlies, N.H., Kvalheim, O.M., Cech, N.B., 2016. Biochemometrics for natural products research: comparison of data analysis approaches and application to identification of bioactive compounds. J. Nat. Prod. 79, 376–386.

Klontz, K.C., DeBeck, H.J., LeBlanc, P., Mogen, K.M., Wolpert, B.J., Sabo, J.L., Salter, M., Seelman, S.L., Lance, S.E., Monahan, C., Steigman, D.S., Gensheimer, K., 2015. The role of adverse event reporting in the FDA response to a multistate outbreak of liver disease associated with a dietary supplement. Publ. Health Rep. 130, 526–532.

Lim, W., Mudge, K.W., Vermeylen, F., 2005. Effects of population, age, and cultivation methods on ginsenoside content of wild American ginseng (Panax quinquefolium). J. Agric. Food Chem. 53, 8498–8505.

Lobb, A., 2009. Hepatoxicity associated with weight-loss supplements: a case for better post-marketing surveillance. World J. Gastroenterol. 15, 1786–1787.

Low, T.Y., Wong, K.O., Yap, A.L.L., De Haan, L.H.J., Rietjens, I.M.C.M., 2017. The regulatory framework across international jurisdictions for risks associated with consumption of botanical food supplements. Compr Rev Food Sci F 16, 821–834.

Lui, E.M.K., Azike, C.G., Guerrero-Analco, J.A., Romeh, A.A., Pei, H., Kaldas, S.J., Arnason, J.T., Charpentier, P.A., 2012. Bioactive polysaccharides of American ginseng panax quinquefolius L. In modulation of immune function: phytochemical and pharmacological characterization. In: Karunaratne, D.N. (Ed.), The Complex World of Polysaccharides. IntechOpen.

Lunsford, K.E., Bodzin, A.S., Reino, D.C., Wang, H.L., Busuttil, R.W., 2016. Dangerous dietary supplements: Garcinia cambogia-associated hepatic failure requiring transplantation. World J. Gastroenterol. 22, 10071–10076.

Ma, J., Pawar, R., Grundel, E., 2017. Validation of an LC-MS/MS method for analysis of anti-diabetic, anti-obesity, and cholesterol-lowering drugs in botanical dietary supplements labelled for blood sugar management. Abstr. Pap. Am. Chem. Soc. 254.

Maan, A.A., Nazir, A., Khan, M.K.I., Ahmad, T., Zia, R., Murid, M., Abrar, M., 2018. The therapeutic properties and applications of Aloe vera: a review. J. Herb. Med. 12, 1–10.

Madhu, S.K., Shaukath, A.K., Vijayan, V.A., 2010. Efficacy of bioactive compounds from Curcuma aromatica against mosquito larvae. Acta Trop. 113, 7–11.

Mathews, F.R., 1932. Locoism in Domestic Animals, Texas Agricultural Experiment Station Bulletin.

McKinney, J.D., Richard, A., Waller, C., Newman, M.C., Gerberick, F., 2000. The practice of structure activity relationships (SAR) in toxicology. Toxicol. Sci. 56, 8–17.

Miousse, I.R., Skinner, C.M., Lin, H.X., Ewing, L.E., Kosanke, S.D., Williams, D.K., Avula, B., Khan, I.A., ElSohly, M.A., Gurley, B.J., Koturbash, I., 2017. Safety assessment of the dietary supplement OxyELITE (TM) Pro (New Formula) in inbred and outbred mouse strains. Food Chem. Toxicol. 109, 194–209.

Nikolic, D., Godecke, T., Chen, S.N., White, J., Lankin, D.C., Pauli, G.F., van Breemen, R.B., 2012. Mass spectrometric dereplication of nitrogen-containing constituents of black cohosh (Cimicifuga racemosa L.). Fitoterapia 83, 441–460.

NTP, 2010. Toxicology and carcinogenesis studies of Goldenseal root powder (Hydrastis Canadensis) in F344/N rats and B6C3F1/N mice (feed studies). In: NTP (Ed.), Technical Report Series. NIEHS/NTP, Research Triangle Park, NC.

NTP, 2011. Toxicology and carcinogenesis studies of Ginseng (CAS NO. 50647-08-0) in F344/N rats and B6C3F1/N mice (gavage studies). In: NTP (Ed.), Technical Report Series. NIEHS/NTP, Research Triangle Park, NC.

NTP, 2012. Toxicology and carcinogenesis studies of Kava Kava extract (CAS NO. 9000-38-8) in F344/N rats and B6C3F1/N mice (gavage studies). In: NTP (Ed.), Technical Report Series. NIEHS/NTP, Research Triangle Park, NC.

NTP, 2013. Toxicology and carcinogenesis studies of *Ginkgo biloba* extract (CAS No. 90045-36-6) in F344/N rats and B6C3F1/N mice (gavage studies). In: NTP (Ed.), Technical Report Series. NIEHS/NTP, Research Triangle Park, NC.

NTP, 2016. Toxicology studies of green tea extract in F344/NTac rats and B6C3F1/N mice and toxicology and carcinogenesis studies of green tea extract in Wistar Han [Crl:WI (Han)] rats and B6C3F1/N mice (gavage studies). In: NTP (Ed.), Technical Report Series. NIEHS/NTP, Research Triangle Park, NC.

Olivon, F., Allard, P.M., Koval, A., Righi, D., Genta-Jouve, G., Neyts, J., Apel, C., Pannecouque, C., Nothias, L.F., Cachet, X., Marcourt, L., Roussi, F., Katanaev, V.L., Touboul, D., Wolfender, J.L., Litaudon, M., 2017. Bioactive natural products prioritization using massive multi-informational molecular networks. ACS Chem. Biol. 12, 2644–2651.

Overk, C.R., Yao, P., Chadwick, L.R., Nikolic, D., Sun, Y.K., Cuendet, M.A., Deng, Y.F., Hedayat, A.S., Pauli, G.F., Farnsworth, N.R., van Breemen, R.B., Bolton, J.L., 2005. Comparison of the in vitro estrogenic activities of compounds from hops (Humulus lupulus) and red clover (Trifolium pratense). J. Agric. Food Chem. 53, 6246–6253.

Powell, S.L., Goedecke, T., Nikolic, D., Chen, S.N., Ahn, S., Dietz, B., Farnsworth, N.R., van Breemen, R.B., Lankin, D.C., Pauli, G.F., Bolton, J.L., 2008. In vitro serotonergic activity of black cohosh and identification of N-omega-Methylserotonin as a potential active constituent. J. Agric. Food Chem. 56, 11718–11726.

Prince, E.K., Pohnert, G., 2010. Searching for signals in the noise: metabolomics in chemical ecology. Anal. Bioanal. Chem. 396, 193–197.

Qiu, F., Cai, G.P., Jaki, B.U., Lankin, D.C., Franzblau, S.G., Pauli, G.F., 2013. Quantitative purity-activity relationships of natural products: the case of anti-tuberculosis active triterpenes from oplopanax horridus. J. Nat. Prod. 76, 413–419.

Ralphs, M.H., Manners, G.D., Pfister, J.A., Gardner, D.R., James, L.F., 1997. Toxic alkaloid concentration in tall larkspur species in the western US. J. Range Manag. 50, 497–502.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M., Latha, L.Y., 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complem 8, 1–10.

Schlag, E.M., McIntosh, M.S., 2006. Ginsenoside content and variation among and within American ginseng (Panax quinquefolius L.) populations. Phytochemistry 67, 1510–1519.

Shao, A., Broadmeadow, A., Goddard, G., Bejar, E., Frankos, V., 2013. Safety of purified decolorized (low anthraquinone) whole leaf Aloe vera (L) Burm. f. juice in a 3-month drinking water toxicity study in F344 rats. Food Chem. Toxicol. 57, 21–31.

Sharma, S.B., Gupta, R., 2015. Drug development from natural resource: a systematic approach. Mini Rev. Med. Chem. 15, 52–57.

Shipkowski, K.A., Betz, J.M., Birnbaum, L.S., Bucher, J.R., Coates, P.M., Hopp, D.C., MacKay, D., Oketch-Rabah, H., Walker, N.J., Welch, C., Rider, C.V., 2018. Naturally complex: perspectives and challenges associated with botanical dietary supplement safety assessment. Food Chem. Toxicol. : an international journal published for the British Industrial Biological Research Association 118, 963–971.

Singh, S., Prasad, B., Savaliya, A.A., Shah, R.P., 2009. Strategies for characterizing sildenafil, vardenafil, tadalafil and their analogues in herbal dietary supplements, and detecting counterfeit products containing these drugs. Trends Anal. Chem. 28, 13–28.

Somerville, S.J., Lewis, M., Kuipers, H., 2005. Accidental breaches of the doping regulations in sport: is there a need to improve the education of sportspeople? Br. J. Sports Med. 39, 512–516 discussion 516.

Soni, M.G., Carabin, I.G., Griffiths, J.C., Burdock, G.A., 2004. Safety of ephedra: lessons learned. Toxicol. Lett. 150, 97–110.

Stickel, F., Shouval, D., 2015. Hepatotoxicity of herbal and dietary supplements: an update. Arch. Toxicol. 89, 851–865.

UNEP, 2016. In: Programme, U.N.E. (Ed.), UNEP Frontiers: 2016 Report: Emerging Issues of Environmental Concern. UNEP Division of Early Warning and Assessment, Nairobi, Kenya.

Waidyanatha, S., Ryan, K., Roe, A.L., Jia, W., Paine, M.F., Ferguson, S., Gurley, B.J., Welch, K., Chow, M.S.S., Devito, M., Rider, C., 2018. Follow that botanical: challenges and recommendations for assessing absorption, distribution, metabolism and excretion of botanical dietary supplements. Food Chem. Toxicol. : an international journal published for the British Industrial Biological Research Association (121), 194–202.

Weller, M.G., 2012. A unifying review of bioassay-guided fractionation, effect-directed analysis and related techniques. Sensors 12, 9181–9209.

Westenburg, H.E., Lee, K.J., Lee, S.K., Fong, H.H., van Breemen, R.B., Pezzuto, J.M., Kinghorn, A.D., 2000. Activity-guided isolation of antioxidative constituents of Cotinus coggygria. J. Nat. Prod. 63, 1696–1698.

Wiklund, S., Johansson, E., Sjostrom, L., Mellerowicz, E.J., Edlund, U., Shockcor, J.P., Gottfries, J., Moritz, T., Trygg, J., 2008. Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models. Anal. Chem. 80, 115–122.

Wu, C.H., Wang, C.C., Kennedy, J., 2011. Changes in herb and dietary supplement use in the U.S. adult population: a comparison of the 2002 and 2007 National Health Interview Surveys. Clin. Therapeut. 33, 1749–1758.