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In silico approach to safety of botanical dietary supplement ingredients utilizing constituent-level characterization

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ABSTRACT

Botanicals used in dietary supplements industry can have toxicology concerns related to endpoint gaps that cannot be fully resolved by a history of use, or existence of conflicting safety data. However, traditional toxicological studies on botanicals are scientifically and pragmatically challenging due to testing of complex mixtures of constituents, cost, time, and animal usage. Alternatively, we developed an in silico decision-tree approach to address data gaps and inform need for further studies by toxicologically evaluating the chemical composition of botanicals. Following advanced multi-detector analytical characterization of a botanical, each chemical constituent is: (a.) quantitatively benchmarked against similar constituents in commonly consumed foods or botanicals with well-established safety profiles, (b.) systematically evaluated for toxicity data utilizing structure-activity relationships, and, (c.) compared to established thresholds of toxicological concern in absence of safety data or structural analogs. Finally, where safety endpoint gaps are identified which cannot be resolved without additional in vitro or in vivo studies, the botanical compositional data are critical to inform on study design. Results with three herbal preparations demonstrate the utility of this novel approach to identify potential hazards and establish safe human use levels for botanicals in a cost efficient and informative manner that minimizes animal use.

1. Introduction

In today's world of commercially prepared herbal ingredients, a wide range of unique botanicals are available in various preparations ranging from traditional to novel extracts. Especially in the last decade, consumer exposures to these ingredients have expanded tremendously in the general population (Blumenthal et al., 2012), which has intensified scrutiny and compliance expectations by regulators, industry stewards and consumers.

While regulators and industry organizations are making great strides to establish hazard profiles for nutritional ingredients, there continues to be many botanicals with toxicity endpoint gaps or conflicting data that need to be addressed. Largely, these assessment gaps result from over-reliance on limited historical information rather than using data to assure safety through empirical investigation. Challenges with historical hazard assessment reviews may include: conflicting results in publications, different exposures or indications of use, anecdotal evidence rather than well-controlled studies, and the myriad of challenges associated with testing complex mixtures including poorly described and characterized test materials, differences between tested materials and commercially used materials, use of highly concentrated extracts, natural variations in botanical composition, and the presence of contaminants. Traditional toxicology studies used to resolve these gaps can be expensive, time consuming, often require the use of animals, and have critical design considerations when investigating complex mixtures, e.g. dose preparation of whole dried leaf vs. extract; antibacterial activity or cytotoxicity; varied physiochemical nature of botanical material; and differences between actives of interest vs. constituents of concern.

The objective of this paper is to describe a novel approach to overcome many of these obstacles by applying state-of-the-art analytical techniques to identify and quantify botanical constituents and then apply a *botanical constituent decision tree*, which uses both food intake levels and established *in silico* toxicology assessment tools, to identify hazards for individual chemical constituents of a botanical. Combining this analysis with appropriate dosing and

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co-exposure considerations is then used to establish a risk characterization for the various constituents of the botanical preparation. This approach provides a versatile means to assess safety for human use, illustrated in this paper through three cascading scenarios: (1) by benchmarking exposures to constituents commonly found in food to exposures via supplement use (see, 3.1); (2) by using constituent analysis to justify bridging safety data between different methods of botanical preparation (see, 3.2); (3) and by establishing exposure thresholds for individual constituents with limited human use data using *in silico* toxicology assessment tools (see, 3.3). The botanical preparations were chosen for these aforementioned case studies to illustrate one or more of these cascading scenarios as the decision tree is applied. Our proposed approach can resolve safety endpoint gaps for complex botanical mixtures or clarifies the specific data needed, using constituent analysis and thereby reducing the extent of testing and focusing the need for more traditional *in vitro* and *in vivo* studies.

2. Methods

2.1. Botanical decision tree

The botanical decision tree (see Fig. 1) was developed specifically to address hazard assessment endpoints of botanicals used in dietary supplements to support safe human use. The decision tree takes advantage of existing food intake data and constituent hazards assessment systems, as well as broadly accepted and well-established expert approaches. These tools, which rely upon confirmed constituent identity and inherent structure-activity relationships (SAR), include: DEREK, Toxtree, Meteor™, Toxnet, (Wu et al., 2010; Blackburn et al., 2011), Threshold of Toxicological Concern (TTC) (Munro et al., 1996; Kroes et al., 2004) and referral to USDA nutrient databases, EFSA food consumption databases, Food Commodity Intake Database (FCID), Phenol-Explorer, NHANES, RIFM/FEMA, PubMed, Scifinder or other primary literature sources.

Once the identity of the plant, including plant part(s) is established, the botanical decision tree begins with an understanding of both the method of botanical preparation and a determination of the estimated human exposure level, i.e. the serving size to be recommended for use in the supplement product. The toxicologist can then conduct a preliminary review of published literature, weighting each publication for both quality, adequate design and appropriate scientific rigor, to determine whether a safety assessment can be completed based on available data and thus derive a supportable safe human exposure level (see Fig. 1, Pre-Work). If published data are inadequate to determine a safe exposure level at or above desire use levels, the next step is to identify any unresolved safety endpoint data gaps and/or understand differences in methods of preparation between reported materials versus the desired preparation being assessed. Next, a sample of the desired preparation under consideration is obtained and analytical characterization (see 2.3) is completed to identify and quantify individual botanical constituents sufficiently to address safety concerns (see Fig. 1, Sample Acquisition & Characterization). Using all of these data, questions A-C are then addressed to determine whether each constituents with known structures are commonly consumed in the diet and, if so, whether the dietary supplement exposure is comparable to food intake. For constituents above food intakes or are not commonly consumed as food, published safety data is also reviewed to determine if the data are sufficient to establish a suitable margin of safety (MoS). Depending on the outcome of each decision point (a)-(e), individual botanical constituents may involve the '*in silico* hazard assessment process' (see Fig. 1, Hazard Analysis: Identify Constituent of the Botanical Driving Safety Assessment). These steps provide the necessary information to address the subsequent safety decision point questions D and E relating to SAR and TTC respectively. For constituents with structures that are not fully elucidated, the assessor skips to Question E where the exposure-based prioritization tool referred to as Threshold of Toxicologic Concern (TTC) allows for a worst-case risk assessment to be performed for specific chemical entities that fit within its constraints.

2.2. Case study materials

The decision tree is illustrated by applying its decision principles to the evaluation of three different botanical preparations. These preparations were obtained from various commercial suppliers. (i) A red clover sample identified by the supplier as red clover herb (whole herb), hereafter referred to as sample TB1, was extracted with 70/30 ethanol water. (ii) Two different extracts were prepared by a commercial supplier from the same starting raw material (*Pelargonium sidoides* root), extracted with either 50% w/w aqueous ethanol (referred to as 50% PE) or 11% w/w aqueous ethanol (referred to as 11% PE). (iii) A sample of *Scutellaria baicalensis* Georgi root that had been extracted with water and then dried was obtained from a commercial supplier. All samples were then subject to analysis.

2.3. Analytical constituent characterization and identification

Samples for each of the three botanical preparations were analyzed using UHPLC separations with UV (Photodiode Array), CAD (Charged Aerosol Detection), and HRMS (high resolution mass spectrometry) in both positive and negative ion mode detection, with electrospray ionization/volatilization. Details on sample preparation and analysis for each of the test case botanical preparations are provided in Supplementary material. The targeted lower limit of detection for constituent characterization was set to enable TTC approaches.

3. Results

The decision tree shown in Fig. 1 was applied to three botanicals, selected to illustrate several scenarios that commonly arise in the course of evaluating the safety of a botanical preparation. Of note, this paper is not intended to present an exhaustive defense of safety of these particular botanical preparations or all of their constituents. As such we combined all three botanicals in the illustration and do not include a detailed review of the existing safety data for each of the botanicals discussed, but instead provide only a brief summary of safety issues/safety data gaps that emerged from the detailed literature reviews of these botanicals or their constituents (summarized in Supplementary material). These botanicals serve only as examples intended to be illustrative of the approach to resolving safety data gaps outlined in the decision tree. Neither was it the intention of this paper to detail the analytical findings for these three scenarios. The detail on a comprehensive characterization of a botanical is sufficient to justify a stand-alone publication. For the purposes of illustration, examples of the data for characterization of individual chemical constituents are provided in the Supplementary material.

3.1. Benchmarking constituent safety between the diet and botanical supplements

Constituents from a botanical with limited human use data can be cleared by an assessor for safe human use if these constituents also occur in common foods and there are adequate dietary intake data to estimate exposure levels that can in turn be used as a