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An innovative approach to the safety evaluation of natural products: Cranberry (*Vaccinium macrocarpon* Aiton) leaf aqueous extract as a case study

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ABSTRACT

Assessment of safety for a food or dietary ingredient requires determination of a safe level of ingestion compared to the estimated daily intake from its proposed uses. The nature of the assessment may require the use of different approaches, determined on a case-by-case basis. Natural products are chemically complex and challenging to characterize for the purpose of carrying out a safety evaluation. For example, a botanical extract contains numerous compounds, many of which vary across batches due to changes in environmental conditions and handling. Key components integral to the safety evaluation must be identified and their variability established to assure that specifications are representative of a commercial product over time and protective of the consumer; one can then extrapolate the results of safety studies on a single batch of product to other batches that are produced under similar conditions. Safety of a well-characterized extract may be established based on the safety of its various components. When sufficient information is available from the public literature, additional toxicology testing is not necessary for a safety determination on the food or dietary ingredient. This approach is demonstrated in a case study of an aqueous extract of cranberry (*Vaccinium macrocarpon* Aiton) leaves.

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1. Introduction

The standard of safety for generally recognized as safe (GRAS) food ingredients (21 CFR 170.3(i)) is based on the reasonable certainty that consumption at the estimated daily intake (EDI) poses a reasonable certainty of no harm to consumers. GRAS is a rigorous process that relies on common knowledge and expert consensus about the safety of the substance for its intended use (Kruger et al., 2011). Safe level of ingestion through scientific procedures can be derived by applying an uncertainty factor to a no observed adverse effect level (NOAEL) derived from a well-conducted toxicology study to derive an acceptable daily intake (ADI). However, when the level of addition of the ingredient to the feed in the animal study exceeds 5% (w/w) untoward physiological effects due to dietary imbalance alone may manifest in such studies (Office of Food Additive Safety, 2000; Kruger and Mann, 2003; Hayes, 2008; Klaassen, 2008). This limitation enters into consideration in the case of natural products, for example, crude extracts, which are composed of a mixture of tens or hundreds of compounds. Many of these compounds are present at such low concentrations that it is impracticable to concentrate the overall extract to such an extent that derivation of an ADI utilizing traditional uncertainty factors applied to NOAELs could be generated on each of the minor constituents. Additionally, these matrix molecules may exert an effect on the bioavailability of the active compounds that are present in the natural product (IFT, 2009). It is also important to note that the concept of the 100-fold uncertainty factor is not appropriate for physiologically active substances. In these cases, the effects seen in animal studies may be due to the physiologic or pharmacologic activity of the active principle and not a classic toxicological response.

In addition to the exaggeration of exposure that presents a difficulty for animal testing, it can also be a challenge to define adequately batch variability for natural products. Chemical characterization that focuses on only the major or readily quantifiable components of a natural product, or those components of particular interest, for example, those considered to be responsible for beneficial effects does not serve to address information needed for risk assessment. This is especially relevant when considering that the composition of natural products, including botanical extracts, is subject to seasonal and other environmental variations over time.

The initial characterization of the raw material and the control of its harvesting and supply over time also play an often overlooked, but critically important, role in establishing the safety of a natural product. Limiting source material to a single species of interest, versus the inadvertent collection of other species, will

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narrow the possibilities of which compounds may be present in the final product. Therefore, species identity must be included as part of the product specification. Also, the chemical content of various plant parts (leaf, stem, flower, fruit, root, aboveground parts, rhizome, seed) may vary, depending on the growth stage of the plant and its defense needs at the time of harvest, in addition to the natural tendency of specialized plant cells to compartmentalize specific compounds (Youngken, 1921). Thus, the concentration of a particular compound is not necessarily assumed to be uniform across all of a plant's parts and one must specify the plant part used as the source material for the final product as part of the established identity criterion. Such "positive" criteria are readily incorporated into product specifications along with the "negative" line item tests that serve to exclude the presence of unwanted contaminants or impurities (i.e., heavy metals, microbes). Natural toxicants that may theoretically be present, but have never been reported to actually occur in the species of interest may be ruled out in source material via negative analytical results on multiple, non-consecutive lots; whereas, known natural toxicants (for example, lupin alkaloids) may be subject to health-based cutoff values in final product specifications.The degree of processing and refinement of the raw material (i.e., dried leaves) will contribute to the resulting chemical diversity and concentration found in the final processed extract or ingredient; in general, less refined products are the most chemically complex (Harborne, 1998). If not properly controlled through specifications and standard operating procedures, the differences in the quality of the botanical source material used, coupled with variations introduced during processing (for example, extraction solvent type, temperature and time; formation of methanolic adducts due to reaction of electrophilic molecules with methanol solvent in the presence of a silica gel chromatography column; Malliard reaction products formed during heated processes), can result in significant chemical variability from batch to batch. It is not necessary for safety evaluation purposes that every individual compound be measured in a final product's specification. Appropriate grouping of selected compounds or classes of compounds for analysis establishes the product composition for purposes of safety evaluation. In short, the high chemical diversity of natural products renders them a challenge to characterize analytically as well as evaluate for safety.

The reasoning behind the choice of cranberry leaf as the case study for the application of our safety determination strategy is that whole cranberry fruit and its juice have a long history of safe consumption as foods, suggesting that the leaf may also be found suitable for consumption in some form. However, despite the prevalent use of cranberry fruit juice and extract as a beverage and in dietary supplements, and publications detailing the chemical content of these, there has been little work published thus far on the chemistry of cranberry leaf. As with other plants, the leaf may have a different chemical profile compared with the fruit and, as such, requires an evaluation to demonstrate its safe use in food products.

An innovative approach to synthesizing relevant information from various disciplines to determine safety of natural products is proposed. This strategy involves (1) review and analysis of existing phytochemical and botanical literature; (2) establishing chemical composition of the raw material and the commercial product; (3) determination of health-based levels of exposure for the identified compounds or compound and; (4) utilization of published toxicology studies to establish safety of exposure to the extract through evaluation of the components/compound classes. We apply our approach in a case study as the basis for the evaluation of an aqueous extract of cranberry (*Vaccinium macrocarpon* Aiton) leaves for use in a beverage.

2. Methods

2.1. Strategy for determining the safety of a botanical extract

An overview of our strategic framework for the safety assessment of a natural product that we developed and applied to the aqueous extract of *Vaccinium macrocarpon* leaf is described in Fig. 1. The initial step encompassed a literature search, beginning with *Vaccinium macrocarpon* Aiton, and widening the search to encompass other members of the genus *Vaccinium* and family Ericaceae as appropriate. Databases included, but were not limited to, PubMed, ToxNet, NAPRALERT, and DialogWeb. Discrimination was necessary at this stage, as certain plant families encompassed tens or hundreds of genera, and each genus contained numerous species. Additionally, it should be noted that the taxonomic relationships between species and genera may be in a state of flux in the case of certain plants; renaming and reclassifications of taxa are not uncommon in the botanical literature. The most recently verified Latin binomial name (www.tropicos.org) should be used when retrieving literature on a species. The taxonomic relationships between species, genera and family for *V. macrocarpon* Aiton are indicated in Fig. 2. It is also recommended to consult the botanical systematics and phytochemistry literature, as it can sometimes clarify taxonomic relationships between specific species, genera, and tribes and contains reports describing the known biosynthetic pathways and the occurrence patterns of certain compound classes in plants. This type of information can provide insight regarding the potential capacity of a species to produce certain structural classes when occurrence reports are not found. For example, the presence of biosynthetic machinery (e.g., specific enzymes controlling key biosynthetic steps) that could produce a particular compound structural class in a species of interest may not be reported in the scientific literature, but multiple reports of such compounds occurring in closely related taxa are suggestive that the compounds may be present in the species of interest.

Once the literature review and data gap analysis for *V. macrocarpon* was completed, appropriate standard operating procedures (e.g., harvesting, processing) were developed to prevent the introduction of undesired species, including weeds. Raw material specifications were established that incorporated a positive identity criterion to ensure the use of the proper species.

Compounds and structural classes identified from literature reports on *Vaccinium* species were classified according their potential health effects. Completing this task required the consideration of indicator compounds when the specific chemicals reported for *V. macrocarpon* or related species did not have sufficient information to allow scientific judgment regarding potential health effects. Choosing a indicator compound depends on its structural similarity with the compound of interest, and ideally results in the choice of a compound for which safety data are available and which could potentially be present in the plant. If one plans to analyze plant material or an extract for presence of the indicator compound, then the choice may be limited depending on the commercial availability of standards. In this case, there must be an understanding that analysis of a plant for the presence or absence of a indicator compound is done as a due diligence step to rule out problematic compounds, but one cannot strictly interpret an absence of the indicator compound in the plant material to imply that the compound of concern is also absent, as the compound of concern was not actually measured. The specific indole and quinolizidine alkaloids of concern reported in the literature for *Vaccinium* species were not commercially available; therefore, the indicator compounds yohimbine and lupinine were chosen, respectively, as reference standards and analyzed for in dried leaf samples.

Regarding the choice of the indicator compounds, compounds were chosen which may be produced by the plant if it contains the biosynthetic machinery thought to be responsible for the biosynthesis of terpene indole and quinolizidine alkaloids. Jankowski originally reported the isolation of the indole alkaloid cannagunine B (Fig. 3) and a related compound (Jankowski, 1973a,b) from an unspecified species of *Vaccinium*. Although this report was never corroborated by other researchers and was, in fact, contested by some chemists (Chemical Society (Great Britain), 1974), we wished to exclude this subclass of indole alkaloid as being present in *Vaccinium macrocarpon*. The choice of the indicator compound in this case was dictated by the observation that cannagunine B appeared to be a product of the terpene alkaloid biosynthesis pathway (Dewick, 1997; Fig. 3), which involves the coupling of tryptamine, derived from tryptophan, and secologanin, an iridoid. This is a logical approach as iridoids have been reported in *V. macrocarpon*, including in this report. The first known biosynthetic intermediate in the terpene indole alkaloid pathway, strictosidine (Misra et al., 1996), was not commercially available for use as a reference standard. Therefore, the downstream biosynthetic product yohimbine was chosen for analysis as its multiple ring system resembled that of the proposed, but contested, structure of cannagunine B. In the case of the quinolizidine alkaloids, myrtine and epimyrtine (Fig. 4) were reported in *V. myrtillus* by Slosse and Hootele (1981). However, since that initial report, little biosynthetic work has been done on these particular compounds, and neither myrtine nor epimyrtine were commercially available as reference standards. Therefore the decision was made to analyze *V. macrocarpon* for the presence of (−)-lupinine, as it is one of the first major downstream products reported for the quinolizidine alkaloid pathway (Dewick, 1997; Fig. 4). The use of indicator compounds is a best practice that can be applied in situations where the actual compounds in question are not available as purified reference standards for analytical use.

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