NTP
National Toxicology Program

Applying In vitro Approaches to Understand Complex Mixtures in Assessing Botanical Safety

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January 26, 2021

Mixtures risk assessment framework

Problem formulation

Is Data Quality Adequate?

no

No quantitative assessment; only qualitative assessment

yes

Whole Mixture Data

Mixture of concern

Sufficiently similar mixture

Group of similar mixtures

Mixture RfD/C; Slope Factor

Comparative Potency

Component Data

Do components interact?

no

yes

Components have similar MOAs

Components have different MOAs

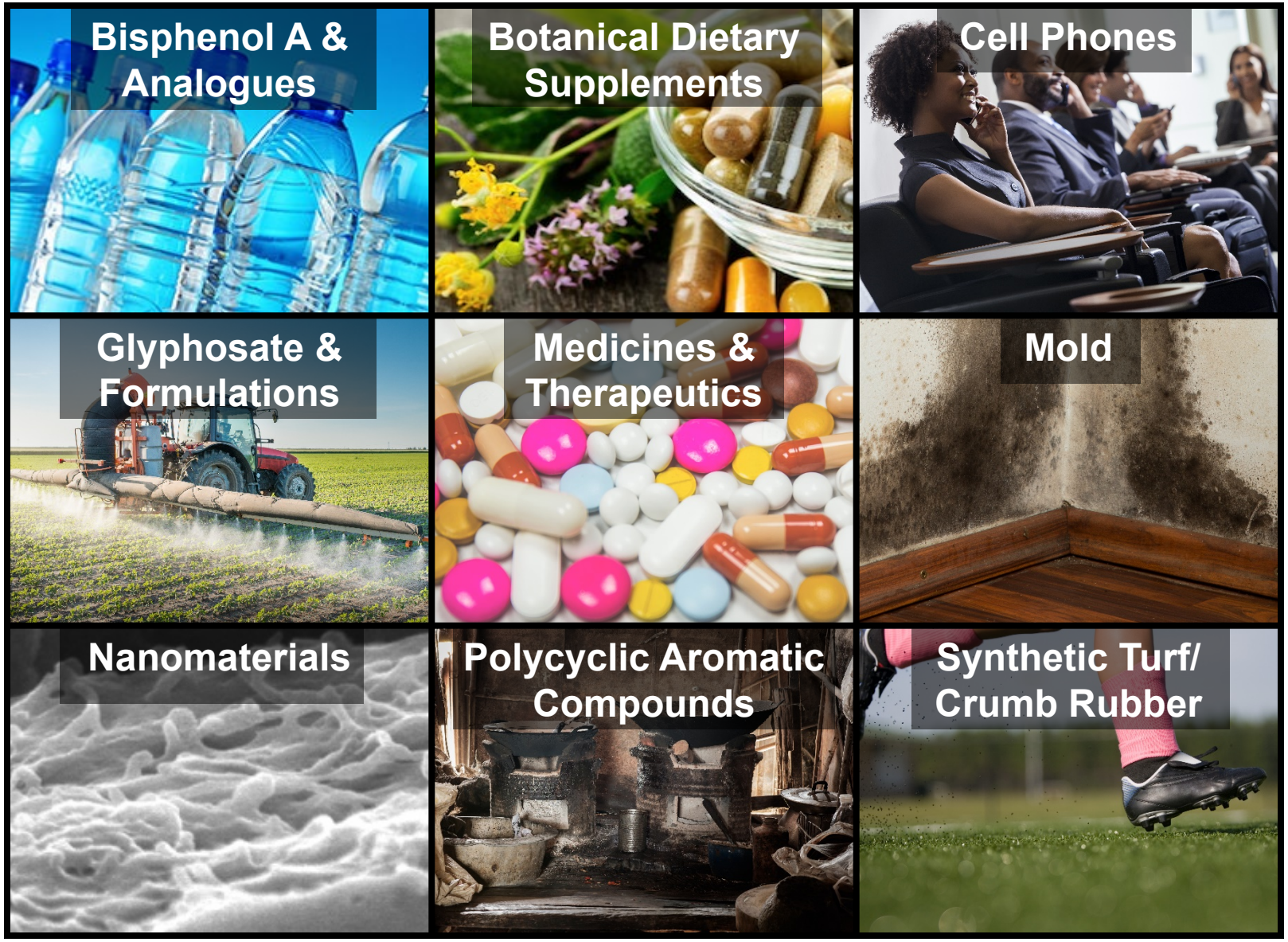
Dose Addition

Response Addition

Interactions Based Hazard Index

Adapted from U.S. EPA (2000) Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures

Current research at NTP



Botanical dietary supplements

Widespread exposure + relatively high doses

- Approximately 18% of adults in the U.S. (~40 million people) used nonvitamin, nonmineral dietary supplements in the past 12 months according to the 2012 National Health Interview Survey
- US consumers spent $9.6 billion on botanical dietary supplements in 2019
- Recommended doses can be in the range of 100s - 1000s mg per day

From: Clarke et al., 2015, Trends in the Use of Complementary Health Approaches Among Adults: United States, 2002-2012.
Smith et al., 2020. US Sales of Herbal Supplements Increase by 8.6% in 2019. *HerbalGram,* 127**,** 54-69.

NTP interest in botanicals

- NTP evaluates substances that are of public health concern
- There is little safety data on most botanicals
- Public concern about the quality and integrity of botanicals available in the marketplace
- NTP has received a number of nominations to study botanical dietary supplements
  - National Cancer Institute (9), NIEHS (5), Private Individuals (3), FDA (2)

Aloe vera

Botanical Supplements Come From Nature, But That Doesn't Mean They're Safe.

Manufacturers of supplements aren't required to demonstrate to the government their products are effective or safe.

Author: Tanya Rivera
Published: 4:37 PM EDT November 1, 2019
Updated: 6:08 PM EDT November 1, 2019

HEALTH NEWS JAN. 2, 2020 / 3:30 AM

Experts: Oversight needed for safety, efficacy of nutritional supplements

By Brian P. Dunleavy

(Botanical) Dietary Supplement Regulation

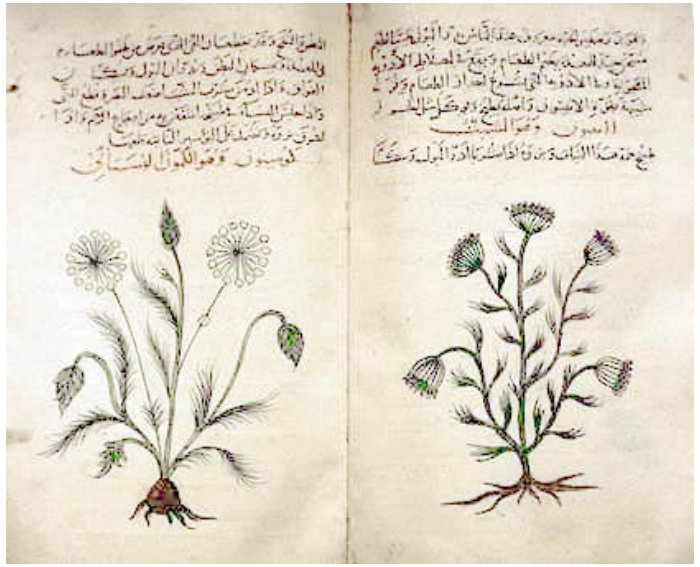
1994 Dietary Supplement Health and Education Act

- Amends the FD&C – created a regulatory framework for dietary supplements
  - Intent: Balance consumer access and consumer protection
  - Defines dietary supplements as foods and excludes them from consideration as food additives
  - Puts the burden of proof for risk on FDA (i.e., FDA has to prove that a dietary supplement is not safe)
  - Clarifies labeling requirements
  - Requires new dietary supplement ingredients to be registered with the FDA
  - Specifies Good Manufacturing Practices for dietary supplements
  - Created the Office of Dietary Supplements at NIH

From: Abdel-Rahman, 2011, Toxicological Sciences 132(2): 333-348.
Dietary Supplement Health and Education Act of 1994. Public Law 103–417, 108 Stat. 4325-4335; October 25 1994.

History of safe use

Discorides' Materia Medica, c. 1334



Toxicology Letters 314 (2019) 10–17

Contents lists available at ScienceDirect

Toxicology Letters

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

Mini review

Development of a consensus approach for botanical safety evaluation – A roundtable report

Corrado L. Galli[a], Nigel J. Walker[b], Nicholas H. Oberlies[c], Amy L. Roe[d], James Edwards[e], Suzanne Fitzpatrick[f], James C. Griffiths[g], A. Wallace Hayes[h], Catherine Mahony[i], Daniel S. Marsman[d], Lara O'Keeffe[i,*]

- Consensus statements on history of safe use:
  - The safety of a botanical cannot be judged based solely on a history of food use unless it can be demonstrated that a comparable composition is ingested on a regular basis across broad geographic and demographic populations
  - In the assessment of a botanical, it is misleading to assume that a history of human use addresses all aspects of safety

Current NTP botanical portfolio

Completed

- *Aloe vera* nondecolorized whole leaf extract
- Bitter orange extract
- *Ephedra* (ma huang)
- Ginseng root extract
- *Ginkgo biloba* extract
- Goldenseal root powder
- Green tea extract
- Gum guggul extract
- Kava kava extract
- Milk thistle extract
- *Senna*

Ongoing

- Black cohosh extract
- Dong quai (root powder or extract)
- *Echinacea purpurea* extract
- Garcinia cambogia
- *Usnea* lichen
- Valerian root

Coneflower
Echinacea purpurea

Testing approach

- Identify knowledge gaps
  - Specific concern: Ephedra and cardiotoxicity
  - General: Lack of toxicity and carcinogenicity data
- Test article selection
- Study design (general)
  - Animals: Male and female B6C3F1/N mice and Sprague Dawley rats (previously F344)
  - Exposure duration: 2-week, 3-month, 2-year
  - Dosing paradigm: typically oral gavage for botanical dietary supplements
  - Endpoints: clinical chemistry, hematology, genotoxicity, sperm motility and vaginal cytology, histopathology

Ginkgo biloba

History of NTP botanical research

Botanical	Male Rats	Female Rats	Male Mice	Female Mice
Aloe vera	Clear	Clear	No	No
Ginkgo biloba	Some	Some	Clear	Clear
Ginseng	No	No	No	No
Goldenseal	Clear	Clear	Some	No
Green tea	No	No	No	No
Kava Kava	Equivocal	No	Clear	Clear
Milk thistle	No	No	No	No
Senna	Not tested	Not tested	No	No
Bitter orange	Increased heart rate and blood pressure			
Ephedra	Cardiotoxicity			

Green tea
Camellia sinensis

Sources of variation

Source material	Processing	Finished product	Exposure
Plant part (aerial, root, whole plant, leaf, seed)	Extraction process*	Manufacturing process*	Dose (use pattern)
Climate	Solvents	Excipients	Length of dosing
Soil conditions	Adulteration	Combination with other botanicals	Life-stage
Season	Contamination	Adulteration	Disease-state
Plant maturity	Storage/shipping conditions	Contamination	Nutritional status
Contaminants (mold, pesticides, metals)		Storage/shipping conditions	Background genetics
Co-harvested materials (other plants, soil)			Co-exposures
Adulteration			

*Proprietary

NTP botanical workshop

April 26-27, 2016, NIH Campus, Bethesda, MD

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U.S. Department of Health and Human Services

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2016

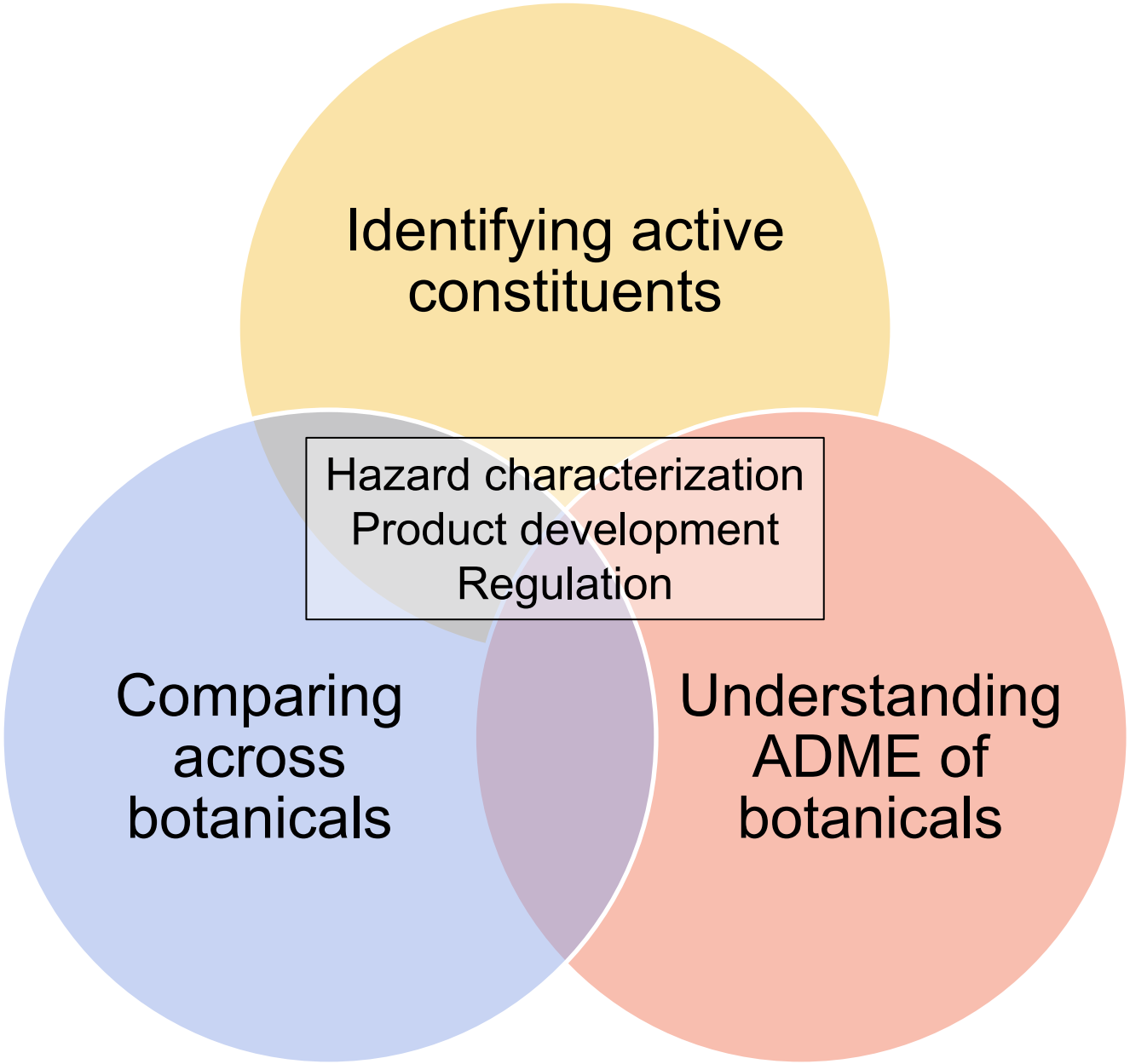
Workshop: Addressing Challenges in the Assessment of Botanical Dietary Supplement Safety

SHARE THIS: 18
https://ntp.niehs.nih.gov/go/workshop_botanicals

April 26-27, 2016
9 a.m. - 5 p.m. EDT
Location: Lister Hill Auditorium
National Institutes of Health (NIH), Bethesda, Maryland

http://ntp.niehs.nih.gov/about/presscenter/events/2016/index.html

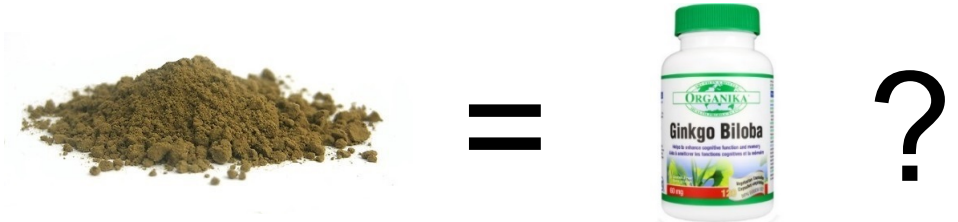
Key challenges in assessing safety



Sufficient similarity

Sufficient similarity = phytoequivalence

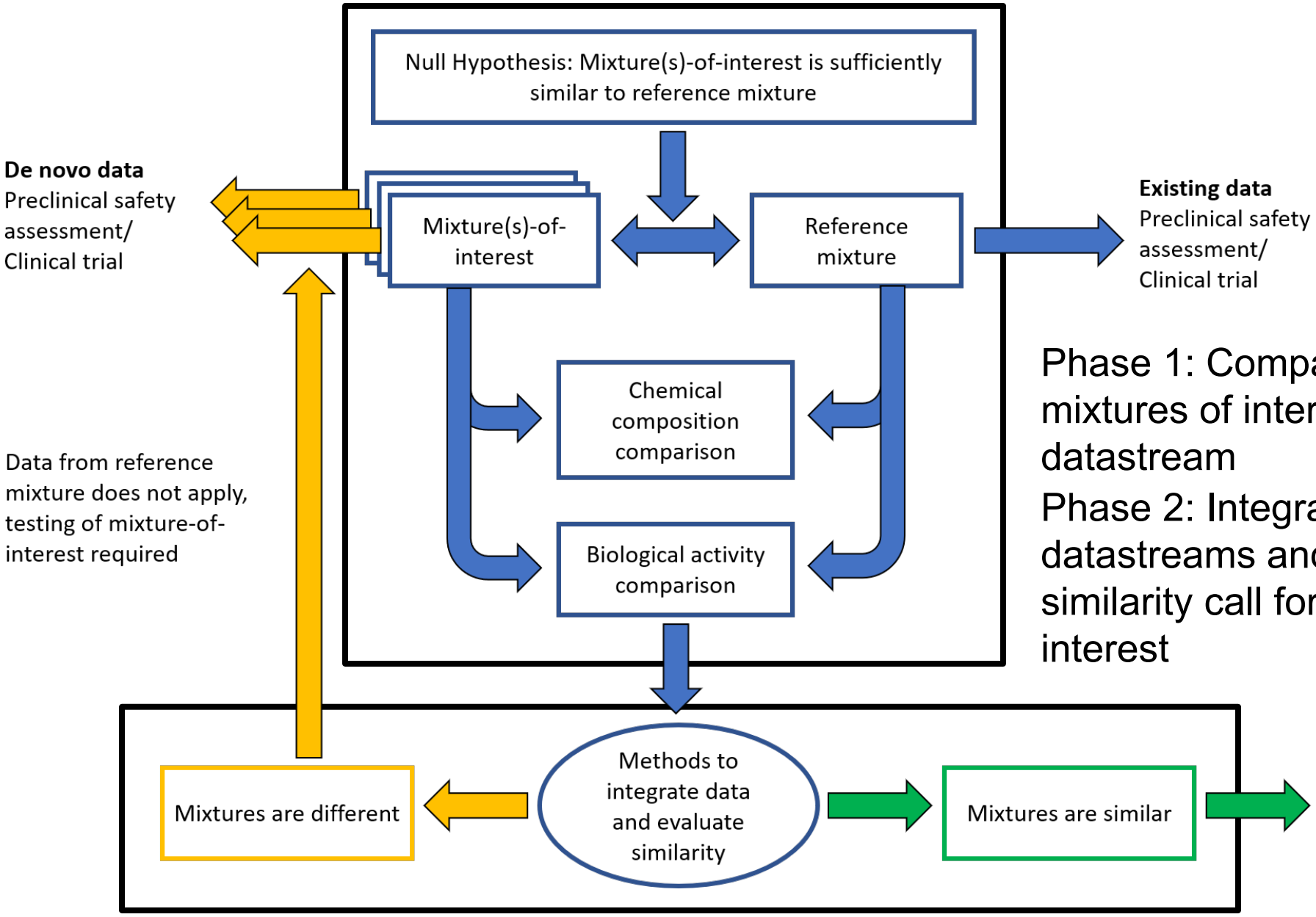
Two mixtures are similar enough that data from one of the mixtures (*reference mixture*) is transferable to the other (*mixture of interest*).



Why is this important?

There are thousands of products in the marketplace and we are not going to test all of them

Sufficient similarity framework



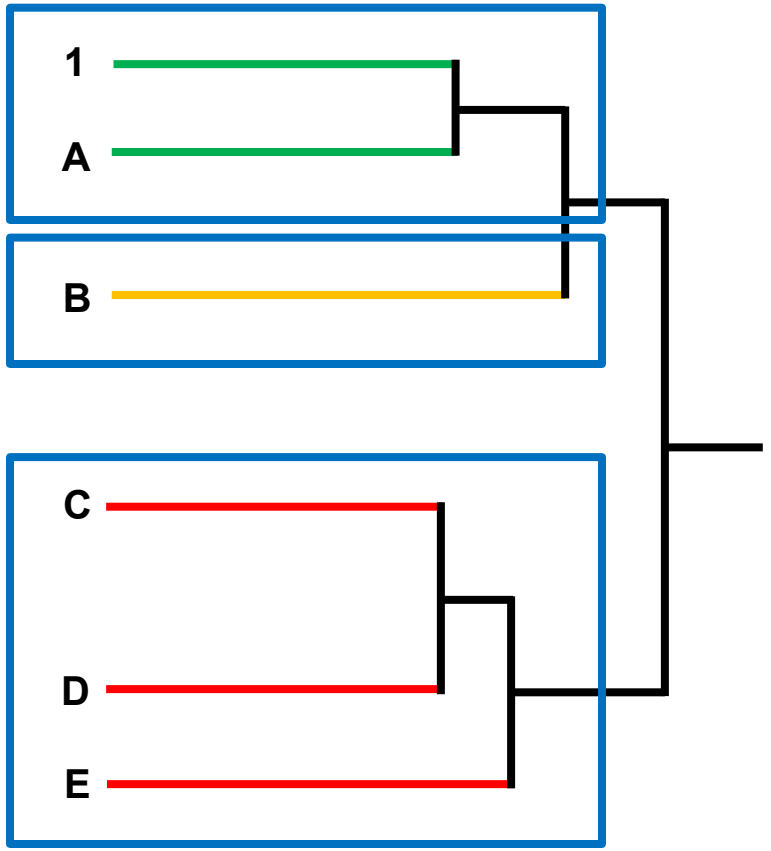
Phase 1: Comparing reference to mixtures of interest within each datastream

Phase 2: Integrating across datastreams and making an overall similarity call for each mixture of interest

Comparing the reference to the mixture(s) of interest

Simple rules

1. Generate data (any kind of data – chemistry, *in vitro*, *in vivo*) on the reference and mixtures of interest
2. Multivariate statistical approaches to analyze large datasets (PCA, hierarchical clustering)
3. Similarity judgment
    a) Mixtures in the same group as the reference are considered “similar”
    b) Mixtures in the most different group are considered “different”
    c) Mixtures in neither the most similar or the most different groups are considered “maybe similar”



Determining sufficient similarity

Chemical data

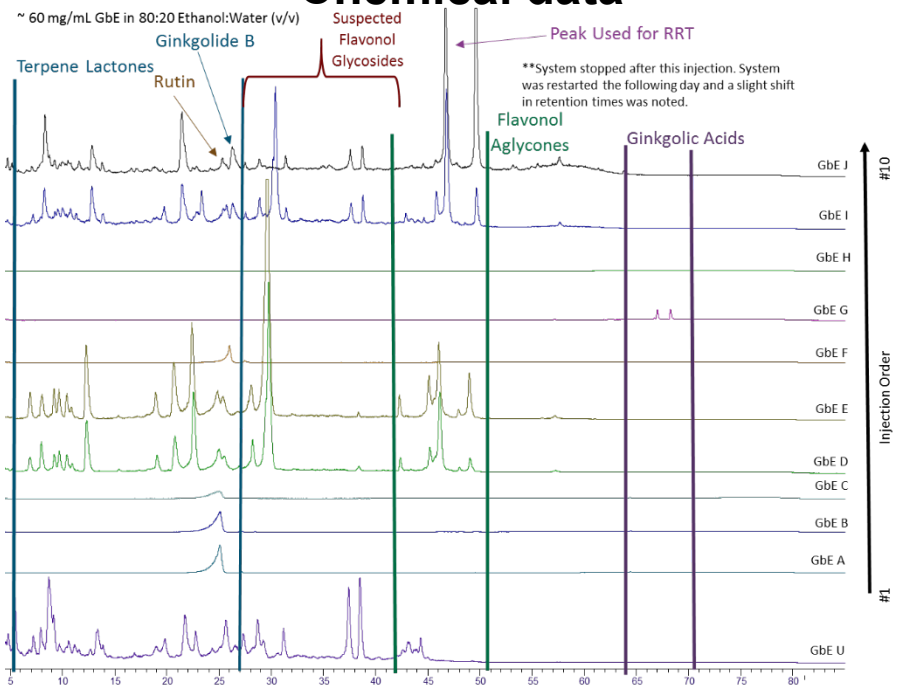
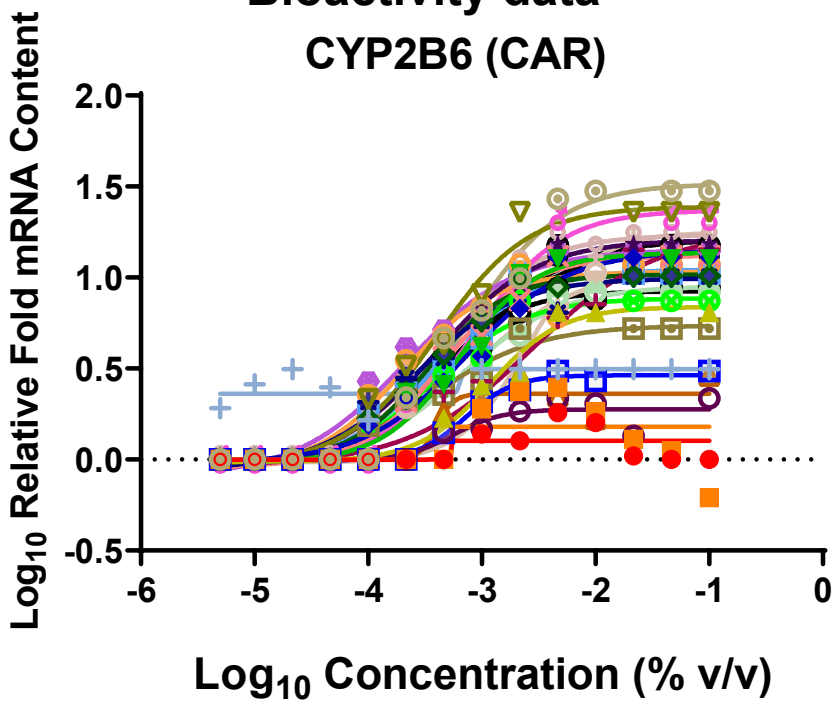
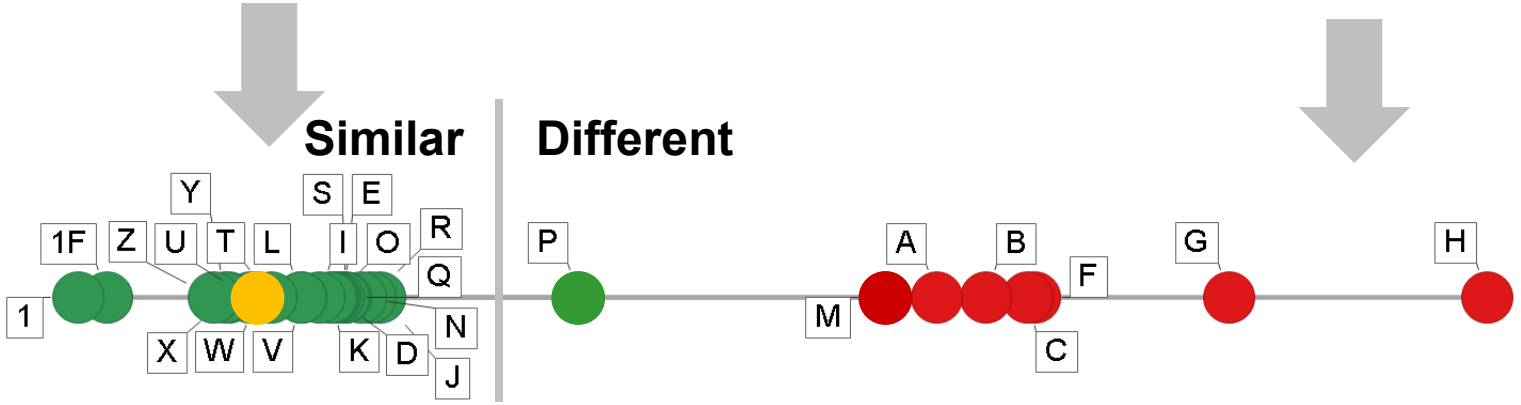


Figure 2. Non-Targeted Fingerprint Chromatograms of First Set of GbE Samples (Not Hydrolyzed), HPLC-ELSD

Bioactivity data





Catlin et al., (2018). How similar is similar enough? A sufficient similarity case study with *Ginkgo biloba* extract. Food Chem Toxicol. 118: 328-339.

Black cohosh (*Actaea racemosa*)

Natural variation, contamination, and adulteration

NIH National Institutes of Health *Turning Discovery Into Health*

Dietary Supplement Label Database

Home | Search | About | Contact Us | Help Menu

Ingredient - Black Cohosh

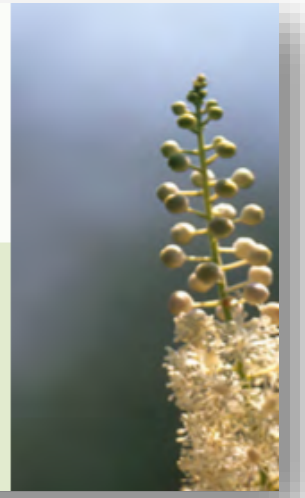
424 product(s) contain the ingredient "Black Cohosh"

ABC AHP NCNPR
Botanical Adulterants Program

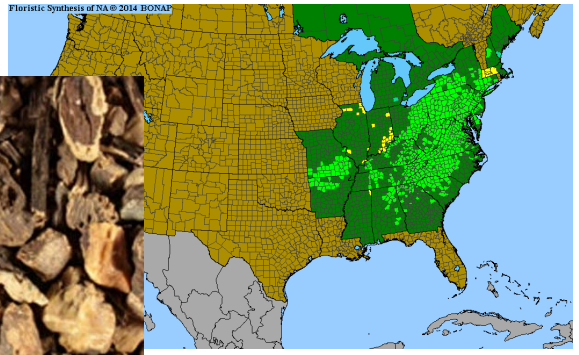
BOTANICAL ADULTERANTS BULLETIN

on Adulteration of *Actaea racemosa*

By Stefan Gafner, PhD*
American Botanical Council, PO Box 144345, Austin, TX 78723
*Corresponding author: email



Black cohosh
Actaea racemosa



Yellow cohosh
Actaea podocarpa

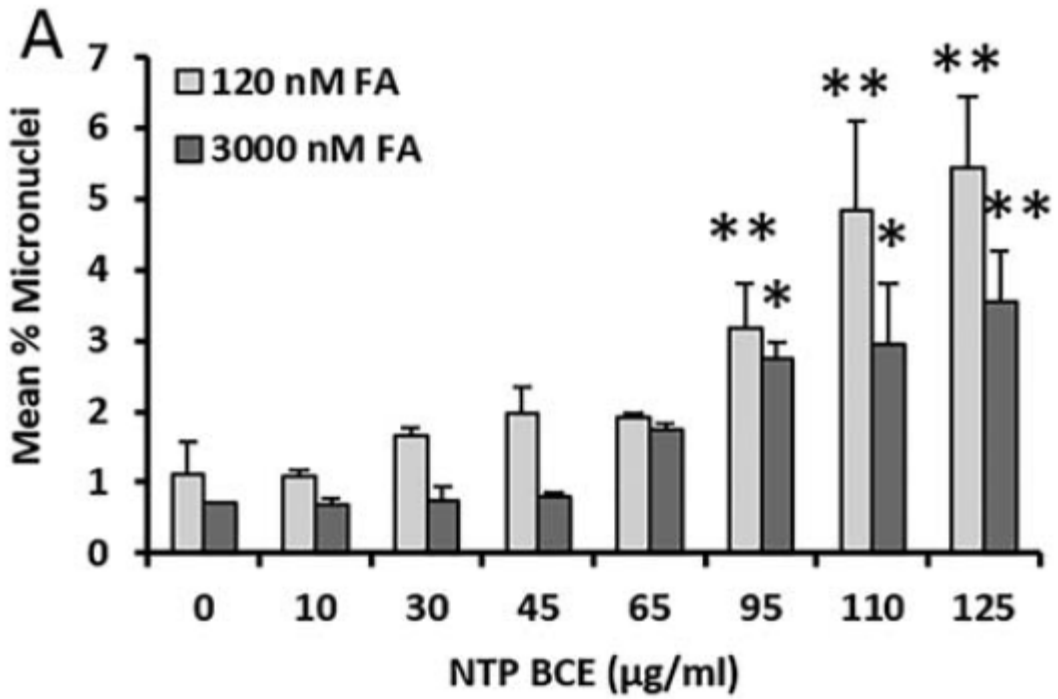
Red cohosh
Actaea rubra

Chinese cohosh
Sheng ma
Actaea dahurica

http://bonap.net/Napa/TaxonMaps/Genus/County/Actaea

Black cohosh (*Actaea racemosa*)

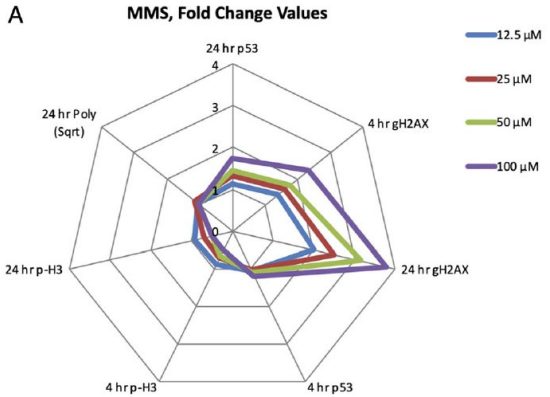
In vitro assessment



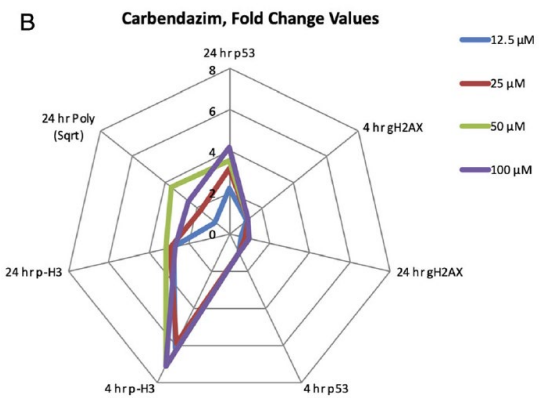
Black Cohosh Extracts and Powders Induce Micronuclei, a Biomarker of Genetic Damage, in Human Cells

Stephanie L. Smith-Roe,[1]* Carol D. Swartz,[2] Kim G. Shepard,[2] Steven M. Bryce,[3] Stephen D. Dertinger,[3] Suramya Waidyanatha,[1] Grace E. Kissling,[1] Scott S. Auerbach,[1] and Kristine L. Witt[1]

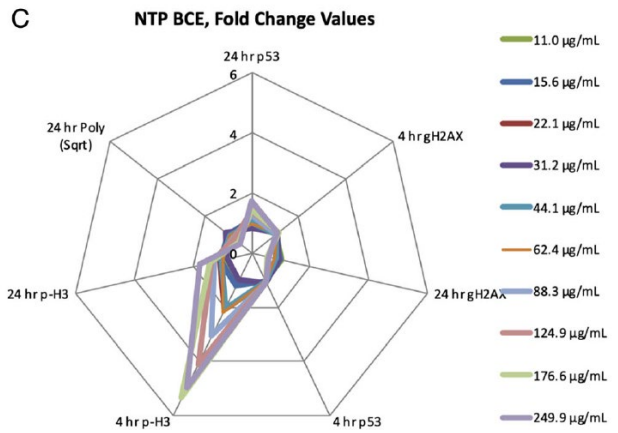
Environmental and Molecular Mutagenesis 59:416–426 (2018)



Clastogen positive control



Aneugen positive control



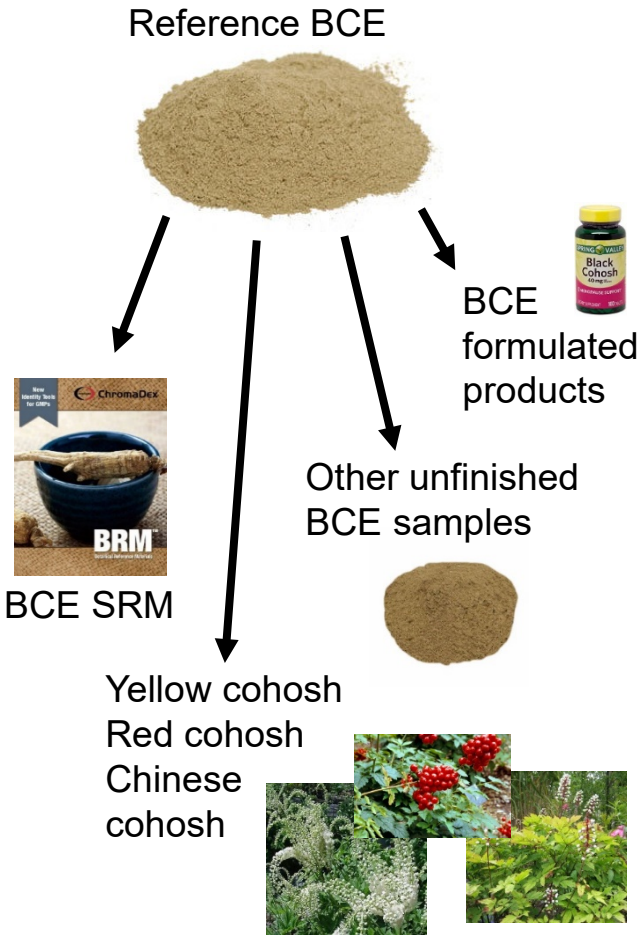
Evidence for an Aneugenic Mechanism of Action for Micronucleus Induction by Black Cohosh Extract

Derek T. Bernacki,[1] Steven M. Bryce,[1] Jeffrey C. Bemis,[1] Stephen D. Dertinger,[1] Kristine L. Witt,[2] and Stephanie L. Smith-Roe[2]*

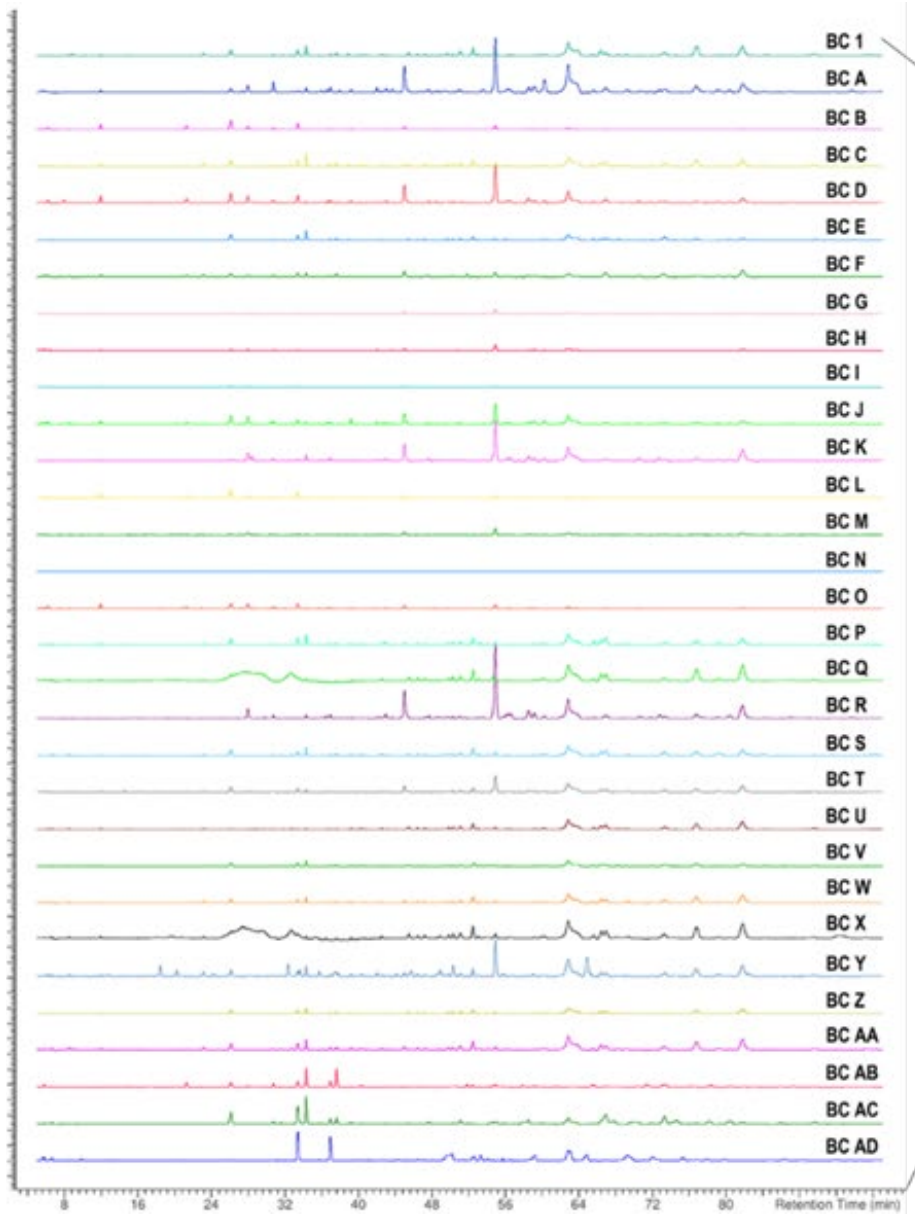
Environmental and Molecular Mutagenesis 60:845–856 (2019)

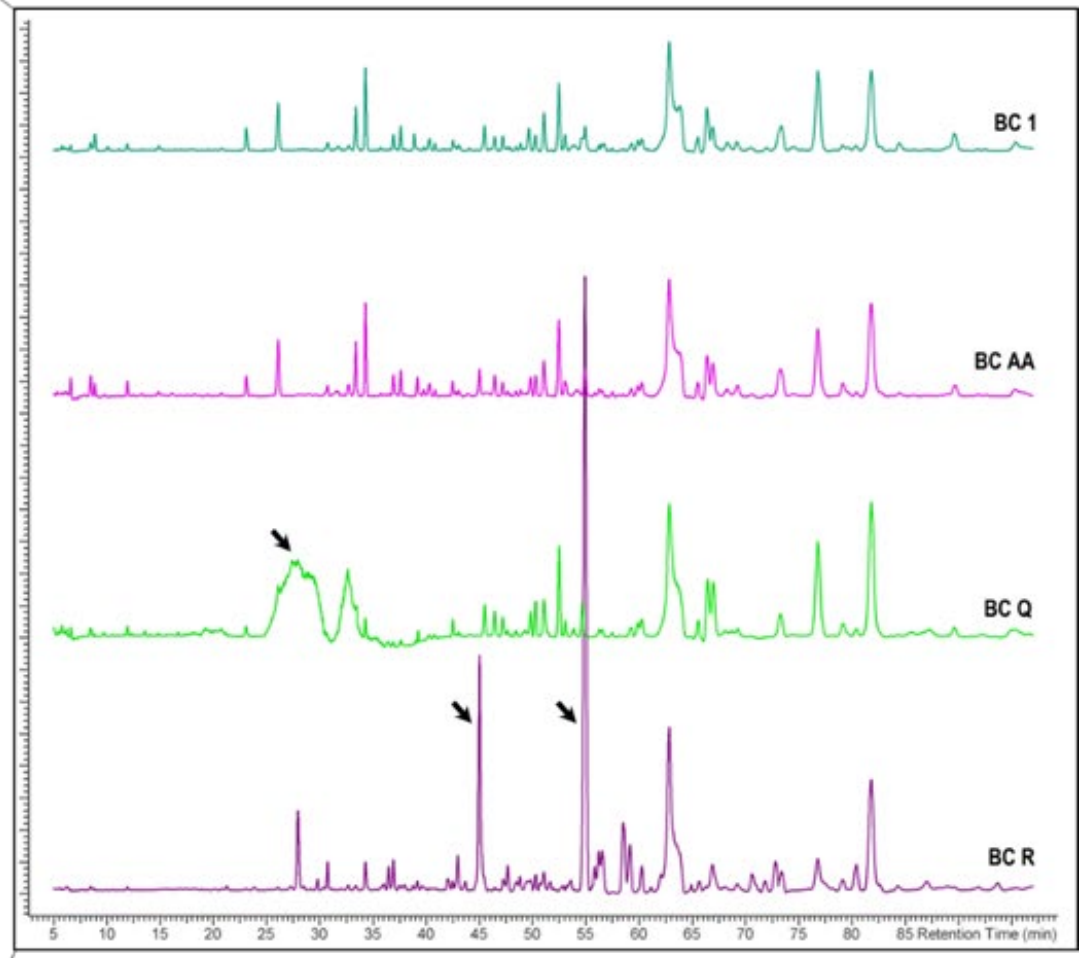
Black cohosh (*Actaea racemosa*)

- What are we comparing?
  - Reference black cohosh extract – assessed in 90-day
  - Black cohosh extract unfinished samples
  - Black cohosh extract Standard Reference Material
  - Other cohosh extract Standard Reference Materials
  - Formulated black cohosh extract products
- How are we comparing?
  - Chemical comparison
    - Non-targeted chemistry – chromatographic profiles
  - Biological comparison
    - *In vitro* assay
      - Human hepatocyte assay (AhR, CAR, PXR, FXR, PPARα)
      - Genotoxicity – micronucleus assay
  - Combining chemical and biological information



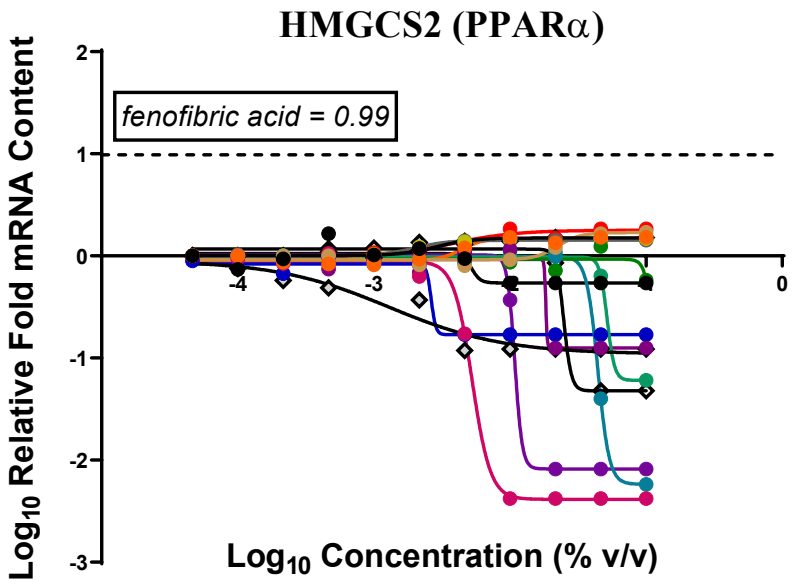
Black cohosh (*Actaea racemosa*)

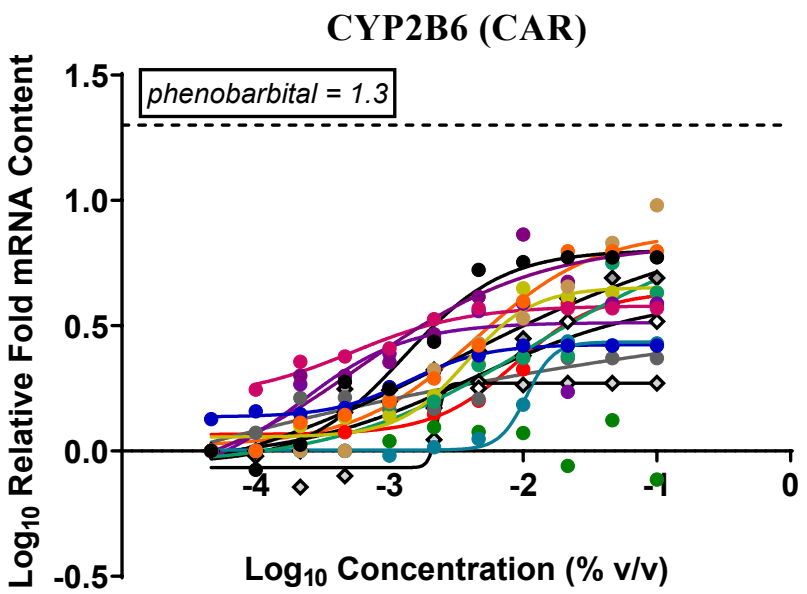


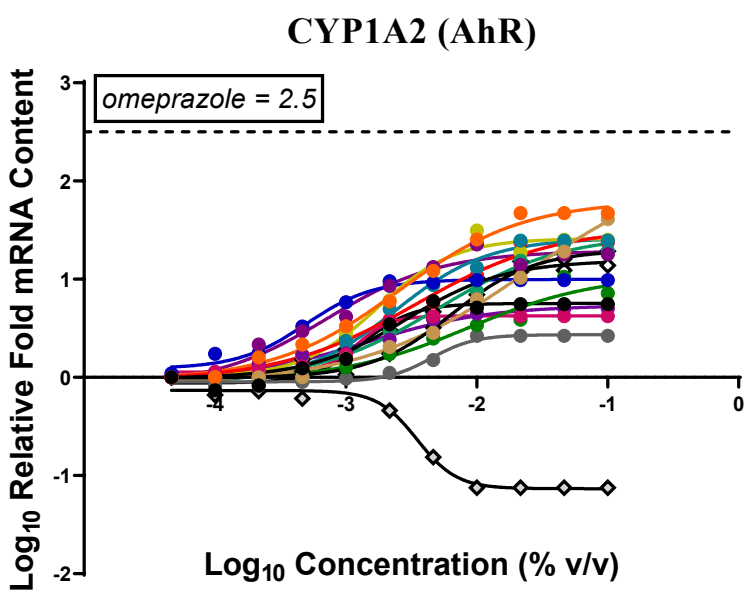


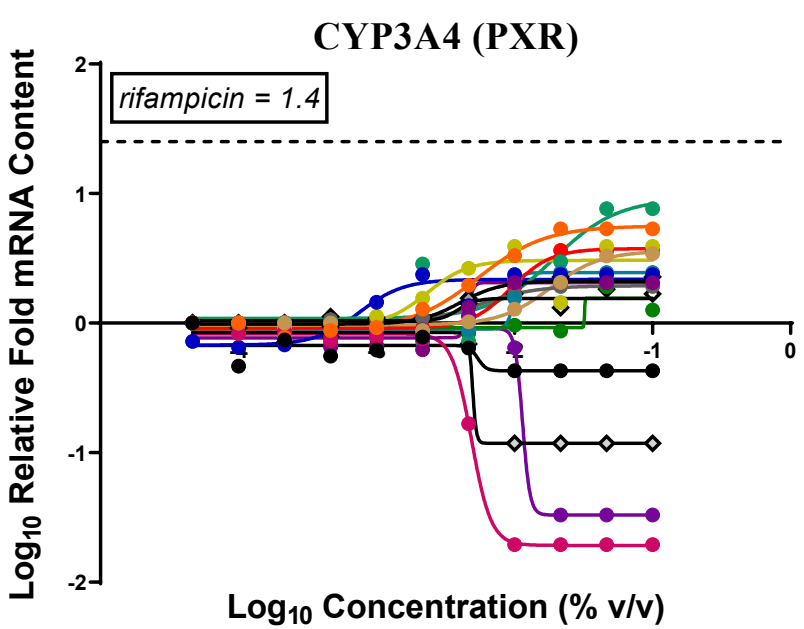
Ryan et al., (2019). Evaluating Sufficient Similarity of Botanical Dietary Supplements: Combining Chemical and In Vitro Biological Data. Toxicological Sciences. 172:316-329.

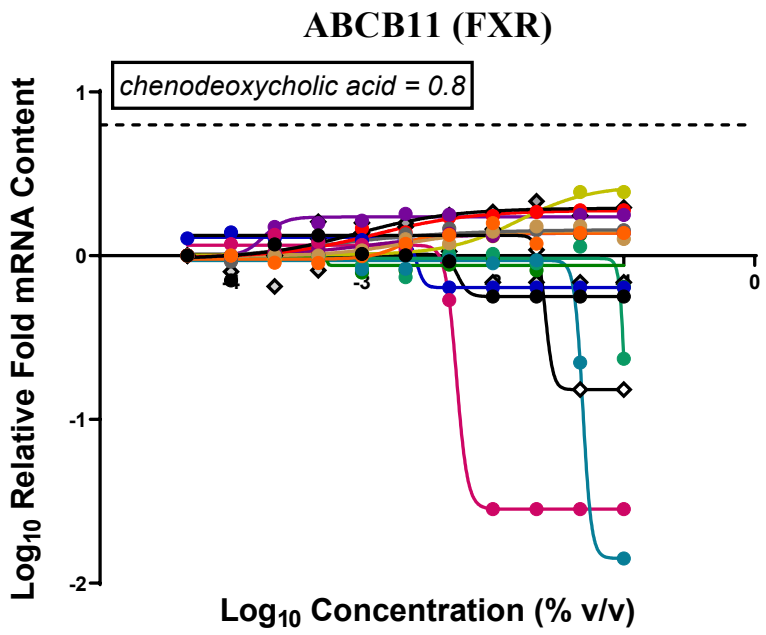
Black cohosh (*Actaea racemosa*)

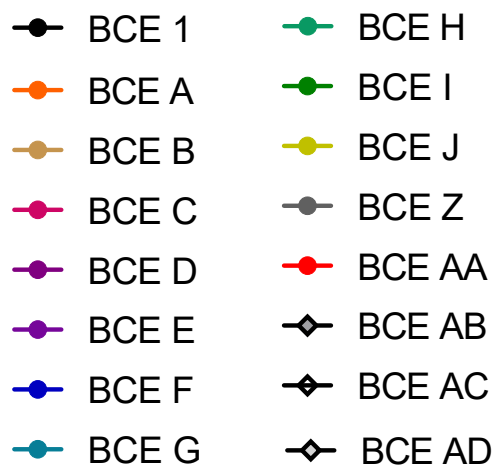










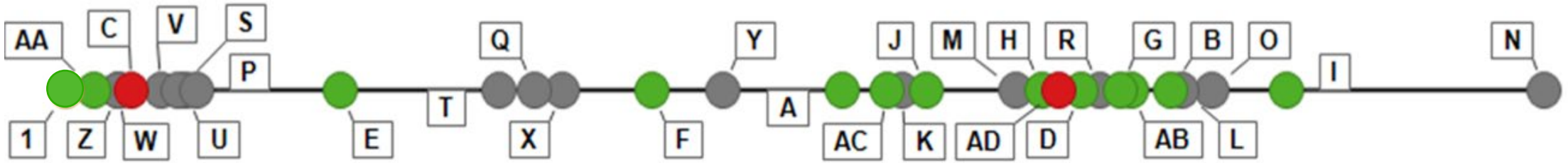


Comparing chemical and bioactivity similarity

Strength of evidence

	A	B	C	D	E	F	G	H	I	J	AA	AB	AC	AD
Nontargeted chemistry	-1	0	1	-1	1	-1	-1	0	0	-1	1	0	0	0
PHH gene expression	1	1	-1	1	1	1	1	1	1	1	1	1	1	-1
Genotoxicity	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Similarity score	0.3	0.7	0.3	0.3	1	0.3	0.3	0.7	0.7	0.3	1	0.7	0.7	0

Visual interval inspection



Black cohosh (*Actaea racemosa*)

Key points

- Micronucleus induction and megaloblastic anemia are the critical endpoints identified in animal studies
- This finding was replicated in human cells (not a rodent-specific finding)
- An aneugenic mechanism was identified, which indicates there is likely a threshold effect
- All cohoshes induced micronucleus formation (not specific to subset of black cohosh samples and active constituent has not been identified)
- The next step is to identify the constituent(s) responsible for the genotoxic effect

Identifying active constituents

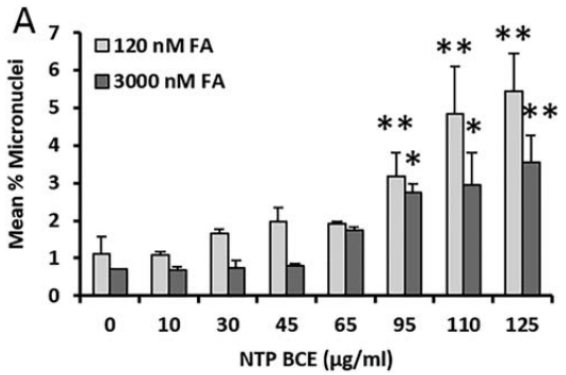
Black cohosh

Extraction

Bioassay

Active extract

Separation



Bioassay

Isolation/
Identification

Chemical Structure

Active fraction

Roberts et al., 2019. Food and Chemical Toxicology. 124: 431-438.
Smith-Roe et al., 2018. Environmental and Molecular Mutagenesis 59:416-426.

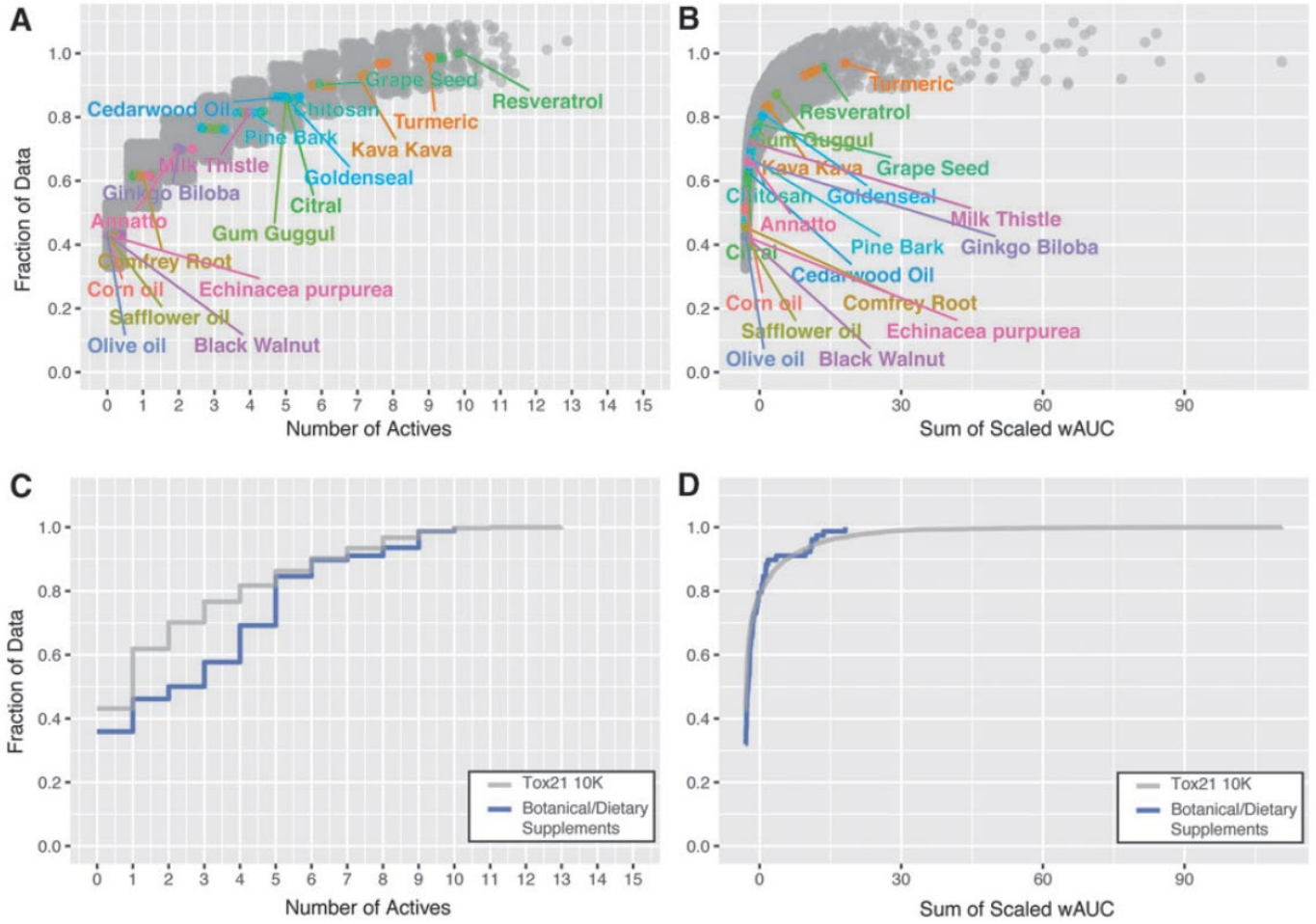
Botanicals in Tox21

- Toxicology in the 21st Century (Tox21) is a federal collaboration between EPA, NIH (National Center for Advancing Translational Sciences and the National Toxicology Program) and the Food and Drug Administration
- Phase 2 involved evaluating the 10k chemical library (8193 unique chemicals) in over 75 quantitative high throughput assays measuring stress response and nuclear receptor activity
- Mostly focused on single chemicals, some defined mixtures included

Can the Tox21 platform be used to evaluate botanical dietary supplements and other complex mixtures?

Botanicals in Tox21

In vitro assay	F-value	Rank
elg1-luc-agonist	20312.81	1
hse-bla-agonist	1425.55	2
mmp-antagonist	25.72	3
aromatase/er-er-agonist	23.55	4
ahr-agonist	21.79	5
er-luc-bg1-4e2-agonist	17.50	6
rt-viability-hepg2-glo	15.85	7
pparg-bla-antagonist	14.20	8
rt-viability-hek293-glo	12.27	9
rt-viability-hepg2-flor	10.57	10



Hubbard et al., 2019, Using Tox21 High-Throughput Screening Assays for the Evaluation of Botanical and Dietary Supplements. Applied *In vitro* Toxicology 5(1):10-25

Botanical safety

FDA STATEMENT

Statement from FDA Commissioner Scott Gottlieb, M.D., on the agency's new efforts to strengthen regulation of dietary supplements by modernizing and reforming FDA's oversight

"*…as with other commodities that the agency regulates, it's critical that FDA continue to work closely with our partners in industry to achieve our primary goal of protecting public health and safety. As the dietary supplement industry develops new products and ingredients, advances new delivery systems and innovates in other ways, the FDA must do more to leverage its existing resources and authorities to evaluate these products. This requires collaborative research and a shared understanding. I'm pleased to announce that we've recently created the Botanical Safety Consortium, a public-private partnership that will gather leading scientific minds from industry, academia and government to promote scientific advances in evaluating the safety of botanical ingredients and mixtures in dietary supplements. This group will look at novel ways to use cutting-edge toxicology tools, including alternatives to animal testing, to promote the goals of safety and effectiveness we share with consumers and other stakeholders.*"

Botanical Safety Consortium

A public-private partnership aimed at developing a toolbox of *in vitro* and *in silico* assays and approaches for evaluating botanical safety

Objective 2.3: Botanical Safety Consortium

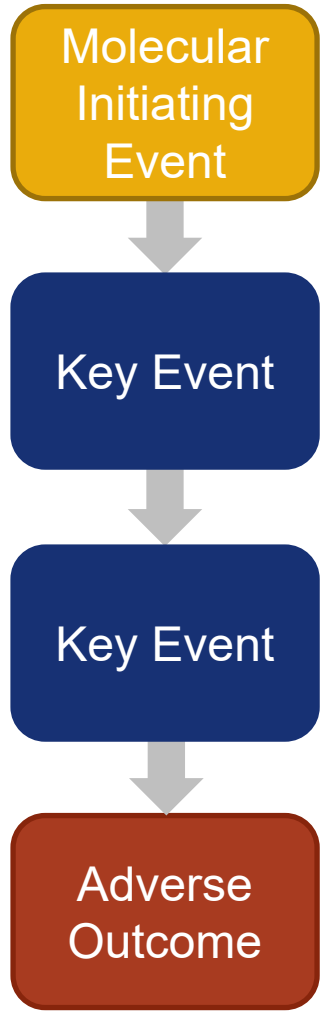
Objectives

BOTANICAL
SAFETY CONSORTIUM

- Engage with a broad group of global stakeholders to leverage the best scientific approaches
- Establish the appropriate levels of chemical characterization for complex botanical ingredients
- Identify pragmatic, fit-for-purpose *in vitro* & *in silico* assays to evaluate botanical safety
- Evaluate the application of these tools via comparison to the currently available safety information
- Integrate these tools and approaches into a framework that will facilitate robust evaluation of botanical ingredients

Challenges

- Better understanding the transition from adaptive to adverse responses in sensitive *in vitro* systems to identify real safety concerns
- Developing recommendations for chemical analysis of complex botanical ingredients and products
- Achieving an appropriate level of biological coverage to identify likely toxicity targets while maintaining a manageable testing platform
- Identifying active constituents and measuring concentrations in *in vitro* assessments to aid in translating findings to humans and comparing across products
- Refining complex mixture read-across methods



Conclusions

- In vitro assays combined with non-targeted chemical analysis were useful in evaluating sufficient similarity of complex mixtures
- In vitro assays can be incorporated into bioassay-guided fractionation approaches to identify active constituents in complex mixtures
- Botanicals evaluated in Tox21 assays point to both challenges and opportunities for complex mixtures
- The Botanical Safety Consortium is actively working to develop a toolkit of in vitro assays and recommended framework for assessing botanical safety

Turmeric
Curcuma longa

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Garcinia cambogia
Garcinia gummi-gutta