NTP
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Challenges and Approaches for Using Animal and Human Data to Evaluate *In vitro* Systems in Botanical Safety Assessment

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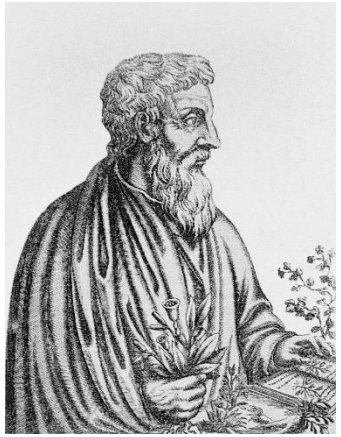
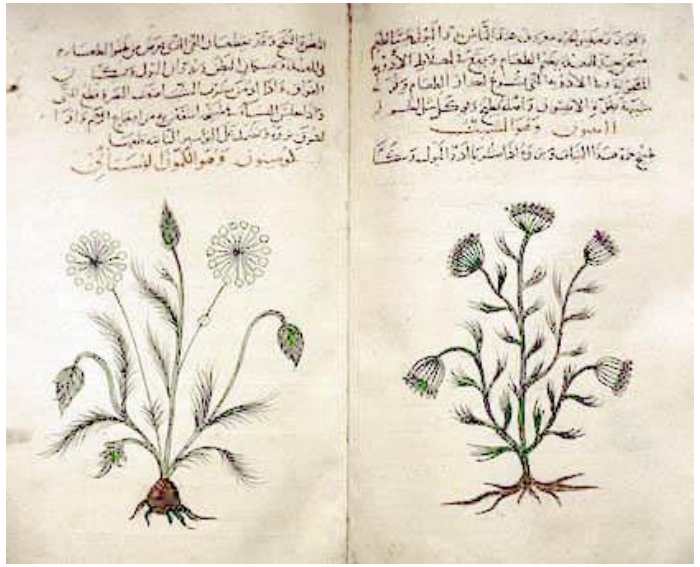
Overview

- Current paradigm for safety evaluation of botanicals
- Case studies comparing across data streams
  - Black cohosh
  - Green tea extract
  - *Ginkgo biloba* extract
- Challenges and next steps

History of safe use



Discorides' Materia Medica, c. 1334



Toxicology Letters 314 (2019) 10–17

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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

Evidence used in safety evaluation

Human

- Data streams
  - Clinical trials
  - Adverse event reporting
- Considerations
  - Genetic diversity
  - Product variability
  - Under-reporting
    - Latency
    - Difficulty proving causality

Animal

- Data streams
  - Toxicity studies in rodents
  - Preclinical safety studies
- Considerations
  - Lack of genetic diversity
  - Single test article
  - Resource intensive

In vitro

- Data streams
  - Untargeted (Tox21)
  - Targeted/mechanistic
- Considerations
  - Isolated systems
  - Human-relevant
  - IVIVE required
  - Can test multiple samples

https://generated.photos

Botanical safety case studies

Black cohosh (*Actaea racemosa*)

Background

- Use: menopausal symptoms, menstrual cramps, premenopausal symptoms
- Recommended dose: 20-40 mg twice daily
- Background on safety:
  - Long history of use (included in the first USP monographs in 1820!)
  - No serious side effects noted in clinical trials (NCCIH)
  - Case reports indicate potential hepatotoxicity
  - Limited animal safety data
  - Commonly adulterated with other cohoshes and should not be confused with blue cohosh (used for stimulating labor and not considered to be safe)

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"

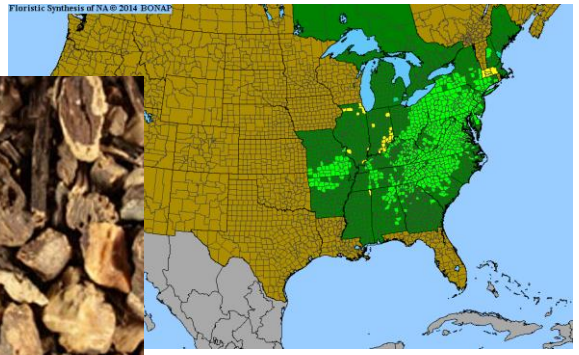
ABC AHP NCNPR
Botanical Adulterants Program

BOTANICAL ADULTERANTS BULLETIN

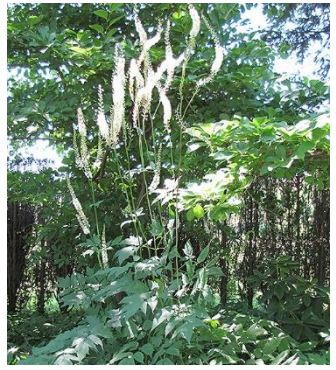
on Adulteration of *Actaea racemosa*

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*)

Animal studies

- 90-day oral gavage toxicity study with ♀ Wistar Han rats: 0, 15, 125, 250, 500, 1000 mg/kg

- 90-day oral gavage toxicity study with ♀ B6C3F1/N mice: 0, 62.5, 125, 250, 500, 1000 mg/kg

Megaloblastic anemia
Micronucelus induction

- 3-day subcutaneous injection immature ♀ CD-1 mouse uterotrophic assay with ♀ B6C3F1/N mice: 0, 0.001, 0.1, 1, 10, 100, 500 mg/kg

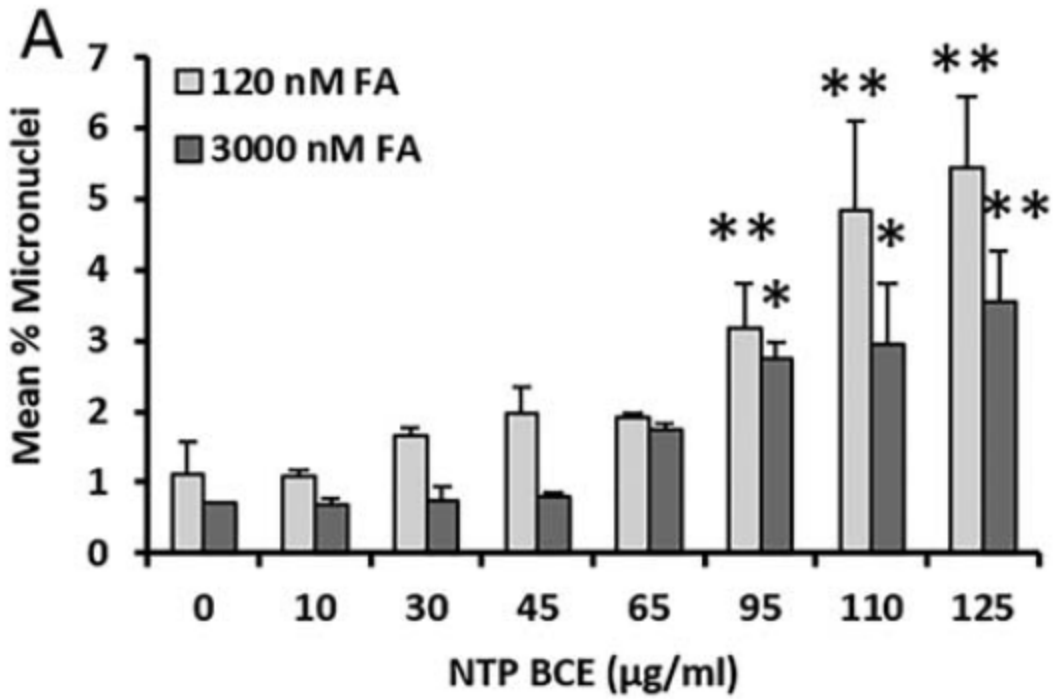
- 90-day oral gavage with ♀ B6C3F1/N mice to evaluate mechanism of megaloblastic anemia: 0, 1000 mg/kg

Mercado-Feliciano et al., (2012). An ethanolic extract of black cohosh causes hematological changes but not estrogenic effects in female rodents. Toxicology and Applied Pharmacology. 263:138-147.

Cora et al., (2017). A black cohosh extract causes hematological and biochemical changes consistent with a functional cobolamin deficiency in female B6C3F1/N mice. Toxicologic Pathology. 45(5): 614-623.

Black cohosh (*Actaea racemosa*)

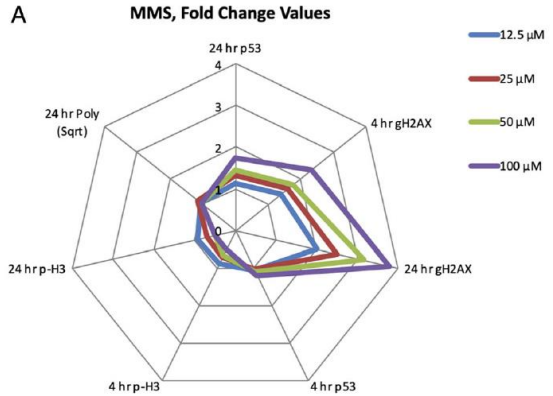
In vitro assessment



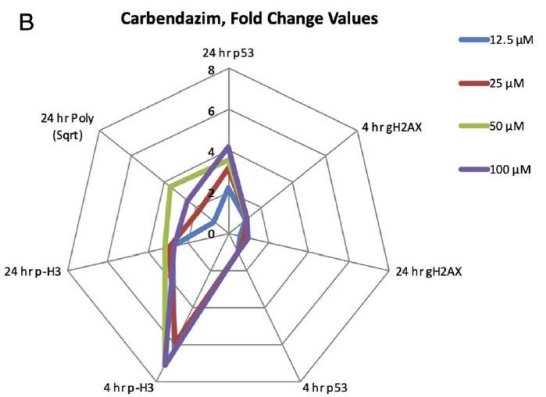
Black Cohosh Extracts and Powders Induce Micronuclei, a Biomarker of Genetic Damage, in Human Cells

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]

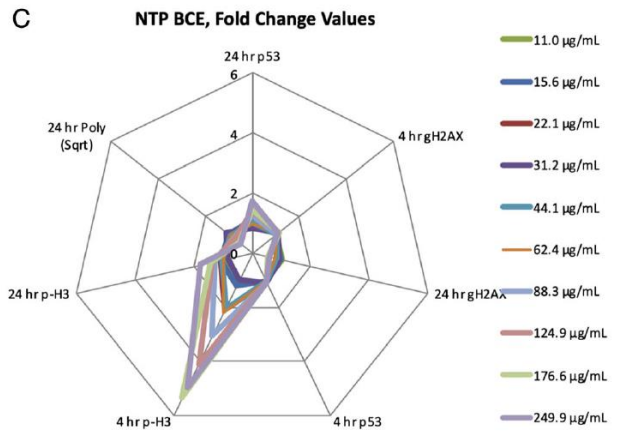
Environmental and Molecular Mutagenesis 59:416–426 (2018)



Clastogen positive control



Aneugen positive control

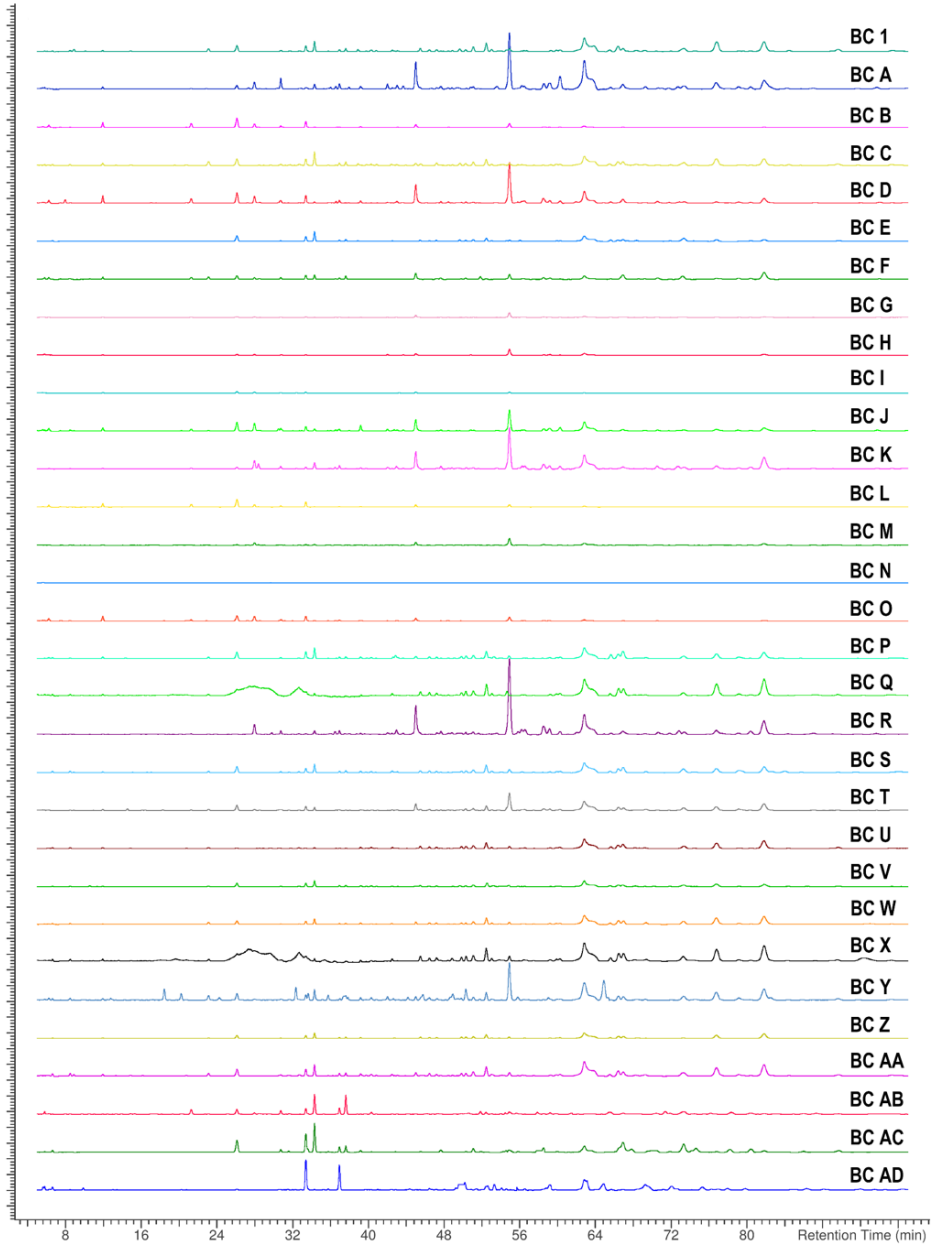


Evidence for an Aneugenic Mechanism of Action for Micronucleus Induction by Black Cohosh Extract

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)

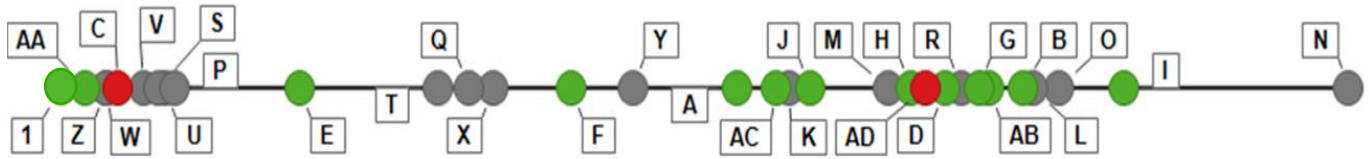
Black cohosh (*Actaea racemosa*)



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



Evaluating Sufficient Similarity of Botanical Dietary Supplements: Combining Chemical and *In Vitro* Biological Data

Kristen R. Ryan,*,[1] Madelyn C. Huang,*,[1] Stephen S. Ferguson ,* Suramya Waidyanatha,* Sreenivasa Ramaiahgari,* Julie R. Rice,* Paul E. Dunlap,* Scott S. Auerbach,* Esra Mutlu,* Tim Cristy,† Jessica Peirfelice,† Michael J. DeVito,* Stephanie L. Smith-Roe,* and Cynthia V. Rider*,[2]

TOXICOLOGICAL SCIENCES, 172(2), 2019, 316–329

Comparing across data streams

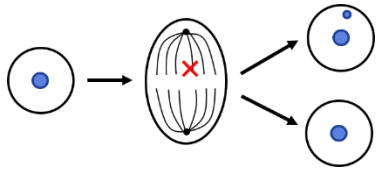
Recommended dose = 40-80 mg/day
40-80 mg / 60 kg (female weight) =
0.67-1.33 mg/kg

human

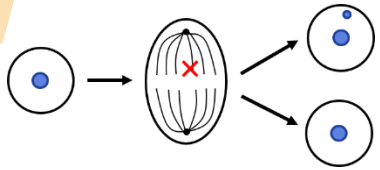
15-30X higher dose in animals than humans

1 black cohosh extract tested

LOAEL = 250 mg/kg (mice)
HED = 20 mg/kg



animal



in vitro

Test article	Supplier
Black cohosh extract XRM	Chromadex
Black cohosh extract	Supplier 1*
Black cohosh extract	Supplier 2
Black cohosh extract	Supplier 3
Black cohosh extract	Supplier 3
Black cohosh extract	Supplier 3
Black cohosh extract	Supplier 4
Black cohosh extract	Supplier 4
Black cohosh extract	Supplier 5
Black cohosh extract	Supplier 6
Black cohosh extract	Supplier 7
Black cohosh root powder	Supplier 8
Chinese cohosh VBRM	Chromadex
Red cohosh VBRM	Chromadex
Yellow cohosh VBRM	Chromadex

Black cohosh (*Actaea racemosa*)

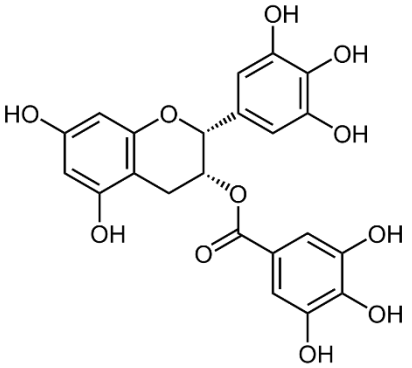
Key points

- 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)
- All cohoshes induced micronucleus formation (not specific to subset of black cohosh samples and active constituent has not been identified)
- An aneugenic mechanism was identified, which indicates there is likely a threshold effect
- Rodent doses were 15-30x above human doses
- Cost-benefit should be considered: some risk, no convincing efficacy data

Green tea (*Camellia sinensis*) extract

Background

- Use: weight loss
- Recommended dose: 400-800 mg daily
- Background on safety:
  - Purported active constituent(s): catechins (Epigallocatechin-gallate)
  - Long history of use of green tea, not green tea extract
  - Liver toxicity noted in clinical trials and adverse event reports
  - Liver identified as a target in animal safety data



EGCG

Green tea (*Camellia sinensis*) extract

Human data

- Liver injury characteristics
  - Typically arises within 3 months; latency of 10 days – 7 months
  - Acute hepatitis-like syndrome
  - Hepatocellular pattern of serum enzyme elevations
  - Liver biopsy findings of necrosis, inflammation, and eosinophils resembling acute hepatitis

CAUSALITY ASSESSMENT RESULTS FOR CASES RELATED TO SIDS

DILIN causality Scale	OVERALL (HDS) score	% of total	Agent-Specific (GTE) Score	% of total	Specific Score - OTHER (drug name)	% of total	RUCAM scale	RUCAM score	% of total
1 = definite	**4**	**11.4**	**4**	**11.4**	**0**	**0.0**			**0.0**
2 = highly likely	**11**	**31.4**	**11**	**31.4**	**0**	**0.0**	**Highly Probable: * or >8**	**6**	**17.1**
3 = probable	**14**	**40.0**	**14**	**40.0**	**0**	**0.0**	**Probably: 6-8**	**23**	**62.9**
4 = possible	5	14.3	5	14.3	5	12.5	Possible: 3-5	6	17.1
5 = unlikely	0	0	0	0	29	87.5	Unlikely: 1-2	0	0.0
6 = insufficient data	1	2.9	1	2.9	1	0	Excluded: 0 or <0	1	2.9
	35	**100.0**	**35**	**100.0**	**35**	**100.0**		**35**	**100.0**

SIDS: single-ingredient dietary supplement, containing only green tea extract as ingredient
DILIN: Drug-Induced Liver Injury Network
HDS: herbal dietary supplement
GTE: green tea extract
Drug name: different drugs were involved, some used alone or concurrently with others
RUCAM: Roussel Uclaf Causality Assessment Method [28, 29].

LiverTox: Clinical and Research Information on Drug Induced Liver Injury [Internet]. Bethesda (MD): National Institute of Diabetes and Digestive and Kidney Diseases; 2012-. Green Tea. [Updated 2018 Mar 12].

Oketch-Rabah et al., (in press). United States Pharmacopeia (USP) Comprehensive Review of the Hepatotoxicity of Green Tea Extracts. Toxicology Reports.

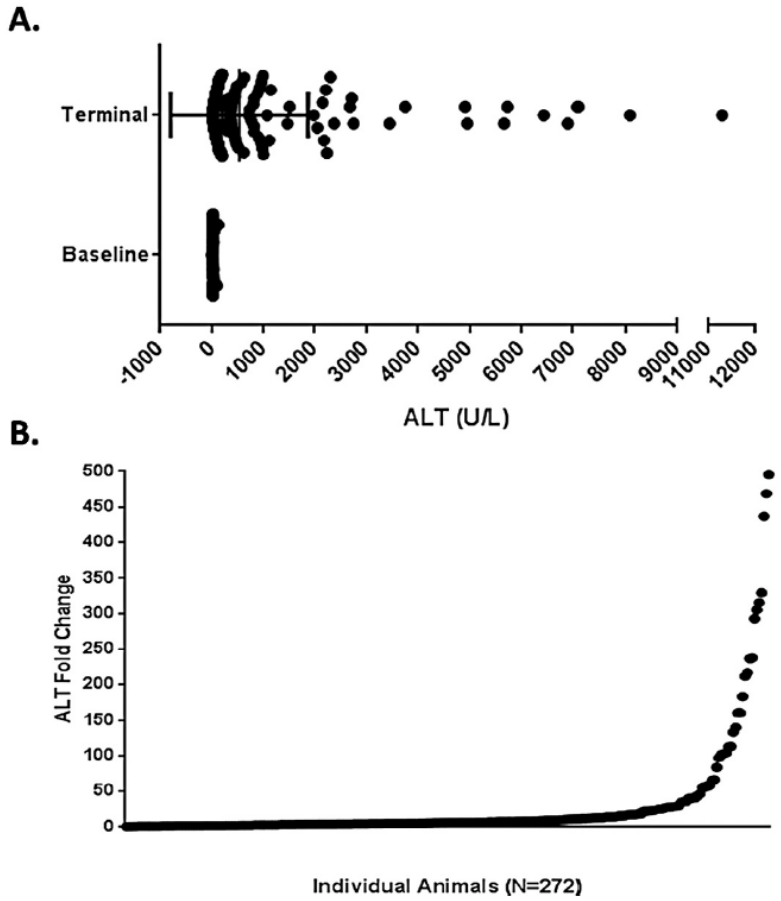
Green tea (*Camellia sinensis*) extract

Animal data

- Fasted versus fed beagle studies
  - Finding: Liver toxicity in fasted animals more severe and occurs at lower doses than in fed animals

- Multiple rodent subchronic studies and chronic studies
  - Convert all exposures to EGCG units to compare across studies
  - Confirm the key toxicity target is the liver

Diversity outbred mice study: Most tolerated 50 mg/kg EGCG, 16% exhibited severe hepatotoxicity



Animal data summarized in: **Oketch-Rabah** et al., (in press). United States Pharmacopeia (USP) Comprehensive Review of the Hepatotoxicity of Green Tea Extracts. Toxicology Reports.

Church et al., (2015). Sensitivity to hepatotoxicity due to epigallocatechin gallate is affected by genetic background in diversity outbred mice. Food and Chemical Toxicology. 76:19-26.

Green tea (*Camellia sinensis*) extract

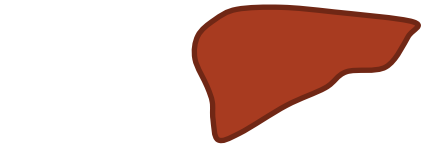
In vitro data

- Model system: rat primary hepatocytes

	EGCG concentration (μmol/L)							
	0	5	10	15	20	30	50	100
LDH leakage ($n = 6$)	16.1 ± 0.9	16.3 ± 0.6	**24.9 ± 1.7*****	**37.7 ± 1.8*****	**50.2 ± 1.3*****	**55.3 ± 1.4*****	**56.5 ± 0.8*****	**66.7 ± 2.6*****
WST-1 ($n = 6$)	100.0 ± 1.8	103.1 ± 2.2	**88.1 ± 3.0***	**51.2 ± 12.5*****	**30.4 ± 8.2*****	**10.5 ± 1.7*****	**2.7 ± 0.2*****	**1.5 ± 0.1*****
Albumin ($n = 6$)	60.0 ± 4.4	**49.9 ± 8.9****	**38.5 ± 3.5*****	**31.3 ± 3.2*****	**21.7 ± 3.8*****	**18.5 ± 0.9*****	**11.6 ± 1.5*****	**6.6 ± 1.4*****
Caspase 3 ($n = 6$)	100 ± 13	121 ± 15	**690 ± 45*****	**1100 ± 77*****	**1212 ± 50*****	**1274 ± 65*****	**1491 ± 44*****	**763 ± 108*****
Caspase 8 ($n = 8$)	100.0 ± 18.5	n/a	108.8 ± 15.7	n/a	n/a	**339.9 ± 34.6*****	**306.2 ± 32.9*****	n/a
Caspase 9 ($n = 8$)	100.0 ± 12.3	n/a	105.6 ± 7.2	n/a	n/a	**116.0 ± 7.0***	**120.0 ± 11.1****	n/a
TBARS ($n = 6$)	0.49 ± 0.08	0.42 ± 0.04	0.45 ± 0.06	0.60 ± 0.23	0.47 ± 0.11	0.44 ± 0.05	0.46 ± 0.09	0.54 ± 0.09
ROS ($n = 8$)	100 ± 8.6	98 ± 8.4	**82 ± 9.5****	**80 ± 8.5****	87 ± 4.6	96 ± 12.5	110 ± 9.0	**131 ± 11.5*****
TNFα ($n = 5$)	80 ± 8.7	19 ± 15.4	71 ± 17.4	130 ± 5.0	209 ± 32.8	**253 ± 23.1***	149 ± 21.8	99 ± 24.9

Kucera et al., (2015). *In vitro* toxicity of epigallocatechin gallate in rat liver mitochondria and hepatocytes. Oxidative Medicine and Cellular Longevity.

Comparing across data streams

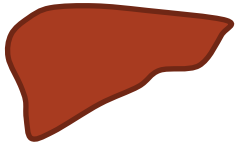


human

Recommended dose = 400-800 mg/day
400-800 mg / 60 kg (female weight) =
6.67-13.3 mg/kg

525 mg EGCG dose in humans led to plasma levels of 4.41 µmol/L (Nakagawa et al. 1997)

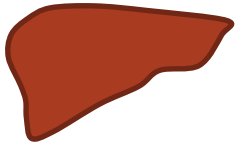
Liver effects observed in animal studies at doses that are relevant to humans



animal

LOAEL = 120 mg/kg
EGCG (fasted dog)
HED = 66.7 mg/kg

LOAEL = 50 mg/kg
EGCG (DO mice)
HED = 4.07



in vitro

Mechanistic insight:

- Pro-apoptotic pathway
- Mitochondrial membrane damage

LOAEL = 10 µmol/L EGCG

Key points

- EGCG is useful in comparing across studies and doses
  - Studies with pure EGCG support that it is the active constituent for liver toxicity
  - Many issues associated with complex mixtures are not relevant to this case study
- Liver toxicity is evident across testing platforms at doses that are relevant to recommended green tea extract doses
- Pharmacokinetics and accompanying changes in internal dose based on fed/fasted state is important in understanding liver toxicity
- There seems to be a genetic (and/or underlying condition) component to green tea extract liver toxicity susceptibility

Ginkgo biloba extract

Background

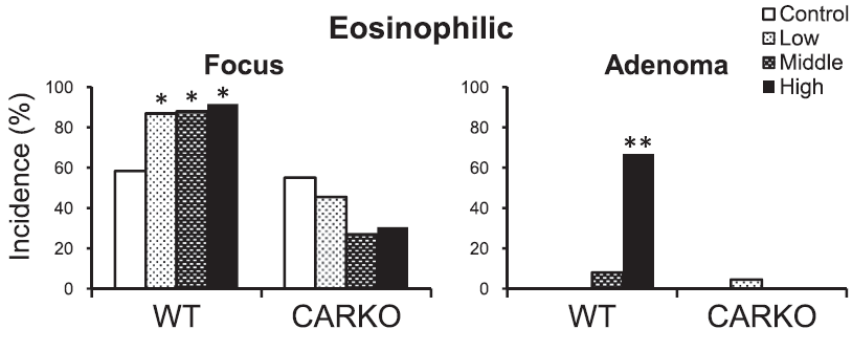
- Use: mental acuity
- Recommended dose: 120 – 240 mg/day
- Background on safety:
  - Long history of use
  - No serious side effects noted in clinical trials
  - Some case studies involving excessive bleeding and inconsistent signals regarding drug-botanical interactions
  - Commonly adulterated with cheaper plants (*Sophora japonica*) or pure flavonols (rutin)

Animal data

- 90-day oral gavage toxicity study with Fisher 344 rats: 0, 62.5, 125, 250, 500, 1000 mg/kg
- 90-day oral gavage toxicity study with B6C3F1/N mice: 0, 125, 250, 500, 1000, 2000 mg/kg
- 2-year oral gavage toxicity study with Fisher 344 rats: 0, 100, 300, 1000 mg/kg
- 2-year oral gavage toxicity study with B6C3F1/N rats: 0, 200, 600, 2000 mg/kg
- Mechanistic studies in gpt Delta and CAR-knockout mice

Carcinogenicity across species and sex

Targets:
- Liver
- Nose
- Thyroid

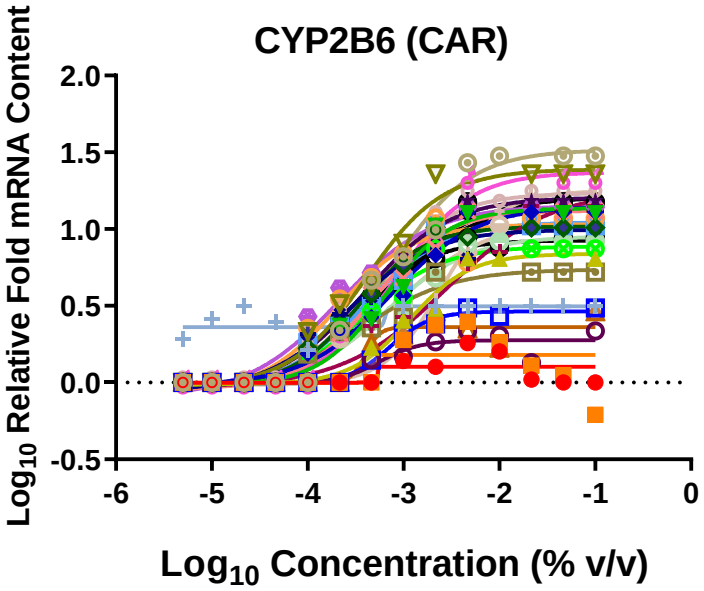


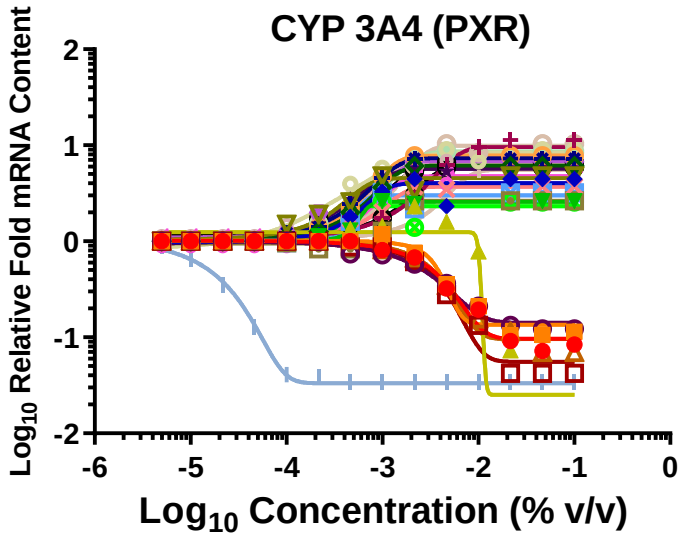
NTP, (2013). Toxicity and carcinogenesis studies of Ginkgo biloba extract in F344/N rats and B6C3F1/N mice.

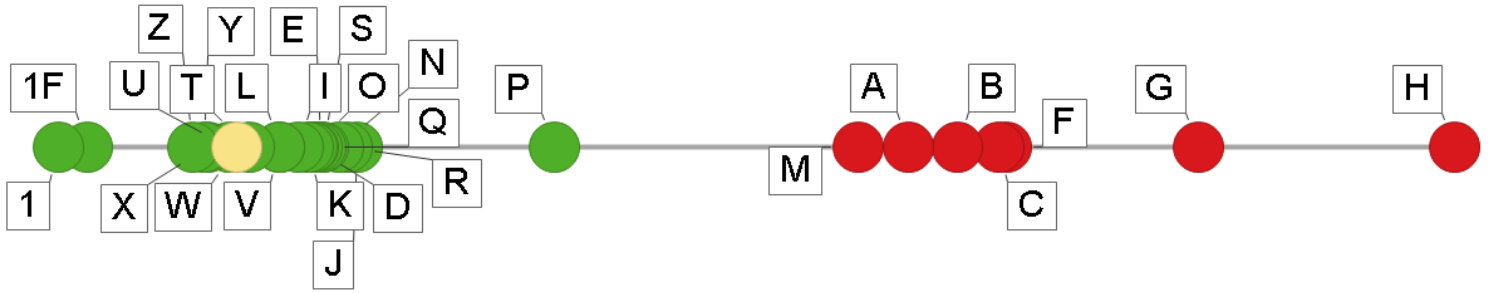
Maeda et al. (2015). Essential role of constitutive androstane receptor in Ginkgo biloba extract induced liver hypertrophy and hepatocarcinogenesis. Food and Chemical Toxicology 83:201-209

Ginkgo biloba extract

In vitro data







Human data

Table 4
Association of ginkgo consumption with liver function, NHANES 2001–2012.[a]

	Non-Consumers		Ginkgo Consumers		Difference (consumers − non-consumers)		P Value
	Mean	SE[b]	Mean	SE	Mean	SE	
Alkaline phosphatase (ALP), U/L							
All	67.3	0.2	67.1	1.0	−0.2	1.0	0.8074
Male	68.4	0.3	67.8	1.3	−0.6	1.3	0.6556
Female	66.2	0.3	66.1	1.6	−0.1	1.6	0.9475
Alanine aminotransferase (ALT), U/L							
All	25.8	0.2	26.7	0.9	0.8	0.9	0.3476
Male	30.3	0.3	30.8	1.5	0.5	1.5	0.7684
Female	21.5	0.2	22.7	0.7	1.3	0.7	0.0767
Aspartate aminotransferase (AST), U/L							
All	25.6	0.1	26.3	0.6	0.8	0.7	0.2503
Male	27.7	0.2	28.2	1.0	0.5	1.0	0.6486
Female	23.5	0.1	24.6	0.7	1.1	0.7	0.1123
Gamma glutamyl transferase (GGT),U/L							
All	28.4	0.4	30.5	2.0	2.1	2.1	0.3182
Male	33.4	0.5	37.9	3.2	4.5	3.2	0.1667
Female	23.5	0.4	22.7	1.6	−0.8	1.8	0.6621
Lactate dehydrogenase (LDH), U/L							
All	128.2	0.4	129.3	1.6	1.1	1.5	0.4673
Male	128.4	0.4	128.9	2.0	0.6	2.0	0.7721
Female	128.1	0.4	129.4	1.9	1.3	1.8	0.4607
Bilirubin, mg/dL							
All	0.76	0.004	0.77	0.01	0.02	0.01	0.1702
Male	0.84	0.01	0.86	0.02	0.01	0.02	0.5049
Female	0.67	0.004	0.69	0.02	0.02	0.02	0.2188

Moderate doses of commercial preparations of *Ginkgo biloba* do not alter markers of liver function but moderate alcohol intake does: A new approach to identify and quantify biomarkers of 'adverse effects' of dietary supplements

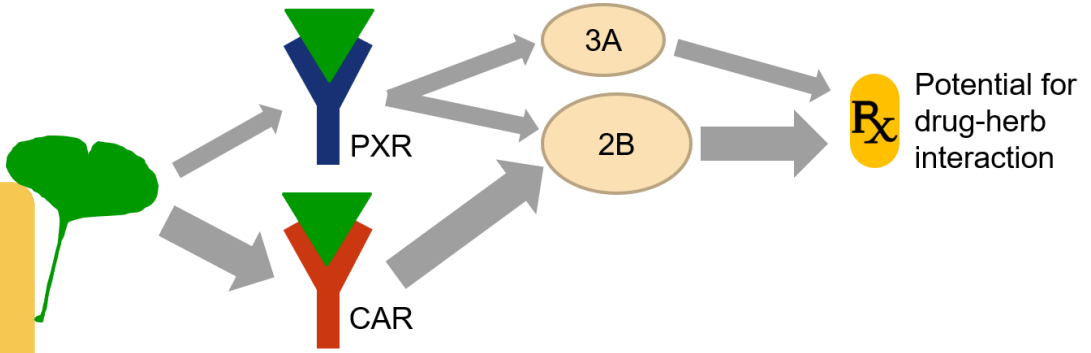
Harris R. Lierberman, Mark D. Kellogg, Victor L. Fulgoni III, Sanjiv Agarwal

Regulatory Toxicology and Pharmacology (2017) 84: 45-53

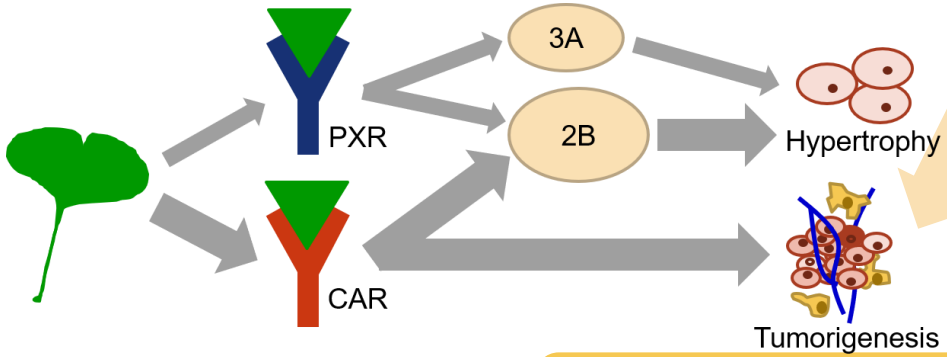
Comparing across data streams

Recommended dose = 120-240 mg/day
120-240 mg / 60 kg (female weight) =
2-4 mg/kg

human



4-8X higher dose
in rat than human



CAR and PXR were
induced in human cells

LOAEL = 100 mg/kg
(rat) HED = 16 mg/kg

animal

in vitro

32% of samples tested did not resemble standardized GBE

Molecular pathways adapted from Maeda 2015

Key points

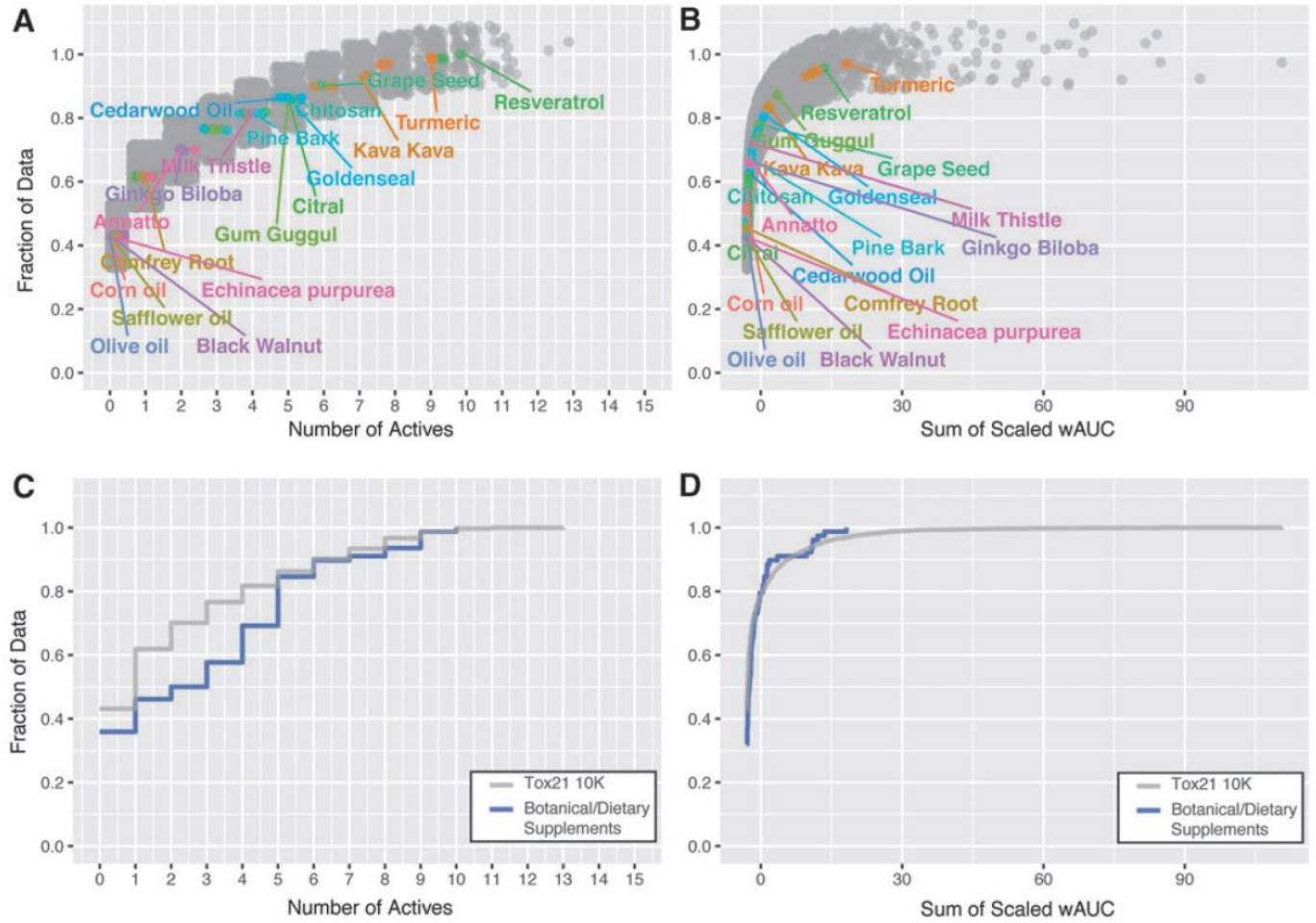
- The CAR/PXR mechanism of action does not result in the same suite of effects in humans as it does in rodents, therefore, the observed carcinogenesis is not directly translatable
- However, this does not mean that gingko is without safety concerns – the changes in drug metabolizing enzymes indicate potential for drug-herb interactions
- Terpene trilactones are the likely active constituents in GBE and there is a wide range of phytochemical compositions among samples

Observations

- There is value in building case studies for evaluating botanical safety that incorporate human, animal, and *in vitro* data
- The current paradigm involves human adverse event signals or animal toxicity studies preceding and triggering *in vitro* research that is typically used to:
  - provide a bridge between human and animal findings (black cohosh and GBE)
  - provide mechanistic insights (green tea extract)
- What considerations are needed to move toward a future where *in vitro* and *in silico* approaches provide a preliminary approach for evaluating botanical safety?
  - Coverage of adequate biological space
  - Decreasing the likelihood of false negatives due to deficiencies in the model system or conditions
  - Note that for most botanicals, we have human use data to identify potential toxicity targets, for completely new ingredients, animal evaluation is likely warranted

Botanicals in Tox21

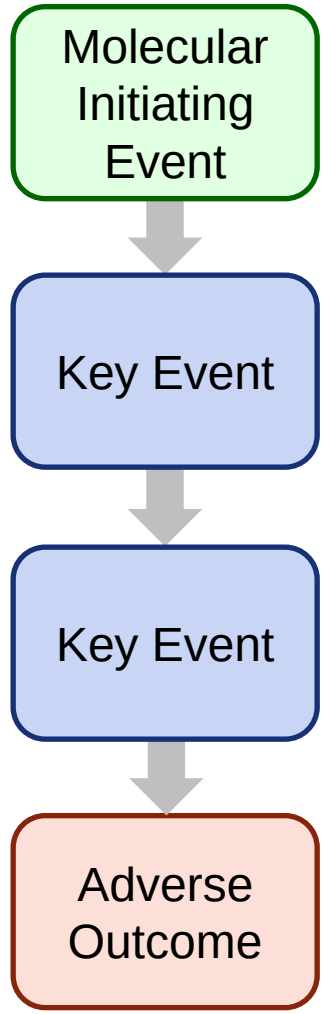
In vitro assay	F-value	Rank
elg1-luc-agonist	20312.81	1
hse-bla-agonist	1425.55	2
mmp-antagonist	25.72	3
aromatase/er-er-agonist	23.55	4
ahr-agonist	21.79	5
er-luc-bg1-4e2-agonist	17.50	6
rt-viability-hepg2-glo	15.85	7
pparg-bla-antagonist	14.20	8
rt-viability-hek293-glo	12.27	9
rt-viability-hepg2-flor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

Challenges

- Better understanding the transition from adaptive to adverse responses in sensitive *in vitro* systems to identify real safety concerns
- 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 aids in translating findings to humans and comparing across products



Next steps

- Continue to evaluate case studies that contain all three data streams
- Botanical Safety Consortium work to identify a suite of recommended assays in target areas (hepatotoxicity, genetic toxicity) and evaluate a library of botanicals in those assays
  - Identify areas that require additional assay coverage (DART, cardiotox, systems toxicity)
  - Characterize the domain of applicability for the assays
  - Compare results to existing data from animal and human studies

BOTANICAL
SAFETY CONSORTIUM

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