

PHYTOFORGE-X / ASD-001

Integrated Evidence, Experimental Validation, Mechanistic, Pharmacokinetic, Statistical, and Reproducibility Framework for Plant-Derived Molecules in Autism Spectrum Disorder Research

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Abstract

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental condition involving multiple biological and behavioral dimensions. This protocol establishes a rigorous preclinical framework for evaluating plant-derived molecules without assuming that any candidate is an effective treatment. PHYTOFORGE-X/ASD-001 integrates systematic evidence synthesis, chemical identity verification, exposure and blood–brain barrier (BBB) assessment, behavioral phenotyping, molecular biology, mechanistic perturbation, statistical rigor, replication, and provenance tracking. A broad candidate universe is subjected to a transparent 100→20→10→5 down-selection process. The provisional five-candidate experimental portfolio consists of sulforaphane (SFN), curcumin (CUR), resveratrol (RSV), apigenin (API), and naringin (NAR). These candidates are not considered clinically validated therapies. Each remains subject to predefined experimental gates. The framework explicitly distinguishes ASD core phenotypes from associated or comorbid outcomes, separates parent compounds from metabolites and conjugates, treats molecular docking and network pharmacology as hypothesis generation rather than causal evidence, and requires exposure plausibility before mechanistic claims are advanced. No combination experiment is permitted to proceed until the corresponding single-agent evidence satisfies predefined validation criteria. All animal experiments are designed around ARRIVE 2.0 principles, including defined experimental units, a priori sample-size justification, randomization, blinding, prespecified outcomes, transparent exclusions, and complete reporting. ARRIVE identifies these elements as essential for interpreting the reliability and reproducibility of animal research [1, 2, 3, 4].

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1 Scope and Scientific Position

1.1 Purpose

The purpose of PHYTOFORGE-X/ASD-001 is to determine whether selected plant-derived molecules produce reproducible, biologically interpretable, exposure-plausible effects in validated preclinical models relevant to specific ASD-associated phenotypes. The framework is deliberately constructed to prevent the following logical errors:

1. interpreting antioxidant activity as proof of ASD efficacy;
2. interpreting docking as evidence of target engagement;
3. interpreting target engagement as proof of causal mechanism;
4. interpreting behavioral changes without motor or anxiety controls;
5. transferring evidence from one flavonoid to another;
6. treating naringin and naringenin as interchangeable;
7. treating curcumin formulations as equivalent to free curcumin;
8. treating parent-compound exposure as equivalent to metabolite exposure;
9. treating within-group change as evidence of treatment efficacy;
10. treating a single animal model as sufficient translational evidence;
11. treating statistical significance as synonymous with biological importance;
12. treating absence of evidence as evidence of safety;
13. combining agents before single-agent validation;
14. converting preclinical observations into clinical recommendations.

1.2 Research Boundary

This document is a preclinical research protocol and evidence framework. It is not a clinical treatment protocol and does not provide human dosing, self-medication instructions, or treatment recommendations. The objective is not to “cure autism.” The objective is to determine, using falsifiable experiments, whether defined molecular interventions produce reproducible effects on defined biological or behavioral endpoints.

1.3 Scientific Principle

The central PHYTOFORGE-X principle is:

No claim without traceable evidence; no result without traceable data; no mechanism without causal testing; no advancement without predefined gates.

2 Conceptual Model of ASD-Relevant Biology

The framework treats ASD-related biology as multifactorial rather than as a single pathway disorder. Potential biological domains include:

- neuroinflammation;
- microglial activation;
- oxidative and nitrosative stress;
- mitochondrial dysfunction;
- synaptic plasticity;
- excitatory/inhibitory balance;
- GABAergic signaling;
- glutamatergic signaling;
- serotonergic signaling;
- dopaminergic signaling;
- PI3K–AKT–mTOR signaling;
- MAPK signaling;
- NF- κ B signaling;
- Nrf2-dependent antioxidant responses;
- SIRT1-associated signaling;
- epigenetic regulation;
- neurogenesis;
- endocannabinoid signaling;
- gut–brain interactions;
- intestinal barrier function;
- microbial metabolites.

No pathway is presumed to be causally responsible for ASD as a whole.

3 Phenotype Hierarchy

A critical distinction is made between ASD core phenotypes and associated or comorbid outcomes.

3.1 Core Phenotype Domains

1. social interaction;
2. social communication;
3. restricted/repetitive behavior.

3.2 Associated or Comorbid Domains

These include:

- irritability;
- hyperactivity;
- anxiety-like behavior;
- sleep;
- gastrointestinal outcomes;
- seizures;
- sensory abnormalities;
- attention;
- locomotor activity;
- stereotypy;
- compulsive-like behavior.

Improvement in an associated outcome must not automatically be described as improvement in a core ASD phenotype.

4 PHYTOFORGE-X Candidate Universe

The initial candidate universe is approximately 100 plant-derived molecules. Candidate classes include:

- isothiocyanates;
- curcuminoids;
- stilbenes;
- catechins;
- flavonols;
- flavones;

- flavanones;
- anthocyanins;
- phenolic acids;
- terpenoids;
- alkaloids;
- ginsenosides;
- soy isoflavones;
- saffron-derived compounds;
- other defined phytochemicals with mechanistic relevance.

The selection process is:

$$100 \rightarrow 20 \rightarrow 10 \rightarrow 5.$$

Each reduction must have a documented evidence trail.

5 Provisional Five-Candidate Portfolio

Table 1: Provisional experimental portfolio

Candidate	Abbreviation	Chemical class	Principal research rationale
Sulforaphane	SFN	Isothiocyanate	Strongest current human ASD evidence; Nrf2/redox/inflammatory hypothesis
Curcumin	CUR	Curcuminoid	Inflammation/redox/mitochondrial hypothesis; major exposure/formulation issue
Resveratrol	RSV	Stilbene	SIRT1/mitochondrial/redox hypothesis with human adjunctive evidence
Apigenin	API	Flavone	α 7-nicotinic acetylcholine receptor and oxidative-stress hypothesis
Naringin	NAR	Flavanone glycoside	CB1/neurogenesis hypothesis; parent/metabolite distinction required

These five candidates constitute a research portfolio, not a ranking of clinical value. Luteolin and ginsenoside Rg1 remain important replication/comparator candidates and may be reintroduced following evidence updates.

6 Candidate Chemical Identity

6.1 Sulforaphane

- Abbreviation: SFN
- Class: isothiocyanate
- Plant sources: Brassicaceae, particularly broccoli-derived sources
- Identity must be confirmed analytically for each experimental batch.

6.2 Curcumin

- PubChem CID: 969516
- CAS: 458-37-7
- Molecular formula: $C_{21}H_{20}O_6$
- Molecular weight: approximately 368.4 g/mol
- Class: curcuminoid
- Parent/conjugate distinction is mandatory.

6.3 Resveratrol

- PubChem CID: 445154
- CAS: 501-36-0
- Molecular formula: $C_{14}H_{12}O_3$
- Molecular weight: approximately 228.24 g/mol
- Experimental material should specify stereochemical/isomeric identity.

6.4 Apigenin

- PubChem CID: 5280443
- CAS: 520-36-5
- Molecular formula: $C_{15}H_{10}O_5$
- Molecular weight: approximately 270.24 g/mol

6.5 Naringin

- PubChem CID: 442428
- CAS: 10236-47-2
- Molecular formula: $C_{27}H_{32}O_{14}$
- Molecular weight: approximately 580.5 g/mol
- Naringin must not be conflated with naringenin.

7 Evidence Vector

Each candidate is represented by:

$$\mathbf{E}_i = (L_i, R_i, M_i, X_i, P_i, S_i, B_i)$$

where:

L evidence level;

R replication;

M mechanistic support;

X exposure plausibility;

P phenotype relevance;

S statistical rigor;

B bias risk.

A provisional evidence score may be represented as:

$$E_i = w_L L_i + w_M M_i + w_R R_i + w_T X_i + w_P P_i + w_S S_i - w_B B_i.$$

Weights must be declared before confirmatory ranking. A single composite score must not replace the underlying evidence vector.

8 Evidence Levels

Table 2: Evidence hierarchy

Level	Interpretation
E0	Chemical plausibility only
E1	In vitro or computational association
E2	Single preclinical experiment
E3	Replicated preclinical evidence
E4	Multiple models/species with mechanistic support
E5	Human observational or uncontrolled evidence
E6	Controlled human evidence
E7	Replicated controlled human evidence

Evidence level does not automatically imply clinical efficacy.

9 Sulforaphane Evidence Audit

Sulforaphane currently has the strongest human evidence among the provisional portfolio. A 2025 meta-analysis reported six randomized controlled trials and pooled improvements in several ASD-related measures, including total symptoms, aberrant behavior, hyperactivity, social interaction, social communication, and restricted/repetitive behavior. However, individual trials have produced mixed results. A randomized trial by Zimmerman et al. included 57 randomized children, with 45 analyzed, and did not demonstrate a significant primary outcome for general autism severity at the prespecified time points. A later randomized study likewise reported improvement in both groups without significant between-group changes on several caregiver-rated measures. An adjunctive risperidone trial reported greater improvement in irritability and selected secondary outcomes when SFN was combined with risperidone. These results establish a human research signal but do not establish monotherapy efficacy.

9.1 SFN Mechanistic Hypothesis

SFN → Nrf2 → antioxidant/redox response → neuroinflammation/mitochondria → neural function → phenoty

The causal chain must be experimentally tested rather than assumed.

9.2 SFN Decisive Experiment

A decisive experiment should test:

1. exposure;
2. target engagement;
3. Nrf2 perturbation;

4. oxidative/redox endpoints;
5. inflammatory endpoints;
6. neural endpoints;
7. behavioral phenotype;
8. both sexes;
9. at least two relevant models;
10. independent replication.

10 Curcumin Evidence Audit

A 2026 randomized double-blind placebo-controlled study reported large changes in inflammatory and gastrointestinal outcomes following a defined curcumin intervention. The reported outcomes included hs-CRP, ESR, fecal calprotectin and an instrument related to gastrointestinal dysfunction. These findings require aggressive replication and formulation/exposure verification. The study should not be interpreted as proof that curcumin improves the canonical ASD core phenotype.

10.1 Curcumin Exposure Problem

Curcumin is characterized by limited oral bioavailability and extensive metabolism. Therefore:

free curcumin $\neq$ curcumin glucuronide $\neq$ curcumin sulfate $\neq$ formulation – enhanced exposure.

Any experiment must explicitly identify which chemical species are being measured.

10.2 CUR Mechanistic Hypothesis

CUR $\rightarrow$ Nrf2/NF- κ B $\rightarrow$ redox/inflammation $\rightarrow$ mitochondrial/neural function $\rightarrow$ phenotype.

11 Resveratrol Evidence Audit

A randomized double-blind human study involving resveratrol as an adjunct to risperidone did not demonstrate a significant primary irritability endpoint, although a secondary hyperactivity/noncompliance measure differed between groups. This is adjunctive evidence rather than monotherapy evidence. A small open-label pilot also reported changes in some behavioral measures, but its design limits causal inference.

11.1 RSV Exposure

Resveratrol undergoes extensive metabolism, including glucuronidation and sulfation. Therefore:

$$C_{\text{brain,parent}} \neq C_{\text{brain,total}}$$

and parent compound and metabolite measurements must be distinguished.

11.2 RSV Mechanistic Hypothesis

$\text{RSV} \rightarrow \text{SIRT1} \rightarrow \text{mitochondrial/redox regulation} \rightarrow \text{neural function} \rightarrow \text{behavior}$.

A causal SIRT1 perturbation experiment is required.

12 Apigenin Evidence Audit

A 2024 preclinical study investigated apigenin in BTBR mice and reported changes in repetitive/compulsive-like behaviors together with oxidative stress-related findings. Electrophysiological experiments provided an $\alpha 7$ -nicotinic acetylcholine receptor hypothesis. However, the study used small experimental groups and a principal model. No convincing controlled human ASD efficacy evidence is currently established for apigenin.

12.1 API Decisive Experiment

The experiment should test:

$\text{API} \rightarrow \alpha 7\text{-nAChR} \rightarrow \text{synaptic/inhibitory signaling} \rightarrow \text{behavior}$.

Required components include:

- receptor perturbation;
- second ASD-relevant model;
- social phenotype;
- repetitive phenotype;
- locomotor control;
- sex-stratified analysis;
- CNS exposure measurement.

13 Naringin Evidence Audit

A 2025 preclinical study reported changes in autistic-like behaviors in BTBR mice together with hippocampal neurogenesis and transcriptomic changes. The authors implicated CB1 signaling. The study remains preclinical and requires independent replication.

13.1 Naringin versus Naringenin

This distinction is mandatory:

$\text{Naringin} \neq \text{Naringenin}$.

Naringin is a glycoside, whereas naringenin is the corresponding aglycone. Evidence concerning naringenin cannot automatically be credited to naringin.

13.2 NAR Decisive Experiment

The causal chain is:

$$\text{NAR} \rightarrow \text{CB1} \rightarrow \text{neurogenesis} \rightarrow \text{neural function} \rightarrow \text{behavior}.$$

The experiment must distinguish:

- parent naringin;
- naringenin;
- conjugated metabolites;
- brain exposure;
- CB1 perturbation;
- neurogenesis perturbation.

14 Cross-Candidate Red-Team Analysis

The five candidates are not mechanistically independent. A major shared domain is:

$$\text{oxidative stress} + \text{inflammation} + \text{mitochondrial dysfunction}.$$

This creates a redundancy problem. If all five candidates produce similar downstream changes, the apparent “multi-pathway” coverage may be substantially less diverse than expected. Therefore candidate selection must consider mechanistic redundancy. Define:

$$P_R = \sum_j \max(0, N_j - N_{\text{target},j}).$$

A high redundancy penalty reduces portfolio diversity.

15 Mechanistic Coverage

For mechanism j :

$$C_j = 1 - \prod_i (1 - M_{ij}),$$

where M_{ij} represents the degree of validated mechanistic coverage provided by candidate i . Importantly, M_{ij} must not be assigned solely from computational predictions. Target engagement requires experimental evidence. Causal mechanism requires perturbation. Replicated causal mechanism requires independent replication.

16 Experimental Architecture

The experimental program proceeds through sequential gates.

1. chemical identity;
2. batch quality;
3. concentration/exposure;
4. cytotoxicity and general toxicity;
5. target engagement;
6. molecular phenotype;
7. cellular phenotype;
8. behavioral phenotype;
9. causal perturbation;
10. second-model replication;
11. independent replication;
12. portfolio decision.

A failure at an upstream hard gate prevents downstream interpretation.

17 Chemical Quality Control

Each experimental batch requires:

- compound identity;
- purity;
- lot number;
- supplier;
- analytical method;
- formulation;
- storage condition;
- preparation date;
- stability information;
- degradation assessment where relevant.

No experimental conclusion may be based on an unidentified or inadequately characterized material.

18 Exposure and BBB Framework

The critical question is not simply whether a molecule has biological activity in vitro. The question is whether the experimentally relevant concentration can be achieved at the biological site of interest. The conventional brain/plasma ratio is:

$$K_{p,\text{brain}} = \frac{C_{\text{brain}}}{C_{\text{plasma}}}.$$

Where possible, unbound concentrations should be considered:

$$K_{p,uu} = \frac{C_{\text{brain,unbound}}}{C_{\text{plasma,unbound}}}.$$

An exposure margin may be expressed as:

$$EM = \frac{C_{\text{achievable,unbound}}}{EC_{50}}.$$

Interpretation requires caution because EC_{50} values can depend strongly on assay conditions and molecular species.

19 Parent Compound and Metabolite Accounting

For candidates with extensive metabolism, every experiment should identify whether measured activity is attributable to:

1. parent molecule;
2. active metabolite;
3. inactive metabolite;
4. glucuronide;
5. sulfate;
6. degradation product;
7. formulation-derived species.

Failure to distinguish these species produces a mechanistic attribution error.

20 ASD Behavioral Assay Matrix

Table 3: Core behavioral domains

Domain	Required considerations
Social interaction	Direct measurement of social preference or interaction
Social communication	Species-appropriate communication endpoints
Repetitive behavior	Repetitive/stereotyped behavioral measures
Locomotion	Required control for activity-related confounding
Anxiety-like behavior	Needed to distinguish anxiety effects from social effects
Motor function	Needed to identify motor impairment or stimulation
Sensory phenotypes	Included where biologically justified
Seizure susceptibility	Included for candidates/models with relevant hypothesis

Behavioral effects cannot be interpreted as ASD-specific without appropriate controls.

21 Molecular Assay Matrix

The molecular panel may include:

- Nrf2;
- HO-1;
- NQO1;
- NF- κ B;
- TNF- α ;
- IL-6;
- IL-1 β ;
- microglial activation markers;
- astroglial markers;
- mitochondrial respiration;
- ATP;
- ROS;
- lipid peroxidation;
- GSH/GSSG;
- SOD;

- catalase;
- synaptic proteins;
- GABAergic markers;
- glutamatergic markers;
- relevant pathway-specific targets.

Only prespecified assays should be treated as confirmatory.

22 Sex as a Biological Variable

Sex must not be treated merely as a demographic covariate. A treatment-by-sex interaction may be represented as:

$$Y = \beta_0 + \beta_1 T + \beta_2 S + \beta_3 (T \times S) + \epsilon.$$

The interaction term β_3 should be examined where the design has adequate power. If the experiment cannot resolve sex-specific effects, this limitation must be reported rather than assumed away.

23 Randomization

Randomization must be computer-generated or otherwise formally documented. Appropriate approaches include:

- blocked randomization;
- stratified randomization;
- randomized complete blocks;
- matched-pair allocation where appropriate.

Blocking variables may include:

- litter;
- cage;
- experimental day;
- body-weight class;
- sex;
- batch.

ARRIVE 2.0 specifically recommends reporting how randomization was generated and what was randomized [3].

24 Blinding

Blinding should be maintained wherever scientifically and operationally possible. Relevant stages include:

1. allocation;
2. treatment administration;
3. behavioral assessment;
4. molecular assays;
5. data cleaning;
6. statistical analysis.

Behavioral assessment should be blinded whenever possible because subjective assessment can introduce bias [4].

25 Sample Size

Sample size must be justified a priori for confirmatory experiments. The protocol must report:

- exact experimental-unit n per group;
- total experimental units;
- total animals;
- anticipated attrition;
- primary endpoint;
- assumed effect size;
- variance estimate;
- desired power;
- alpha;
- multiplicity strategy.

ARRIVE explicitly requires reporting exact experimental-unit counts and the basis for sample-size decisions [2]. For pilot or feasibility experiments, sample size may instead be justified by operational objectives, but pilot data should not automatically be used as if they were definitive estimates of treatment effect.

26 Experimental Unit

The experimental unit is the entity independently exposed to the intervention. It may be:

- individual animal;
- litter;
- cage;
- another independently treated unit.

Subsamples from the same experimental unit must not automatically be treated as independent observations. This prevents pseudoreplication.

27 Inclusion and Exclusion Criteria

Criteria must be specified before analysis. Every exclusion requires:

- experimental-unit identifier;
- predefined or post hoc status;
- reason;
- decision-maker;
- effect on primary analysis;
- final analysis n .

All losses must remain visible in the provenance record. ARRIVE recommends exact reporting of n for every analysis and transparent accounting of exclusions [5].

28 Primary Endpoint

Each confirmatory experiment must have one prespecified primary endpoint unless a justified hierarchical endpoint strategy is registered. The primary endpoint determines:

- sample-size calculation;
- primary hypothesis;
- primary statistical model;
- confirmatory interpretation.

Secondary and exploratory outcomes must not be silently promoted to primary status after data inspection.

29 Statistical Analysis Plan

The default analysis hierarchy is:

1. inspect data integrity;
2. verify experimental-unit structure;
3. assess missingness;
4. inspect distributions;
5. evaluate model assumptions;
6. conduct prespecified primary analysis;
7. conduct multiplicity-controlled secondary analysis;
8. conduct exploratory analyses separately;
9. report effect sizes and confidence intervals;
10. perform sensitivity analyses.

29.1 Effect Size

Statistical significance alone is insufficient. Results should report:

effect size + 95% confidence interval + p -value + n + variability.

30 Failure Gates

The following failure codes are defined.

Code	Failure	Interpretation
F01	Identity	Chemical identity unresolved
F02	Purity/stability	Material unsuitable or unstable
F03	No concentration response	No interpretable exposure-response relationship
F04	Cytotoxicity	Apparent efficacy confounded by toxicity
F05	No phenotype	Prespecified phenotype not demonstrated
F06	Motor confound	Behavioral result explained by locomotor effects
F07	Target not engaged	Proposed target not experimentally engaged
F08	Perturbation failure	Causal perturbation does not support mechanism
F09	Inadequate exposure	Relevant tissue exposure not demonstrated
F10	Parent/metabolite ambiguity	Active species unresolved
F11	Sex interaction unresolved	Important sex-specific effect cannot be interpreted
F12	Model-specific	Effect limited to one model

F13	Replication failure	Same-laboratory replication fails
F14	Independent-lab failure	Independent replication fails

31 Replication Ladder

Table 5: Replication ladder

Level	Requirement
R0	Initial experiment
R1	Same-laboratory replication
R2	Independent experiment with meaningful protocol confirmation
R3	Independent laboratory replication
R4	Translational validation pathway

A candidate should not be described as robustly validated solely because an R0 experiment is positive.

32 Decision States

Each candidate receives one of four states:

GO Evidence satisfies the predefined advancement gate.

HOLD Evidence is promising or informative but incomplete.

STOP A predefined failure criterion has been reached.

REDESIGN The experiment is uninterpretable and requires methodological modification.

The decision must reference:

- evidence snapshot;
- experimental data snapshot;
- replication level;
- exposure status;
- mechanism status;
- phenotype status;
- failure status.

33 Combination Experiments

Combination experiments are prohibited until single-agent validation requirements are satisfied. Mechanistic complementarity is not equivalent to pharmacological synergy.

33.1 Bliss Independence

For fractional effects E_A and E_B :

$$E_{AB}^{Bliss} = E_A + E_B - E_A E_B.$$

The observed synergy deviation is:

$$\Delta_{Bliss} = E_{AB}^{obs} - E_{AB}^{Bliss}.$$

33.2 Highest Single Agent

$$E_{HSA} = \max(E_A, E_B)$$

and:

$$\Delta_{HSA} = E_{AB}^{obs} - E_{HSA}.$$

33.3 Loewe Additivity

For two agents:

$$\frac{d_A}{D_A} + \frac{d_B}{D_B} = 1.$$

The appropriate model depends on the available concentration-response data. A single favorable combination result is insufficient to establish synergy.

34 Multi-Agent Optimization

For a candidate combination vector $\mathbf{x}$:

$$\max_{\mathbf{x}} [E(\mathbf{x}) - \lambda_T T(\mathbf{x}) - \lambda_I I(\mathbf{x}) + \lambda_S Synergy(\mathbf{x})].$$

Here:

- E = biological efficacy;
- T = toxicity burden;
- I = interaction burden;
- $Synergy$ = experimentally demonstrated synergy.

No synergy term should be credited solely because two compounds affect different pathways.

35 Safety and Toxicology Framework

The safety score is conceptualized as:

$$S_i = S_{\text{tox}} + S_{\text{interaction}} + S_{\text{PK}} + S_{\text{organ}} + S_{\text{cardiac}} + S_{\text{disease-specific}}.$$

“Natural” is not equivalent to safe. Unknown safety must be coded as unknown rather than safe. Relevant concerns include:

- cytotoxicity;
- hepatotoxicity;
- nephrotoxicity;
- cardiovascular effects;
- seizure threshold;
- developmental effects;
- endocrine activity;
- CYP interactions;
- P-glycoprotein;
- UGT metabolism;
- formulation-specific toxicity.

36 Evidence for Each Candidate

36.1 SFN

Human randomized evidence exists but is heterogeneous. The 2025 meta-analysis reported pooled improvements in several ASD domains, while individual randomized studies have produced null or mixed primary outcomes. Current status:

ADVANCE – high-priority validation

Required evidence:

- exposure;
- target engagement;
- Nrf2 causality;
- multiple models;
- both sexes;
- independent replication.

36.2 CUR

A recent randomized study reported substantial inflammatory/GI biomarker changes, but these findings require replication, formulation verification, and clearer linkage to ASD core phenotypes. Current status:

ADVANCE – replication/exposure gate

36.3 RSV

Human adjunctive evidence exists, but primary endpoints have not established monotherapy efficacy. Current status:

ADVANCE – mechanistic comparator

36.4 API

Current evidence is primarily preclinical. Current status:

ADVANCE – independent preclinical replication

36.5 NAR

Current evidence is primarily preclinical and based on a BTBR model. Current status:

ADVANCE – replication and parent/metabolite gate

37 Provenance Graph

Every scientific claim must be represented as a provenance chain:

$$Plant \rightarrow Molecule \rightarrow Batch \rightarrow Study \rightarrow Model \rightarrow Endpoint \rightarrow Mechanism \rightarrow Evidence \rightarrow Candidate \rightarrow 1$$

A claim is invalid if any essential link cannot be traced.

38 Research Database Architecture

The canonical database contains the following entities:

1. STUDY
2. COMPOUND
3. MODEL
4. BATCH
5. EXPERIMENT

6. ANIMAL
7. EXPERIMENTAL_UNIT
8. RANDOMIZATION
9. BLINDING
10. TREATMENT
11. ENDPOINT
12. BEHAVIOR
13. SAMPLE
14. ASSAY
15. PK_MEASUREMENT
16. MECHANISM
17. PERTURBATION
18. ANALYSIS
19. RESULT
20. FAILURE
21. REPLICATION
22. LITERATURE_EVIDENCE
23. DECISION

The database must preserve separation between:

raw data → validated data → derived data → statistical analysis → decision.

39 Data Integrity Rules

The following invariants apply:

1. Every animal belongs to a defined experiment.
2. Every experimental unit has an explicit unit type.
3. Every treatment references a compound or control condition.
4. Every assay references a sample.
5. Every sample references an experimental unit through its animal.

6. Every result references an analysis.
7. Every analysis references a dataset version.
8. Every decision references an evidence snapshot.
9. Every failure references an experiment.
10. Every literature claim references a verifiable source identifier.

40 Literature Evidence Registry

The literature registry must record:

- PMID;
- DOI;
- publication type;
- model;
- species;
- sample size;
- endpoint;
- effect;
- exposure;
- limitations;
- replication status;
- verification date.

No DOI or PMID should be entered without verification.

41 Search Strategy

The literature search should include at minimum:

- PubMed/MEDLINE;
- Crossref;
- Europe PMC;
- relevant toxicology databases;
- pharmacokinetic databases;

- systematic-review databases where available.

Example query structure:

(autism OR "autism spectrum disorder" OR ASD)

AND

(sulforaphane OR curcumin OR resveratrol OR apigenin OR naringin)

Additional queries should address:

- animal models;
- social behavior;
- repetitive behavior;
- neuroinflammation;
- microglia;
- oxidative stress;
- mitochondria;
- BBB;
- pharmacokinetics;
- metabolites;
- toxicity;
- null results;
- adverse effects;
- failed replication.

The search log should record:

- database;
- date;
- exact query;
- filters;
- inclusion criteria;
- exclusion criteria;
- number retrieved;
- number screened;
- number included.

42 Publication Bias

Positive findings are more likely to attract publication and attention than null findings. Therefore the evidence audit must actively search for:

- registered but unpublished trials;
- conference abstracts;
- negative studies;
- failed replication;
- protocol deviations;
- adverse-event reports;
- contradictory findings.

Publication bias must be treated as an uncertainty source.

43 Bayesian Updating

Where sufficient independent evidence exists, Bayesian updating may be used. Conceptually:

$$P(H | D) = \frac{P(D | H)P(H)}{P(D)}.$$

However, apparent independent studies may share:

- investigators;
- laboratories;
- datasets;
- animal sources;
- protocols;
- analytical pipelines;
- publication bias.

Therefore evidence cannot automatically be treated as statistically independent.

44 Sensitivity Analysis

Candidate selection should be tested against alternative reasonable weights. Let:

$$R_i = w_E E_i + w_M B_i + w_{Me} Me_i + w_S Sel_i + w_{PK} PK_i + w_{Sy} Sy_i - w_T T_i - w_I I_i - w_U U_i.$$

Where:

- E = evidence;
- B = biological relevance;
- Me = mechanistic support;
- Sel = phenotype selectivity;
- PK = exposure plausibility;
- Sy = validated synergy;
- T = toxicity;
- I = interaction burden;
- U = uncertainty.

The ranking should be considered robust only if conclusions remain stable across reasonable weighting scenarios.

45 Pareto Analysis

Rather than forcing all objectives into one score, candidates may be represented on a multi-objective Pareto frontier. Objectives include:

- phenotype relevance;
- evidence strength;
- replication;
- exposure;
- mechanistic support;
- safety;
- uncertainty;
- translational plausibility.

A candidate is Pareto-dominated if another candidate is at least as good on all defined objectives and better on at least one. No single “best” candidate should be declared solely from a composite score.

46 Red-Team Falsification Program

Every positive finding must be challenged. Required questions include:

1. Could the result be caused by sedation?
2. Could it be caused by hyperactivity?
3. Could it be caused by motor impairment?
4. Could it be caused by altered anxiety?
5. Could it reflect general sickness?
6. Could the assay be sensitive to experimenter bias?
7. Could the result be batch-specific?
8. Could the result be model-specific?
9. Could the active species be a metabolite?
10. Could the exposure be pharmacologically unrealistic?
11. Could the molecular assay be nonspecific?
12. Does target perturbation eliminate the effect?
13. Does an independent experiment reproduce the finding?
14. Does an independent laboratory reproduce the finding?

A positive experiment that survives these challenges becomes substantially more informative.

47 Replication Criteria

Replication must preserve the essential causal structure of the original experiment while avoiding unnecessary dependence on identical experimental noise. Replication should examine:

- independent batch;
- independent animal cohort;
- blinded assessment;
- prespecified analysis;
- same primary endpoint;
- second model where justified;
- independent laboratory at later stage.

48 Combination Gate

No combination experiment should proceed unless each constituent candidate has independently satisfied:

$$\boxed{Identity \wedge Exposure \wedge Phenotype \wedge Mechanism \wedge Replication}$$

Only then should combinations be investigated.

49 ARRIVE 2.0 Compliance

The protocol is structured around ARRIVE 2.0. The ARRIVE Essential 10 include:

1. study design;
2. sample size;
3. inclusion/exclusion criteria;
4. randomization;
5. blinding;
6. outcome measures;
7. statistical methods;
8. experimental animals;
9. experimental procedures;
10. results.

ARRIVE also recommends information covering background, objectives, ethical statement, housing, animal care, interpretation, generalisability, protocol registration, data access, and conflicts of interest [1]. The official ARRIVE guidance states that its planning resources are intended to improve experimental design, minimize bias, support appropriate sample sizes, and improve statistical analysis [6].

50 Ethical Requirements

Before any live-animal experiment:

- institutional animal-care approval must be obtained;
- applicable Canadian and institutional requirements must be satisfied;
- humane endpoints must be defined;
- distress monitoring must be specified;
- refinement and reduction principles must be applied;

- personnel must be appropriately trained.

This document does not substitute for institutional ethical approval.

51 Go/Hold/Stop Decision Matrix

Table 6: Decision matrix

Decision	Minimum interpretation
GO	Required gates satisfied and evidence supports next-stage validation
HOLD	Signal exists but one or more critical evidence domains remain unresolved
STOP	Predefined failure criterion is met
REDESIGN	Data cannot answer the intended question because of methodological limitations

52 Current Portfolio Status

Table 7: Current research status

Candidate	Status	Principal unresolved issue
SFN	ADVANCE	Mixed human outcomes; mechanism/exposure/replication
CUR	ADVANCE	Formulation, exposure, replication, phenotype specificity
RSV	ADVANCE	Monotherapy evidence and causal SIRT1 mechanism
API	ADVANCE	Independent replication and CNS exposure
NAR	ADVANCE	Parent/metabolite distinction and independent replication

These statuses mean “advance for further validation,” not “proven effective.”

53 Minimum Experimental Dataset

Every confirmatory experiment should produce:

1. chemical QC record;
2. batch identifier;
3. animal registry;
4. experimental-unit registry;
5. randomization record;
6. blinding record;
7. treatment record;

8. raw behavioral data;
9. raw molecular data;
10. PK data where applicable;
11. assay QC;
12. predefined statistical analysis;
13. effect sizes and confidence intervals;
14. exclusion log;
15. protocol-deviation log;
16. failure log;
17. replication record;
18. final decision record.

54 Data Release Architecture

The project should maintain:

```
PHYTOFORGE-X-ASD-001/  
|  
+-- DATABASE/  
|   +-- asd001.sqlite  
|   +-- schema.sql  
|   +-- seed.sql  
|  
+-- CSV/  
|   +-- study.csv  
|   +-- compound.csv  
|   +-- experiment.csv  
|   +-- animal.csv  
|   +-- behavior.csv  
|   +-- sample.csv  
|   +-- assay.csv  
|   +-- pk_measurement.csv  
|   +-- mechanism.csv  
|   +-- result.csv  
|   +-- failure.csv  
|   +-- replication.csv  
|   +-- decision.csv  
|
```

```
+-- PROTOCOL/
|   +-- preregistration.tex
|   +-- statistical_analysis_plan.tex
|
+-- LITERATURE/
|   +-- search_log.csv
|   +-- evidence_registry.csv
|
+-- ANALYSIS/
|   +-- raw/
|   +-- validated/
|   +-- derived/
|   +-- figures/
|
+-- QC/
|   +-- validation_report.txt
|
+-- RELEASE/
|   +-- SHA256SUMS.txt
```

55 No-Fabrication Rule

This project must never manufacture:

- experimental observations;
- sample sizes;
- p-values;
- effect sizes;
- animal counts;
- pharmacokinetic measurements;
- target-engagement measurements;
- replication results;
- safety conclusions.

When data do not exist, the database field must remain empty or explicitly marked as unavailable.

56 Interpretation Rules

The following hierarchy must be maintained:

association < replication < target engagement < causal mechanism < multi-model replication < independent replication

Likewise:

in vitro activity $\neq$ animal efficacy $\neq$ human efficacy.

And:

statistical significance $\neq$ biological significance $\neq$ clinical significance.

57 Final Research Decision Framework

For candidate i , advancement requires:

$$G_i = I_i \wedge Q_i \wedge X_i \wedge P_i \wedge M_i \wedge R_i \wedge S_i,$$

where:

I identity verified;

Q quality verified;

X exposure plausible;

P phenotype demonstrated;

M mechanism supported;

R replication achieved;

S safety/interpretability requirements satisfied.

A candidate failing a hard gate cannot receive a “GO” decision merely because other domains score highly.

58 Conclusions

PHYTOFORGE-X/ASD-001 provides a structured framework for investigating plant-derived molecules in ASD-relevant preclinical research. The provisional portfolio of SFN, CUR, RSV, API, and NAR is a research portfolio rather than a ranking of therapies. The principal methodological objective is to replace loosely connected claims about phytochemicals with traceable causal experiments. The framework therefore requires:

Chemical Identity $\rightarrow$ *Exposure* $\rightarrow$ *Target Engagement* $\rightarrow$ *Molecular Effect* $\rightarrow$ *Neural Effect* $\rightarrow$ *Phenotype*

Only findings that survive this sequence should be considered sufficiently robust for subsequent translational investigation.

Research Integrity Statement

This protocol is designed to distinguish hypothesis generation from demonstrated evidence. Computational predictions, molecular docking, network pharmacology, pathway enrichment, single-model behavioral findings, and uncontrolled observations are not independently sufficient to establish efficacy. All positive findings must remain falsifiable.

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