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BIOACTIVITY AND SAFETY OF HYDRO-ETHANOLIC LYOPHILISED WITHANIA SOMNIFERA FOR THE POTENTIAL MANAGEMENT OF THE BIOLOGICAL END POINTS OF STRESS

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ABSTRACT: Introduction & Objective: Chronic stress activates the HPA axis and the sympathetic nervous system, leading to inflammation and oxidative toxic stress (OTS). Conventional therapies face bottlenecks like high costs, limited accessibility, and side effects. This study evaluates the chemical safety and metabolomics of Zimbabwean *Withania somnifera*. **Methodology:** Dried *Withania somnifera* roots were extracted via 70%hydroethanolic maceration and lyophilized. Phenolic and flavonoid contents were mapped. Acute oral toxicity was evaluated in female Sprague-Dawley rats up to 5000" mg/kg" (OECD 425). *In-vitro* anti-inflammatory and antioxidant activities were quantified via egg albumin denaturation and DPPH assays. **Results:** A 12% yield of lyophilised extract was obtained from 258g of raw root powder. The metabolomics verified high-density withanolides, alkaloids, tannins, saponins, and polyphenols. Acute testing established an exceptionally wide safety threshold (LD₅₀>5000 mg/kg), with normal weight gain and zero anomalies. The extract demonstrated significant anti-inflammatory inhibition (80% inhibition at 1000 µg/mL compared to 83% diclofenac). A robust anti-oxidant profile showed mean IC₅₀=124.33 µg/mL compared to 20.5µg/mL pure ascorbic acid. The total phenolic content was calculated at 69mg gallic acid equivalents/g extract and the flavonoid content at 32.47mg quercetin equivalents per gram. **Conclusion:** Naturalized Zimbabwean *Withania somnifera* is a safe, metabolite-dense source phytochemicals which can be used for the alleviation of the biologic end points of stress.

INTRODUCTION: The formal concept of biological "stress" originated with Hans Selye in the 1930s, who characterized it as the body's non-specific physiological response to external demands^{1,2}.

Modern medical frameworks classify these physiological disruptions into acute, episodic acute, and chronic states based on duration and frequency of occurrence³.

An immediate threat rapidly triggers the immediate Sympathetic Adrenal Medullary (SAM) axis, signalling pre-autonomic circuits to stimulate chromaffin cells in the adrenal medulla to release systemic catecholamines, comprising roughly 80% epinephrine and 20% norepinephrine, to drive cardiac output, bronchodilation, and glucose

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mobilization for the fight-or-flight response⁴. If the environmental, psychological, or physical threat persists, the sustained Hypothalamic-Pituitary-Adrenal (HPA) axis is deployed^{4, 5}. The hypothalamic paraventricular nucleus secretes corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), which stimulate the anterior pituitary gland to cleave pro-opiomelanocortin (POMC) into adrenocorticotrophic hormone (ACTH). ACTH travels via venous circulation to bind MC2R receptors in the adrenal cortex's zona fasciculata, accelerating steroidogenesis to release cortisol. Under chronic stress, the continuous hypersecretion of cortisol induces widespread allostatic load, the cumulative

cellular wear and tear that manifests across physical, behavioral, and emotional domains^{3, 5}.

Crucially, this sustained elevation triggers glucocorticoid receptor resistance (GCR) across peripheral tissues⁶. The down-regulation of receptor sensitivity breaks normal negative feedback loops; this disables the receptor's capacity to cross-talk with and suppress the master transcription factor nuclear factor-kappa B (NF- κ B), directly causing uncontrolled systemic inflammation and driving pathologies like clinical depression, anxiety, type 2 diabetes, and severe cardiovascular atherosclerosis^{3, 6}.

***Withania somnifera*:**



FIG. 1: WITHANIA SOMNIFERA PLANT

The naturalized Zimbabwean *Withania somnifera* (Ashwagandha) is a metabolite-dense therapeutic plant classified under the *Solanaceae* (nightshade) family, whose evolutionary development as a vascular, seed-bearing flowering angiosperm enables the specialized biosynthesis of key adaptogenic compounds within its roots, foliage, and stems⁷.

Secondary Metabolites: The *Withania Somnifera* pharmacological efficacy against the biological endpoints of chronic stress is driven by a complex matrix of four secondary metabolite macro-classes: steroidal lactones, particularly withanolides like Withanolide A and Withaferin A, which suppress hypothalamic-pituitary-adrenal (HPA) axis cortisol

pathways and promote neurite outgrowth^{8, 9}; basic alkaloids such as tropine and anaferine that modulate central GABA receptors to provide anxiolytic sedation¹⁰; complex glycowithanolides (sitoindosides VII–X) that drive systemic macrophage mobilization; and a rich polyphenolic/flavonoid fraction, including quercetin and gallic acid, which acts as an immediate free-radical scavenger to suppress pro-inflammatory Nuclear Factor-kappa B (NF- κ B) cascades¹¹.

To optimize the recovery of both these lipophilic steroidal frameworks and polar polyphenols from the raw, insoluble lignocellulosic biomass, a solid-liquid thermodynamic extraction using a

moderately polar 70% hydroethanolic solvent mixture is utilized¹². This is followed by low-temperature vacuum lyophilization (freeze-drying) to protect the thermolabile structures via ice sublimation, successfully yielding a standardized, highly stable, and bioactive porous powder suited for integration into modern nano-delivery systems and functional dosage forms¹³.

Objectives of the Study:

Problem Statement: Conventional pharmacological agents for chronic stress management face significant clinical bottlenecks, including severe adverse side effects, high costs, and limited accessibility in resource-constrained regions. Although *Withania somnifera* is an established adaptogen, the biosafety, chemical profile, and toxicological thresholds of the specific naturalized Zimbabwean eco-species remain completely uncharacterized.

Research Questions:

1. What are the optimal extraction yield and parameters for isolating bioactive fractions from Zimbabwean *Withania somnifera* roots using hydro-ethanolic maceration
2. What is the qualitative and quantitative phytochemical composition (specifically total phenolic and flavonoid content) of this regional eco-species?
3. Does the lyophilized crude extract demonstrate significant *in-vitro* antioxidant and anti-inflammatory activity?
4. What is the acute oral toxicological profile (LD_{50}) of the extract in an *in-vivo* rodent model under OECD Guidelines?

Aim and Specific Objectives:

Main Aim: To evaluate the phytochemistry, *in vitro* bioactivity, and acute *in-vivo* safety of a naturalized Zimbabwean *Withania somnifera* root extract.

Specific Objectives:

1. **Phytochemical Profiling and Standardization:** To perform qualitative screening and quantitative determination of

total phenolic content (TPC) and total flavonoid content (TFC) in the lyophilized root extract to establish a chemical baseline for standardization.

2. ***In-vitro* Efficacy Evaluation:** To quantify the antioxidant capacity (via DPPH free-radical scavenging assay) and anti-inflammatory potential (via egg albumin protein denaturation inhibition) of the extract relative to standard reference drugs.
3. ***In-vivo* Safety Profiling:** To determine the acute oral toxicity threshold (LD_{50}) and evaluate preliminary clinical safety parameters of the crude extract in female Sprague-Dawley rats using a modified OECD Guideline 425 protocol.

MATERIALS AND METHODS:

Materials, Equipment and Facilities:

Animal use Approval: Prior to the investigations, animal use and research ethics approvals were obtained from the Joint Parirenyatwa Research Ethics Committee (JREC) which is the local research Institutional Review board for the University of Zimbabwe.

Plant Material Collection and Extraction

Processing: *Withania somnifera* (L.) Dunal roots were collected in Gweru, Zimbabwe in the month of November 2025 at the following coordinates,

<p>◦ Latitude: -19.5765 (19° 34' 35" S)</p> <p>◦ Longitude: 29.9880 (29° 59' 17" E)</p>

and formally authenticated at the National Herbarium and Botanical Gardens in Harare. The roots were washed, shade-dried for three weeks at maximum day temperatures of 32 °C and pulverized using a commercial coffee grinder (Hamilton Beach Model 80410). Biogenic extraction was executed by macerating 258 g of the powdered root matrix in 1000 mL of a 70% (v/v) hydroethanolic mixture for 3 days with continuous mechanical shaking (3 min, twice daily). The crude mixture underwent sequential filtration *via* muslin cloth and a vacuum pressure setup using Whatman No. 1 filter paper. The resulting filtrate was concentrated under reduced pressure (56 Pa) at a controlled temperature using a rotary evaporator

(Rotavapor R300, Buchi, Switzerland) to eliminate excess ethanol, followed by complete vacuum lyophilization to yield a highly stable dry powder.

Qualitative and Quantitative Phytochemical Screening: Standard colorimetric assays were performed on the root extract to identify major secondary metabolite fingerprints.

Detection for Alkaloids by the Wagner Test: To 2 mL of the extract, a few drops of Wagner's reagent (iodine in potassium iodide reagent) were added along the sides of a test tube. (Alemu *et al.*, 2024)¹⁴.

Detection of Tannins by the Ferric Chloride Test: To 3 mL of *Withania somnifera* extract, 4 drops of 10% ferric chloride solution were added (Kebede *et al.*, 2021). The presence of tannins was confirmed by conversion of the solution to a blue-green colour¹⁴.

Detection of Flavonoids by the Ammonia Test: To 2 mL of *Withania somnifera* extract solution, 2 mL of dilute ammonia solution was added, followed by a few drops of concentrated sulfuric acid. (Jaafar *et al.*, 2012). The emergence of a yellow colour indicates the presence of flavonoids¹⁴.

Detection of Glycosides by the Keller-Killani Test: To 2 mL of *Withania somnifera*, 1.5 mL of glacial acetic acid was added, followed by 1 drop of 5% ferric chloride and 2 drops of concentrated sulfuric acid (Brainly, 2025) along the side of test tube). The presence of glycosides was confirmed by the emergence of a blue coloured solution in mixture acetic acid layer¹⁵.

Detection of Phenolic Compounds by the Gelatin Test: Phenolic compounds were detected using the Gelatin test. In this assay, 2ml the lyophilised extract solution was added to 5ml of a 1% gelatin solution and 5 drops of a 10% NaCl were further added. Phenolics were identified by the appearance of a white precipitate¹⁶.

Detection of Saponins by the Simplified Foam Test: The simplified foam test was used to determine the presence of saponins. In this assay 2ml of the extract was added to 20ml distilled water. The mixture was shaken in a graduated

cylinder for 15 minutes. The presence of saponins would be confirmed by the formation of foam with a head height of at least 1cm^{17, 18, 19}.

Total Phenolic Content: To prepare a calibration curve, phenol (Gallic acid) stock solution (5 mg/mL) was added into 100mL volumetric flasks, and then diluted to volume with distilled water. From each calibration solution, 0.25 mL was mixed with 1.25 mL of a 10-fold-diluted Folin-Ciocalteu's phenol reagent (1 mL Folin reagent and 9 mL deionized water) and allowed to react for 5 minutes. Then, 2 mL of 7.5% Na₂CO₃ solution was added, and the final volume was made up to 5 mL with deionized water. After 1 hour of reaction at room temperature, the absorbance at 760 nm was measured spectrophotometrically (Lambda 35 UV/Vis Spectrometer, PerkinElmer Instruments). The test was done in triplicate. Calibration curves were plotted to determine the level of phenolics and tannins in the samples. The total phenolic content was calculated on the basis of the calibration curve of gallic acid and expressed as gallic acid equivalents (GAE), in milligrams per gram of the sample²⁰.

Total Flavonoid Content: Total Flavonoid Content (TFC) of the lyophilized *Withania somnifera* was estimated spectrophotometrically at 510 nm. In the test, 1mg of extract was dissolved in 2mL of distilled water. To this solution, 0.5 mL of 1 M sodium nitrite was added, along with 2 mL of a 1 M NaOH solution. Distilled water was then added to bring the volume to 10 mL. The solution was shaken, allowed to stand at room temperature for 15 min, and then the absorbance was measured. The total flavonoid content was estimated as mg of quercetin equivalent (mg QE/g extract) on a dry weight basis using the standard curve¹².

Detection of Sterols by Salkowski's Test: To 2 mL of extract, add 2ml of chloroform then add a few drops of concentrated H₂SO₄ (shake well and allow to stand¹⁴.

Detection of Terpenoids using the Salkowski Test: 2 mL chloroform + 2 mL plant extract (evaporated on a water bath) + 2 mL conc. H₂SO₄ (boiled on a water bath). A positive result for terpenoids shows a sharp, colored ring (ranging

from golden yellow to deep reddish-brown) right at the interface of the 2 liquids ¹⁴.

Qualitative Antibacterial Profiling: Qualitative antibacterial profiling was performed via the agar well diffusion method on nutrient agar plates inoculated with *Escherichia coli* and *Staphylococcus aureus* using calibrated extract concentrations (150, 250, and 500mg) against a positive doxycycline control.

Ethical In-vivo Acute Oral Toxicity Evaluation:

The acute oral toxicity evaluation was reviewed and approved by the Joint Research Ethic Committee JREC under the reference number JREC/470/2025 approved on the 12 November 2025. In vivo safety and acute oral lethality thresholds were established in strict compliance with a modified OECD Guideline 425 Up-and-Down Procedure ²⁰. Twelve healthy, nulliparous, female Sprague-Dawley rats (8–12 weeks old, 200–250 g) were individually housed and acclimatized for 5 days with free access to standard rodent chow and water. Following an overnight fast, the test group received sequential oral gavage dosing at concentrations of 50, 500, 1000, 2500, and 5000 mg/kg body weight at 48-hour intervals, while control animals received equivalent volumes of distilled water. Clinical signs of toxicity (tremors, salivation, diarrhea, or somnolence) were monitored continuously for the first 12 hours post-dose and daily for 14 days. Somatic changes were recorded via body weight measurements on days 1, 7, and 14. At day 14, all surviving animals were humanely euthanized by cervical dislocation according to AVMA guidelines.

In-vitro Anti-Inflammatory and Antioxidant Bioassays:

The anti-inflammatory performance of the extract was verified using the egg albumin denaturation protection assay. Serial dilutions of the extract and a positive control (diclofenac) were prepared (in 0.4% DMSO). The reaction matrix, comprising 0.4 mL fresh egg albumin, 0.5 mL test sample, and 3 mL phosphate-buffered saline, was incubated at 37°C for 20 min and then heated at 65°C for 30 min in a water bath to drive protein denaturation. Absorbance was recorded at 660 nm ²¹.

Equation 1:

$$\text{Inhibition \%} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}} \times 100$$

Where, $\text{Abs}_{\text{sample}}$ = absorbance of sample, $\text{Abs}_{\text{control}}$ = absorbance of control.

Concurrently, antioxidant capacity was measured using the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay. Aliquots of 100 of the plant extract were mixed with 0.1 mM methanolic stock solution, incubated in total darkness at 22°C for 30 min, and analyzed at 517 nm (Lambda 35, PerkinElmer) against an L-ascorbic acid standard reference curve to determine the half-maximal inhibitory concentration value ^{22, 23}.

Equation 2:

$$\text{DPPH Scavenging percentage effect} = (A^0 - A) / A^0 \times 100$$

Where A^0 is the absorbance of negative control (0.004% DPPH solution), and A is the absorbance in the presence of extract.

The results were reported as IC_{50} values and ascorbic acid equivalents (AAE, mg/g) of *W. somnifera*.

RESULTS AND DISCUSSION:

Harvest Drying Dynamics and Extraction Metabolomics:

32g of lyophilised extract was obtained, a 12% yield.

Final yield calculation ($\text{yield\%} = \text{weight of extract} / \text{weight of starting material} \times 100\%$)

$$= 32\text{g} / 258\text{g} \times 100\%$$

$$= 12\%$$

Shade drying preserved fragile secondary metabolites by shielding the harvested biomass from direct solar ultraviolet (UV) radiation and intense thermal stress, preventing the photo-oxidation of thermosensitive withanolides (e.g., Withaferin A) and volatile essential oils ²⁶. Dehydration kinetics showed that while open sun drying ²⁷ accelerates moisture removal (3–5 days), shade drying under controlled, well-ventilated ambient convection (7–14 days) completely prevented opportunistic mould development and non-enzymatic browning, preserving a high-quality light-beige root profile ^{28, 29}. Qualitative and quantitative evaluation of the 70% hydroethanolic

extract revealed a dense chemical profile of primary and secondary metabolites, characterized by a robust distribution of alkaloids, flavonoids, and saponins **Table 1**.

TABLE 1: PHYTOCHEMICAL SCREENING PROFILING OF WITHANIA SOMNIFERA HYDROETHANOLIC EXTRACT

Phytochemical Class	Screening Technique	Observational Sign / Color Shift	Relative Abundance
Alkaloids	Wagner test	Brown precipitate formation	+++
Flavonoids	Ammonia test	Distinct yellow coloration	+++
Saponins	Simplified foam test	Stable foam formation (6 cm)	+++
Phenolic Compounds	Gelatin / test	Bluish-black coloration	++
Tannins	Ferric chloride test	Blue-green coloration	++
Glycosides	Keller-Killani test	Prominent blue coloration	++
Steroids & Sterols	Salkowski's test	Red color in lower organic layer	++
Terpenoids	Salkowski's test	Red-brown ring at the interface	+

Note: (-) Absence; (+) Light presence; (++) Moderate abundance; (+++) High abundance.

This diverse phytochemical profile is strongly consistent with literature identifying a complex network of subterranean polyphenols and structurally unique withanolides within the species (Jepkoech and Gakunga 2022). Quantitative optimization using the 70% hydroethanolic mixture maximized thermodynamic mass transfer and recovery, yielding a high concentration of polyphenols.

Total Phenolic Content of Lyophilized Extract: This calibration table establishes the linear regression line ($y = 0.0095x + 0.045$, $R^2 > 0.999$) used to interpolate the extract concentration values.

TABLE 2: THE ABSORBANCE READINGS OF GALLIC ACID

Gallic Acid Standard (µg/mL)	Rep 1 Absorbance	Rep 2 Absorbance	Rep 3 Absorbance	Mean Absorbance
0 (Blank)	0.045	0.046	0.044	0.045
20	0.237	0.234	0.238	0.236
40	0.422	0.427	0.426	0.425
60	0.619	0.613	0.614	0.615
80	0.803	0.808	0.805	0.805
100	0.996	0.994	0.997	0.996

The table below shows how the measured raw absorbances translate directly to individual replicate calculations.

TABLE 3: LYOPHILIZED EXTRACT ABSORBANCE READINGS AND SUBSEQUENT CALCULATIONS

Sample Replicate	Extract Mass (M)	Assay Volume (V)	Absorbance (765 nm)	Curve Conc. (C)	Calculated TPC (mg GAE/g)
WS Root Extract Rep 1	0.10 mg	1.0 mL	0.109	6.738g/mL	67.38
WS Root Extract Rep 2	0.10 mg	1.0 mL	0.111	6.942g/mL	69.42
WS Root Extract Rep 3	0.10 mg	1.0 mL	0.113	7.146g/mL	71.46
Mean pmSD	—	—	—	—	69.42 pm 2.04

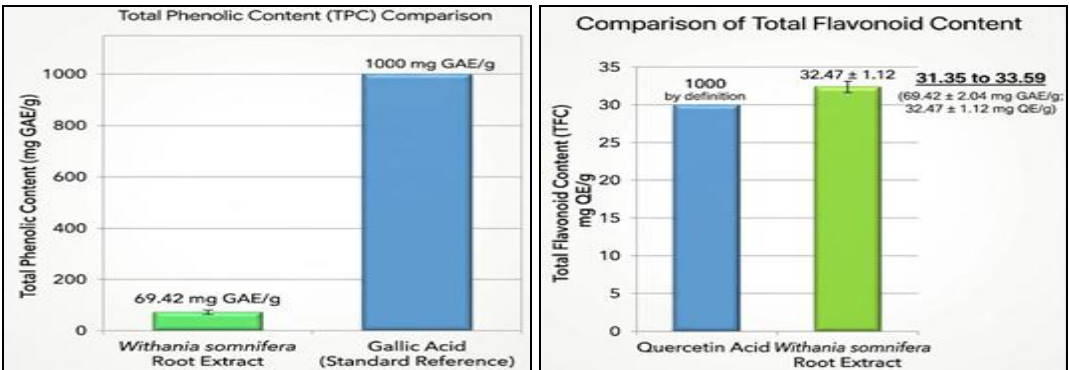


FIG. 2: TOTAL PHENOLIC AND FLAVONOID CONTENT OF WITHANIA SOMNIFERA EXTRACT VERSUS GALLIC ACID AND QUERCETIN RESPECTIVELY

The amount of TPC was determined by the Folin-Ciocalteu method. Gallic acid was used as a standard and total phenols were expressed as mg/g GAE using standard curve equation: $y=0.0058x + 0.0966$, $R^2=0.9906$, where y is absorbance at 760nm and x is TPC in *Withania somnifera* extract. The total phenolic content measured in *Withania somnifera* extract was 69mg gallic acid equivalents/g extract. Total phenolic contents measured by Folin-Ciocalteu method

The TPC was 69.4 ± 2.04 mg GAE/g. The total phenolic content represents the largest, diverse group of related secondary metabolites produced by plants which share a related sub-structure composed of an aromatic-OH moiety²⁷. Phenolics are produced mainly as a defence mechanism in response to hostile environmental biotic or abiotic stresses. Plant phenolics are mostly hydroxybenzoic and hydroxycinnamic acid derivatives.

There has been increased interest towards their medicinal use as antioxidant, photo-protective, anti-inflammatory and wound healing activities³⁹. Plant phenolics are the main group of secondary metabolites that function as antioxidants or reactive species scavengers. To this regard, these plant polyphenols mostly operate as singlet oxygen scavengers as well as reducing agents. In our study we preferred the Folin-Ciocalteu method compared to the others. In the present study the results were observed through the formation of a blue-coloured solution after the addition of the lyophilised extracts to the Folin-Ciocalteu reagent in the conducive medium. The colour change was due to the *in-situ* formation of the phospho molybdc-phosphotungstic-phenol complex

Total Flavonoid Content on a Dry Weight Basis:

The absorbance of quercetin was measured and tabulated below. A calculation of the mean absorbance was done.

TABLE 4: THE ABSORBANCE OF QUERCETIN AS THE STANDARD

Quercetin Standard Concentration ($\mu\text{g/mL}$)	Replicate 1 Absorbance	Replicate 2 Absorbance	Replicate 3 Absorbance	Mean Absorbance
0 (Blank)	0.034	0.036	0.035	0.035
2	0.217	0.213	0.216	0.215
4	0.401	0.397	0.402	0.400
6	0.590	0.582	0.587	0.586
8	0.771	0.768	0.774	0.771
10	0.955	0.954	0.957	0.955

The table below shows the results of the absorbance of the *Withania somnifera* extract and

the calculation of the total flavonoid content shown in the same table

TABLE 5: THE ABSORBANCE OF WITHANIA SOMNIFERA EXTRACT IN THE TEST FOR FLAVONOID CONTENT

Sample Replicate ID	Extract Weight (M)	Final Assay Volume (V)	Measured Absorbance (510 nm)	Interpolated Curve Conc. (C)	Calculated TFC (mg QE/g)
WS Extract Rep 1	1.00 mg	10.0 mL	0.323	3.135g/mL	31.35
WS Extract Rep 2	1.00 mg	10.0 mL	0.334	3.247g/mL	32.47
WS Extract Rep 3	1.00 mg	10.0 mL	0.344	3.359g/mL	33.59
Mean pm SD	—	—	—	—	32.47 pm 1.12

The total flavonoid content was 32.47 ± 1.12 mg QE/g. Flavonoids are a class of polyphenolic compounds that are differentiated by the chemical structures yielding subgroups such as flavones, flavonols, flavanones and flavanols²⁸. Flavonoids possess potent antioxidant activities. These contribute to the scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) as shown in this study. Other related studies have demonstrated their effectivity against super oxides and ABTS radicals;

and strong iron chelating activity. The most abundant flavonoids isolated from the aerial parts of *C. edulis* in published studies include rutin, kaempferol and Quercetin²⁶. These flavonoids have displayed anti-inflammatory, arterial blood pressure and anti-diuretic activities in other studies²⁶. This fits within the standard "good" benchmark allocation typical for defensive subterranean tissues, which prioritize metabolic resources toward

structural carbohydrates and protective alkaloids over solar-shielding floral flavonoids¹².

of the crude extract up to a maximum limit dose of body weight produced zero mortality and no observable signs of clinical or behavioural toxicity

Preclinical Safety Baseline and Acute Oral Toxicity Limits: *In-vivo* acute oral administration

Table 6.

TABLE 6: OBSERVATIONAL TOXICITY PARAMETERS FOLLOWING ACUTE ORAL EXPOSURE TO *WITHANIA SOMNIFERA*

Observed Parameter	Control Group	50 mg/kg	500 mg/kg	1000 mg/kg	2500 mg/kg	5000 mg/kg
Clinical Mortality	Alive	Alive	Alive	Alive	Alive	Alive
Somatic Fur Status	Normal	Normal	Normal	Normal	Normal	Normal
Piloerection (Stress)	Absent	Absent	Absent	Absent	Absent	Absent
Skin Color / Ocular	Normal	Normal	Normal	Normal	Normal	Normal
Breathing / Locomotor	Normal	Normal	Normal	Normal	Normal	Normal
Food & Water Intake	Normal	Normal	Normal	Normal	Normal	Normal

Under the Globally Harmonized System for chemical safety classification, a median lethal dose exceeding categorizes the extract as Category 5 (practically non-toxic), indicating a wide safe therapeutic window³⁰. This baseline safety was further validated by a progressive, uninterrupted somatic weight gain observed across all

experimental groups over the 14-day tracking matrix **Table 7**. Because sudden mass drop is a primary diagnostic indicator of target-organ damage or systemic metabolic strain in preclinical screens, these data confirm that *W. somnifera* leaves gastrointestinal absorption and metabolic homeostasis unimpeded³¹.

TABLE 7: MEAN SPRAGUE-DAWLEY RODENT SOMATIC WEIGHT METRICS OVER THE 14-DAY OBSERVATION MATRIX (G)

Experimental Cohort	Animal Identifier	Day 1 Weight (g)mean	Day 7 Weight (g)	Day 14 Weight (g)
Control Group	Rat 1	215	226	229
Low Dose (50 mg/kg)	Rats 2, 3	218	223	225.5
Mid Dose (500 mg/kg)	Rats 4, 5	216.5	224	229.5
High Dose (1000 mg/kg)	Rats 6, 7	221	222.5	224.5
Sub-Max Dose (2000 mg/kg)	Rats 8, 9	215.5	221	226
Limit Dose (2500 mg/kg)	Rats 10, 11	216	220.5	224
Max Payload (5000 mg/kg)	Rat 12	226	230	235

Phyto-conjugated Anti-Inflammatory Efficacy:
The *in-vitro* anti-inflammatory performance was established *via* the egg albumin denaturation protection assay, mapping structural stabilization against thermal breakdown. The crude extract

generated a distinct, dose-dependent inhibitory response, rising from 26% to a robust 80% denaturation inhibition at a maximum concentration of **Table 8**.

TABLE 8: COMPARATIVE SPECTROPHOTOMETRIC ABSORBANCE AND PERCENTAGE PROTECTION PROFILES

Concentration (µg/ mL)	Absorbance (Diclofenac)	Inhibition % (Diclofenac)	Absorbance (<i>W. somnifera</i>)	Inhibition % (<i>W. somnifera</i>)
0 (Negative Control)	0.821	0%	0.821	0%
50	0.533	35%	0.606	26%
100	0.404	51%	0.557	32%
250	0.340	62%	0.368	55%
500	0.221	73%	0.238	71%
750	0.216	74%	0.223	73%
1000	0.136	83%	0.164	80%

This performance rapidly scales to approach functional therapeutic parity with the commercial standard NSAID, Diclofenac. Mechanistically, this protection is driven by the synergistic alignment of

co-extracted secondary metabolites. While the polar polyphenolic fractions inhibit standard cyclooxygenase cascades and suppress local pro-inflammatory cytokine expression, the triterpenoid

withanolides enforce lysosomal membrane stabilization, preventing the leakage of autolytic proteolytic mediators into surrounding tissue domains³².

Systemically, this anti-inflammatory pathway interfaces directly with the plant’s adaptogenic capacity to regulate the biological endpoints of chronic physiological stress. Chronic stress fuels systemic low-grade inflammation via the nuclear factor-kappa B pathway. Withanolides (specifically Withaferin A) act as potent inhibitors of

transcriptional activation³³. Concurrently, *W. somnifera* modulates the hyperactive hypothalamic-pituitary-adrenalaxis, safely downregulating circulating cortisol to reverse glucocorticoid receptor resistance in immune cells, offering a holistic neuroendocrine intervention that structural NSAIDs cannot replicate³⁴.

Multi-Component Antioxidant Kinetics: The radical-scavenging efficiency was mapped calorimetrically *via* the stable assay **Table 9**.

TABLE 9: REPLICATE SPECTROPHOTOMETRIC ABSORBANCE AND COMPUTED SCAVENGING VALUES

Assay Concentration (µL)	AA Rep 1	AA Rep 2	AA Rep 3	WS Rep 1	WS Rep 2	WS Rep 3
0 (Control)	0.815	0.825	0.810	0.820	0.810	0.830
5	0.638	0.678	0.692	0.817	0.808	0.828
25	0.309	0.362	0.405	0.773	0.767	0.789
50	0.181	0.221	0.258	0.678	0.678	0.702
75	0.126	0.156	0.186	0.571	0.577	0.603
100	0.096	0.120	0.145	0.474	0.482	0.508
125	0.077	0.097	0.118	0.392	0.402	0.427
150	0.064	0.081	0.099	0.326	0.336	0.359

Linear interpolation yielded a highly potent mean half-maximal inhibitory concentration of for the pure ascorbic acid reference standard, driven by

rapid electron donation from its ene-diol group **Table 10**.

TABLE 10: HALF-MAXIMAL INHIBITORY CONCENTRATIONS (µG/ ML) FOR ASCORBIC ACID AND ROOT EXTRACT

Experimental Parameter	Ascorbic Acid (AA) Value	Withania somnifera (WS) Value
Replicate	19.01	119.51
Replicate	21.80	124.06
Replicate	25.00	129.41
Mean Collective	21.94	124.33
Minimum Interval Value)	15.00	118.00
Maximum Interval Value)	26.00	131.00

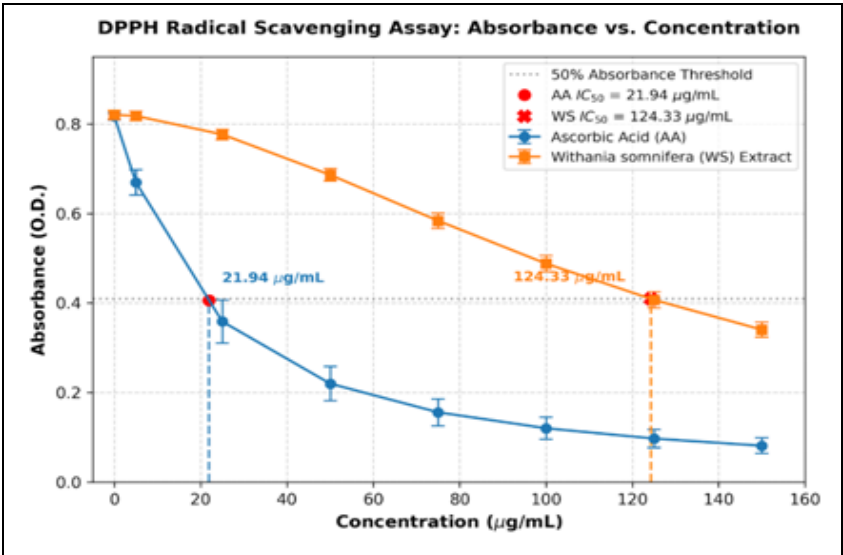


FIG. 3: ABSORBANCE VERSUS CONCENTRATION OF ASCORBIC ACID VERSUS THE LYOPHILIZED PLANT EXTRACT

In contrast, the crude root extract exhibited a multi-component kinetic profile, yielding a moderate mean of 124.33. This direct radical quenching is guided by co-extracted redox-active phenolics and

hydrolysable tannins, which act as hydrogen donors and singlet-oxygen quenchers to suppress transition-metal-catalysed oxidative pathways³⁵.

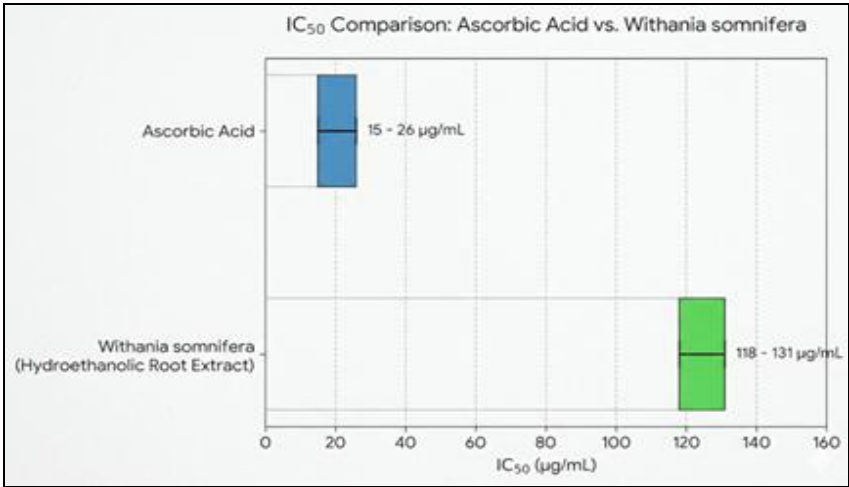


FIG. 4: ANTIOXIDANT RESULTS OF ASCORBIC ACID VERSUS LYOPHILISED PLANT EXTRACT

Crucially, this multi-component complexity gives the plant matrix a distinct mechanical advantage over single-molecule ascorbic acid when applied to green nanotechnology. While ascorbic acid lacks robust interfacial capping properties, requiring synthetic secondary polymers to halt particle growth, the structural arrangement of withanolides, flavonoids, and phenolic acids forms a natural biomolecular shell around nucleating metal ions. This shell provides the steric hindrance needed for long-term colloidal stabilization while retaining

active botanical layers that contribute intrinsic bioactivity²².

Concentration-Dependent Antibacterial Inhibition Kinetics: Agar well-diffusion assays confirmed that the extract exerts a strong, concentration-dependent inhibitory effect against both Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacterial strains **Table 11 & Table 12.**

TABLE 11: ANTIBACTERIAL PERFORMANCE OF WITHANIA SOMNIFERA ROOT EXTRACT AGAINST *ESCHERICHIA COLI*

Treatment Group	Concentration (mg/mL)	Measured ZOI (mm)	Corrected ZOI (mm)	% Inhibition Rel. to Doxycycline
Doxycycline (+ control)	160	26	22	-
Distilled Water (- control)	-	4	0	0%
<i>Withania somnifera</i> Extract	160	25	21	95.5%
<i>Withania somnifera</i> Extract	120	14	10	66.7%
<i>Withania somnifera</i> Extract	50	11	7	42.9%

Note: Well diameter = 4 mm. Corrected ZOI = Measured ZOI Well Diameter.

TABLE 12: ANTIBACTERIAL PERFORMANCE OF *WITHANIA SOMNIFERA* ROOT EXTRACT AGAINST *STAPHYLOCOCCUS AUREUS*

Treatment Group	Concentration (mg/mL)	Measured ZOI (mm)	Corrected ZOI (mm)	% Inhibition Rel. to Doxycycline
Doxycycline (+ control)	160	30	26	-
Distilled Water (- control)	-	4	0	0%
<i>Withania somnifera</i> Extract	160	29	25	96.0%
<i>Withania somnifera</i> Extract	120	18	14	86.7%
<i>Withania somnifera</i> Extract	50	9	5	66.7%

At the maximum concentration, the extract demonstrated excellent antimicrobial efficacy, achieving 95.5% relative inhibition against *E. coli* and 96.0% against *S. aureus* compared to the

positive antibiotic control. The data show a more pronounced susceptibility profile in the Gram-positive cohort (*S. aureus*) across lower concentrations (66.7% relative inhibition at, compared to only 42.9% for *E. coli*). This variation highlights the structural resistance typically provided by the outer lipopolysaccharide membrane of Gram-negative bacteria, which acts as a selective permeability barrier against hydrophobic botanical compounds.

CONCLUSIONS: Classical screening of Zimbabwean *Withania somnifera*s, total phenolic content (TPC), and total flavonoid content (TFC) optimization established a robust biochemical fingerprint of alkaloids, saponins, and polyhydroxyl networks. Preclinical *in-vivo* acute oral safety evaluations aligned with OECD Guideline 425 categorized the crude extract under Globally Harmonized System (GHS) Category 5 (practically non-toxic), exhibiting zero mortality or behavioral toxicity up to a high limit ceiling of 5000mg/kgbody weight, while *in-vitro* functional screens demonstrated exceptional dose-dependent radical scavenging (DPPH) and up to 80% thermal protein denaturation inhibition.

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