RESEARCH PAPER

Quality-by-Design Development of a Dual-Marker Standardised *Achyranthes aspera* Oral Suspension and Its Dose-Dependent Hepatoprotection in HepG2 Cells and Carbon Tetrachloride-Intoxicated Rats

Alok Kumar Singh[1*], Anil Ahuja [2], Amit Kumar Sharma[3]

[1]Research Scholar, (PHD) School of Pharmaceutical Studies, Dr. K.N. Modi University, Newai, Rajasthan 304021, India*
(Corresponding Author) Email: alokkumarpatel81@gmail.com
[2]Professor, School of Pharmaceutical Studies, Dr. K.N Modi University, Niwai ,Rajasthan 304021, India
[3]Associate Professor, Department of Applied Sciences Dr. K. N. Modi University, Newai, Rajasthan

ABSTRACT

Background: Hepatoprotective activity has been repeatedly documented for *Achyranthes aspera* Linn. (Amaranthaceae) in preclinical rodent models; however, virtually all prior investigations employed crude or operationally defined extracts administered by gavage at doses that remain untraceable to discrete phytochemical entities, and no study has delivered the plant's constituents in a pharmaceutically characterised dosage form.

Objective: To develop, within a Quality-by-Design (QbD) framework, an orally administered suspension that concurrently delivers two standardised biomarkers of *A. aspera*—oleanolic acid and achyranthine—and to interrogate whether this formulation affords concentration-dependent cytoprotection in HepG2 cells and dose-dependent hepatoprotection in a prophylactic carbon tetrachloride (CCl_4) rat model.

Methods: A quality target product profile, critical quality attributes (CQAs) and failure mode and effects analysis (FMEA) identified xanthan gum (X_1), polysorbate 80 (X_2) and poloxamer 188 (X_3) as critical formulation variables. A 20-run rotatable central composite design (CCD) was applied against four response variables: sedimentation volume (Y_1), redispersibility (Y_2), apparent viscosity (Y_3) and cumulative drug release at 30 min (Y_4). The optimised suspension underwent ICH stability assessment, in vitro cytotoxicity and cytoprotection profiling against tert-butyl hydroperoxide (t-BHP) in HepG2 cells, and in vivo hepatoprotection evaluation in Wistar rats challenged with CCl_4 at oleanolic acid equivalent doses of 10, 20 and 40 mg/kg, benchmarked against silymarin (100 mg/kg).

Results: All four quadratic response-surface models were statistically significant ($p < 0.0001$) with non-significant lack of fit ($R^2 = 0.975–0.991$). The optimised suspension (xanthan gum 0.50%, polysorbate 80 0.20%, poloxamer 188 0.30% w/v) satisfied every predefined CQA, with measured responses within 3.8% of model predictions, 95.4% drug release at 30 min, content uniformity RSD of 2.14% and ≥96.7% marker retention after 12 months at 25°C/60% RH. The formulation was non-cytotoxic up to 100 µg/mL and augmented HepG2 viability from 56.8% to 87.9% following t-BHP insult, in a concentration-dependent manner, with concordant modulation of LDH, ROS, GSH and MDA. In the CCl_4 rat model, formulation administration yielded no mortality up to 2000 mg/kg and dose-dependently attenuated ALT elevation by 25–61%, hepatic lipid peroxidation, antioxidant enzyme depletion and histological injury (all linear trend $p < 0.0001$), with the highest dose achieving performance indistinguishable from silymarin.

Conclusion: A QbD-engineered, dual-marker standardised *A. aspera* suspension confers dose-dependent hepatoprotection that is attributable to quantified phytochemical constituents delivered from a characterised dosage form, furnishing a reproducible preclinical platform for subsequent pharmacokinetic and chronic-injury investigations.

Keywords: ; oleanolic acid; achyranthine; Quality by Design; central composite design; oral suspension; hepatoprotection; carbon tetrachloride; HepG2; oxidative stress

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1. INTRODUCTION

Chronic hepatic disease imposes a substantial and escalating global burden, accounting for approximately two million fatalities annually, with epidemiological trends shifting progressively from viral aetiologies toward alcohol-related and metabolic dysfunction-associated disorders [4]. Pharmacological strategies for mitigating hepatocellular injury remain conspicuously limited. Silymarin, the most extensively employed hepatoprotective botanical, possesses a well-documented experimental efficacy record yet yields inconsistent clinical outcomes, attributable in part to compositional variability and suboptimal oral bioavailability [29]. This deficit has sustained enduring scholarly interest in plant-derived therapeutic agents that target the oxidative and membrane-damaging events underpinning the predominant forms of hepatotoxic insult.

Achyranthes aspera Linn. (Amaranthaceae) occupies a prominent position in Ayurvedic and folk pharmacopoeias for hepatic and inflammatory ailments, and its hepatoprotective potential has been substantiated across diverse rodent models. A whole-plant extract attenuated N-nitrosodiethylamine/CCl_4-induced hepatocarcinogenesis at 100–400 mg/kg [14]; an ethanolic seed extract conferred protection against CCl_4 injury to a degree comparable with silymarin [20]; root and bark

preparations mitigated CCl_4-induced transaminase elevations [6]; and a seed saponin fraction ameliorated hepatic and antioxidant indices in hypercholesterolaemic rats [15]. The plant's principal pentacyclic triterpenoid, oleanolic acid, is an established hepatoprotective agent that activates Nrf2-dependent antioxidant defence pathways [18, 27, 33], whilst the betaine-type alkaloid achyranthine constitutes a characteristic water-soluble constituent of the species [8].

Two critical lacunae constrain the translational utility of this body of evidence. First, the materials tested have consistently been operationally defined extracts whose active constituent content was not quantified, rendering the effective dose inexpressible in terms of any specific chemical entity and precluding batch-to-batch reproducibility. Second, no investigation has delivered

The present investigation addresses the second gap and interrogates whether the standardised markers retain their biological activity upon formulation. The two markers present antithetical formulation challenges: oleanolic acid is practically insoluble in water and exhibits poor membrane permeability, whereas achyranthine is freely water-soluble and ionisable. An oral suspension accommodates the insoluble marker as dispersed microparticles whilst dissolving the soluble species, permits weight-adjusted volumetric dosing and is suitable for patients unable to swallow solid dosage forms; however, its physical stability is governed by interacting, partially antagonistic compositional variables. Accordingly, the formulation was developed under the QbD framework of ICH Q8(R2) and Q9(R1) [11, 12], and the optimised product was subsequently evaluated in HepG2 cells and a prophylactic CCl_4 rat model, with all doses expressed as marker equivalents. Three central propositions were tested: (i) a suspension capable of satisfying all pre-specified CQAs and whose optimum performance is accurately predictable from fitted response-surface models can be engineered; (ii) at non-cytotoxic concentrations the formulation shields hepatocytes from oxidative injury in a concentration-dependent fashion, and this cytoprotection is attributable to the markers rather than the excipients; and (iii) in vivo hepatoprotection is dose-dependent and coherent across serum biochemical, hepatic oxidative-stress and histopathological outcome domains.

2. MATERIALS AND METHODS

2.1 Drug Substance, Reagents and Excipients

Oleanolic acid (98.4% HPLC purity) and achyranthine (97.6%) were isolated from the methanol fraction of authenticated

2.2 Quality Target Product Profile, Critical Quality Attributes and Risk Assessment

The QTPP was defined prior to any formulation preparation: an oral suspension formulated for adults at risk of hepatic dysfunction, presenting as a 5 mL unit dose containing oleanolic acid at 10 mg/mL and achyranthine at 16 mg/mL, with a target pH of 6.0–7.5, packaged in amber glass with a child-resistant closure. Target strengths were derived from efficacious literature extract doses converted to marker equivalents, the quantity of each marker recoverable by isolation, and the concentration achievable in a physically stable suspension at a practical dose volume. Five CQAs were designated, each assigned a numerical acceptance criterion formulated a priori: content uniformity (90–110% of label; RSD ≤5% across top, middle and bottom container samples); particle size distribution (D50 <5 μm, D90 <10 μm); rheology (apparent viscosity 50–150 cP, thixotropic index 1.2–2.0); physical stability (sedimentation volume F≥0.90 at 14 days; complete redispersion within 10 inversions); and chemical stability (≥95% marker retention; total degradation products ≤2%).

Candidate formulation variables were systematically enumerated by Ishikawa cause-and-effect analysis and prioritised by FMEA [12], with severity, occurrence and detectability each scored on 10-point scales and the risk priority number ($RPN = S \times O \times D$) compared against a pre-defined threshold of 100. Variables meeting or exceeding this threshold were incorporated into the experimental design; the remainder were controlled by specification or fixed as process parameters.

2.3 Pre-formulation Studies

Equilibrium aqueous solubility of each marker was determined by the shake-flask method (excess solid, 37°C, 48 h, 0.45 μm filtration, HPLC assay; n = 3) in water, pH 1.2, 4.5, 6.8 and 7.4 buffers, and in polysorbate 80 (0.2% w/v), poloxamer 188 (0.3% w/v) and their combination. Drug–excipient compatibility was evaluated using 1:1 w/w binary admixtures of each marker with each excipient, the two markers in combination, and the complete placebo incorporating both markers, stored at 40°C/75% RH for four weeks. Admixtures were examined for appearance, assay retention, FTIR spectral band integrity and DSC endotherm retention.

2.4 Experimental Design and Optimisation

Range-finding trials (sixteen compositions comparing xanthan gum with sodium carboxymethylcellulose and varying surfactant concentrations) were assessed at 48 h for sedimentation, caking, redispersibility and pourability to delineate the feasible design region. A three-factor rotatable CCD was subsequently executed in randomised order: eight factorial points (±1), six axial points (±α, $\alpha = 1.682$) and six centre-point replicates (20 runs total). The factors were xanthan gum (X_1, 0.30–0.70% w/v at ±1), polysorbate 80 (X_2, 0.15–0.25% w/v) and poloxamer 188 (X_3, 0.25–0.35% w/v). Response variables were sedimentation volume at 14 days (Y_1, target ≥0.90), redispersibility (Y_2, ≤10 inversions), apparent viscosity (Y_3, 50–150 cP) and cumulative release at 30 min (Y_4, ≥80%).

Each response was fitted to a full quadratic model ($Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X_i^2$) reduced by sequential sum of squares. Model acceptance required simultaneous satisfaction of four criteria: ANOVA significance ($p < 0.05$), non-significant lack of fit against pure error ($p > 0.05$), adjusted and predicted R^2 within 0.2 of each other, and adequate precision exceeding 4.0. Residuals were scrutinised on normal-probability plots and against predicted values and run order. The optimum composition was identified by Derringer–Suich desirability-based numerical optimisation [3], maximising Y_1 and Y_4, minimising Y_2 and targeting Y_3 within its prescribed range. The optimum was independently manufactured as a confirmation batch, and observed responses (n = 3) were compared with model predictions; an absolute prediction error exceeding 10% for any response would have constituted grounds for model rejection.

2.5 Manufacture

Batches of 500 mL were prepared by a six-stage validated procedure: (i) xanthan gum was dispersed in approximately

40% of the total water volume using a high-shear mixer (5000 rpm, 15 min) and hydrated for 2 h; (ii) polysorbate 80 and poloxamer 188 were dissolved in approximately 20% of the water at 40°C and cooled; (iii) the isolated markers were wet-milled with ceramic media (60 min, 70% media loading) to D90 < 10 µm, verified in-process; (iv) the milled markers were dispersed in the surfactant solution and homogenised at 15,000 psi for two cycles; (v) the dispersion was incorporated into the hydrated gum base under planetary mixing (200 rpm, 30 min) and pH adjusted to 6.5 ± 0.1 with citrate buffer; and (vi) preservatives, sweetener and flavourants were incorporated, the batch made to volume, verified in-process and filled into amber glass bottles. Critical process parameters and their proven acceptable ranges were: mixer speed 4500–5500 rpm; hydration time 1.5–2.5 h; milling time 45–75 min; media loading 65–75%; homogenisation pressure 12,000–18,000 psi for 2–3 cycles; final mixing 180–220 rpm for 25–35 min; and final pH 6.4–6.6. The master formula is presented in Table 4.

2.6 Characterisation of the Suspension

All determinations were performed in triplicate. Sedimentation volume ($F = H_u/H_0$) was recorded in 50 mL stoppered graduated cylinders from 1 h to 14 days; redispersibility as the number of 180° inversions required to achieve a uniform dispersion after 14 days undisturbed. Apparent viscosity was measured using a rotational viscometer at 25 ± 0.5°C with a fixed spindle and speed, and the thixotropic index computed from ascending and descending shear-rate rheograms. pH was measured potentiometrically. Particle size distribution was determined by laser diffraction in a saturated solution of the markers, and zeta potential by electrophoretic light scattering following dilution in the formulation's own supernatant. Drug release was characterised in USP Apparatus II (50 rpm, 900 mL phosphate buffer pH 6.8 with surfactant to maintain sink conditions, 37 ± 0.5°C) on a 5 mL dose, with sampling at 5–60 min and HPLC assay; dissolution profiles were fitted to zero-order, first-order, Higuchi [9] and Korsmeyer–Peppas [17, 30] kinetic models. Content uniformity was assessed within containers (top, middle and bottom thirds post-redispersion) and between ten containers. Palatability was rated blind by a small trained panel on a five-point hedonic scale. Microbiological quality was assessed per USP <61>/<62> and preservative efficacy per USP <51>.

2.7 Stability Assessment

The confirmation batch, in its intended container-closure system, was stored at 25°C/60% RH (long-term: 0, 3, 6, 9 and 12 months) and 40°C/75% RH (accelerated: 0, 1, 2, 3 and 6 months) in accordance with ICH Q1A(R2) [10], and evaluated for appearance, pH, viscosity, sedimentation volume, redispersibility, assay, degradation products (stability-indicating HPLC) and microbial count. Photostability was investigated per ICH Q1B. The study was designed as indicative, and no shelf-life claim is assigned.

2.8 In Vitro Evaluation in HepG2 Cells

HepG2 cells (ATCC HB-8065) were cultivated in high-glucose DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO_2, and utilised between passages 8 and 16. Identity and mycoplasma-negative status were confirmed prior to and during the experimental work. Three investigational articles were evaluated in parallel: the optimised suspension, the isolated markers without matrix (API control) and the complete drug-free formulation (blank matrix control); the last was essential because polysorbate 80 and poloxamer 188 are membrane-active. Concentrations are expressed throughout as marker equivalents.

Cytotoxicity: Cells seeded at 1.0×10^4 cells/well (96-well plates) were exposed for 24 h to 1–100 µg/mL of each article; viability was measured by MTT reduction [22], with doxorubicin (1 µM) as cytotoxic positive control. The non-cytotoxic window was defined as the concentration range over which viability remained ≥80% of vehicle control. ***Insult calibration:*** t-BHP (100–750 µM) and CCl_4 (5–20 mM) were each tested over 1–24 h to identify an exposure reducing viability to 50–60%. ***Cytoprotection:*** Cells were pre-treated for 2 h with the formulation (10, 25 and 50 µg/mL), the API control at matched marker-equivalent concentration, the blank matrix or silymarin (reference), washed, and exposed to the calibrated insult. Viability (MTT), membrane integrity (LDH release as a percentage of total releasable activity), intracellular ROS (DCFH-DA, excitation/emission 485/530 nm, normalised to protein), reduced glutathione [5] and MDA as thiobarbituric acid-reactive substances were measured on parallel plates and normalised to protein [19]. Each condition was run in six wells across three independent experiments; the independent experiment served as the unit of replication, and edge wells were excluded.

2.9 In Vivo Evaluation

Ethics and animals: The protocol was approved by the Institutional Animal Ethics Committee constituted under CCSEA, Government of India (approval IAEC/2025/February/A01/025, 24 February 2025), and is reported according to ARRIVE 2.0 [25]. Male Wistar rats (8–10 weeks, 180–220 g; n = 48) were acclimatised for 7 days and housed ≤3 per cage at 22 ± 3°C, 45–65% RH and a 12 h light/dark cycle, with standard chow and water ad libitum. Humane endpoints (>20% weight loss, inability to access food or water, persistent recumbency) were pre-defined.

Acute oral toxicity: The formulation was evaluated by the Acute Toxic Class method (OECD 423) [23] at 5, 50, 300 and 2000 mg/kg (n = 3 per step; ≤10 mL/kg), with observation for 14 days, body weight recording on days 0, 7 and 14, and gross necropsy.

Hepatoprotection study: Group size was derived by power calculation using serum ALT as the primary endpoint (Cohen's d = 2.0, two-sided α = 0.05, power 0.80; n = 5, conservatively increased to 6). Rats were stratified by body weight and randomised to six groups (n = 6): I, normal control (vehicle 10 mL/kg p.o.; olive oil 1.25 mL/kg i.p.); II, toxin control (vehicle; CCl_4); III, silymarin 100 mg/kg; IV–VI, formulation at 10, 20 and 40 mg/kg oleanolic acid equivalent (with 16, 32 and 64 mg/kg achyranthine, fixed by the 10:16 mass ratio of the formula). Treatments were administered orally once daily from day 7 to day 1, and on day 0 groups II–VI received CCl_4:olive oil (1:1 v/v, 1.25 mL/kg i.p.). Low, mid and high formulation doses were selected to correspond to the lowest in vitro cytoprotective concentration, the approximate literature extract dose converted to an oleanolic acid equivalent, and the high dose by geometric progression below the deliverable maximum, respectively. Silymarin was required to differ significantly from the toxin control before formulation groups were evaluated.

Sampling and assays: Rats were euthanised under anaesthesia 24 h post-challenge. Serum was stored at −80°C and assayed

for ALT and AST [28], ALP, total bilirubin, total protein and albumin with commercial kits. The liver was weighed (relative liver weight = [liver/terminal body weight] × 100); the left lateral lobe was fixed in 10% neutral buffered formalin and a portion of the right lobe homogenised (10% w/v, ice-cold phosphate buffer) and centrifuged at 10,000 × g for 15 min at 4°C. In the post-mitochondrial supernatant, MDA [24], GSH [5], SOD [21] and catalase [1] were quantified and expressed per mg protein [19]. Paraffin sections (5 µm, H&E) were scored by a blinded pathologist for necrosis (0–4), inflammatory infiltration (0–4), steatosis (0–3) and regeneration (0–3) in ten high-power fields per section, employing a rubric adapted from established hepatic scoring practice [16]. Body weight, feed intake and clinical signs were recorded daily.

2.10 Statistical Analysis

Continuous data are presented as mean ± SD. Normality (Shapiro–Wilk) and variance homogeneity (Levene) were verified; groups were compared by one-way ANOVA with Tukey's HSD (all pairwise comparisons) or Dunnett's test (versus a single control). Dose- or concentration-dependence across the three formulation levels was assessed by linear contrast employing the pooled error variance from the full ANOVA. Histopathological scores are reported as median (IQR) and compared by Kruskal–Wallis with Dunn's test and Bonferroni correction. Statistical significance was set at $p < 0.05$. Because group sizes were powered to detect protection rather than marginal differences between active treatments, a non-significant difference between the high-dose formulation and silymarin is interpreted as indicative of comparable performance, not formal therapeutic equivalence.

3. RESULTS

3.1 Pre-formulation and Risk Assessment

The two markers exhibited diametrically opposing solubility profiles (Table 1). Oleanolic acid solubility was 0.12 µg/mL in water and 0.47 µg/mL at pH 7.4, whereas achyranthine was freely soluble across the pH range (8.42–11.38 mg/mL). The combined surfactant system enhanced oleanolic acid solubility approximately 200-fold over water (24.82 µg/mL) but still more than 400-fold below its 10 mg/mL target strength, confirming that oleanolic acid would be delivered predominantly as dispersed particles with achyranthine in solution. All fourteen binary and ternary admixtures retained ≥97.5% assay content, characteristic FTIR absorption bands and DSC endotherms following four weeks at 40°C/75% RH, and no excipient was excluded on compatibility grounds.

FMEA identified three formulation variables at or above the RPN threshold of 100: xanthan gum concentration (RPN 168), polysorbate 80 concentration (RPN 126) and poloxamer 188 concentration (RPN 126). Milling time and media loading, homogenisation pressure, xanthan hydration time, order of addition, pH, preservative level, marker purity, excipient grade and storage conditions (RPN 72–96) were controlled by specification or process parameters. In range-finding experiments, xanthan gum demonstrated a superior balance of settling resistance and redispersibility relative to sodium carboxymethylcellulose at equivalent concentrations, attributable to its pronounced pseudoplasticity, and was carried forward exclusively.

Table 1. Equilibrium solubility of the isolated markers at 37°C (mean, n = 3).

Medium	Oleanolic acid (µg/mL)	RSD (%)	Achyranthine (mg/mL)	RSD (%)
Purified water	0.12	4.17	8.42	2.97
0.1 M HCl, pH 1.2	0.14	3.57	8.16	3.06
Acetate buffer, pH 4.5	0.18	5.56	8.74	2.52
Phosphate buffer, pH 6.8	0.31	3.23	10.26	2.44
Phosphate buffer, pH 7.4	0.47	4.26	11.38	2.11
Polysorbate 80, 0.2% w/v	8.64	2.78	12.74	1.96
Poloxamer 188, 0.3% w/v	17.36	2.65	13.82	1.81
Combined surfactant system	24.82	2.38	15.46	1.55

3.2 Response-Surface Modelling

Across the 20 CCD runs (Table 2), sedimentation volume ranged from 0.86 to 0.98, redispersibility from 4 to 7 inversions, apparent viscosity from 43 to 142 cP and 30-min release from 91.8% to 97.8%. All four reduced quadratic models satisfied every adequacy criterion (Table 3). The fitted equations in coded units were:

$$Y_1\ (F) = 0.945 + 0.0253X_1 + 0.0096X_2 + 0.0066X_3 - 0.0078X_1^2$$

$$Y_2\ (\text{inversions}) = 5.00 - 0.246X_1 - 0.539X_2 - 0.123X_3$$

$$Y_3\ (\text{cP}) = 91.22 + 26.69X_1 - 0.19X_2 + 1.74X_3 - 0.25X_1X_2 + 1.15X_1^2$$

$$Y_4\ (\%) = 95.69 + 0.85X_1 + 0.89X_2 + 0.52X_3 - 0.28X_1^2$$

Xanthan gum dominated both sedimentation volume and apparent viscosity: its linear coefficient for viscosity exceeded all other terms by more than an order of magnitude, and the negative X_1^2 term for sedimentation volume indicated diminishing returns at elevated gum concentrations, consistent with a self-supporting flocculated network. Polysorbate 80 exerted the most pronounced influence on redispersibility, more than twice that of the remaining factors, consistent with its role in preventing compact sediment formation. Drug release increased with all three factors; the negative X_1^2 term revealed that the anticipated antagonism between viscosity and release becomes measurable only near the upper extreme of the xanthan concentration range. Response-surface contour plots are presented in Figure 1.

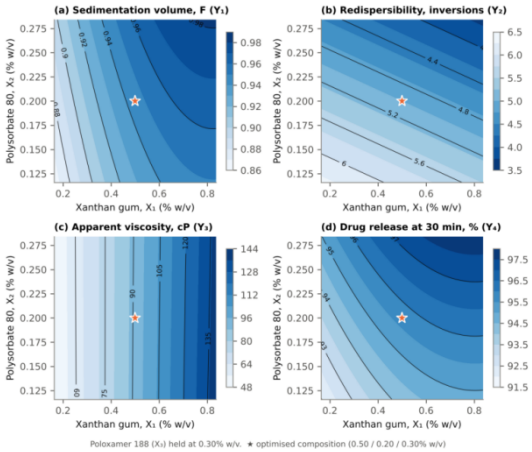


Figure 1. *Response-surface contour plots derived from the fitted quadratic models for (a) sedimentation volume (Y_1), (b) redispersibility (Y_2), (c) apparent viscosity (Y_3) and (d) drug release at 30 min (Y_4), as functions of xanthan gum (X_1) and polysorbate 80 (X_2) concentrations with poloxamer 188 (X_3) held at its centre level (0.30% w/v). The star symbol marks the optimised composition.*

Table 2. Central composite design matrix (actual factor levels, % w/v) and observed responses. F = factorial; A = axial

Run	Type	Xanthan gum X_1 (%)	Polysorbate 80 X_2 (%)	Poloxamer 188 X_3 (%)	Y_1 F	Y_2 inv.	Y_3 cP	Y_4 %
1	F	0.300	0.150	0.250	0.91	5	68	94.2
2	F	0.300	0.150	0.350	0.92	5	71	95.1
3	F	0.300	0.250	0.250	0.93	4	67	96.0
4	F	0.300	0.250	0.350	0.94	4	70	96.8
5	F	0.700	0.150	0.250	0.95	5	118	95.4
6	F	0.700	0.150	0.350	0.96	5	121	96.2
7	F	0.700	0.250	0.250	0.97	4	116	97.1
8	F	0.700	0.250	0.350	0.98	4	119	97.8
9	A	0.164	0.200	0.300	0.86	7	43	91.8
10	A	0.836	0.200	0.300	0.97	5	142	96.1
11	A	0.500	0.116	0.300	0.93	6	89	93.8
12	A	0.500	0.284	0.300	0.96	4	91	97.0
13	A	0.500	0.200	0.216	0.92	6	87	94.0
14	A	0.500	0.200	0.384	0.95	5	94	96.3
15–20	C	0.500	0.200	0.300	0.94–0.95	5	90–92	95.3–95.6

(α = 1.682); C = centre point.

Table 3. Adequacy criteria for the fitted response-surface models.

Criterion	Requirement	Y_1 (F)	Y_2 (inversions)	Y_3 (cP)	Y_4 (%)
Model p-value	< 0.05	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Lack-of-fit p-value	> 0.05	0.2864	0.4127	0.1738	0.3486
R^2	Report	0.9826	0.9748	0.9912	0.9784
Adjusted R^2	Report	0.9689	0.9551	0.9842	0.9611
Predicted R^2	Within 0.2 of adj. R^2	0.9417	0.9186	0.9635	0.9298
Adequate precision	> 4	24.86	21.43	31.72	23.18
CV (%)	Report	1.74	5.62	4.18	1.21

3.3 Optimisation and Model Verification

Desirability optimisation returned xanthan gum 0.50% w/v, polysorbate 80 0.20% w/v and poloxamer 188 0.30% w/v as the optimal composition, which was adopted as the master formula (Table 4). In the independently manufactured confirmation batch, the absolute prediction error was ≤3.78% for every response variable, and all observed values resided within their respective 95% prediction intervals (Table 5). The models were accordingly retained.

Table 4. Master formula of the optimised suspension (500 mL batch).

Component	Function	Quantity per 500 mL	% w/v
Oleanolic acid (isolated)	Active	5.0 g	1.00
Achyranthine (isolated)	Active	8.0 g	1.60
Xanthan gum	Suspending agent	2.5 g	0.50
Polysorbate 80	Wetting agent	1.0 g	0.20
Poloxamer 188	Dispersing agent	1.5 g	0.30
Sodium benzoate	Preservative	0.5 g	0.10
Potassium sorbate	Preservative	0.25 g	0.05
Citric acid monohydrate	pH modifier	0.3 g	0.06
Sodium citrate dihydrate	Buffer	0.7 g	0.14
Sucralose	Sweetener	0.1 g	0.02
Orange flavour	Flavouring	0.5 g	0.10
Vanilla extract	Flavouring	0.25 g	0.05
Purified water	Vehicle	q.s. to 500 mL	—

Table 5. Predicted versus observed responses for the optimised composition.

Response	Predicted	Observed (mean ± SD, n = 3)	95% PI	Absolute error (%)
Y_1 Sedimentation volume (F)	0.960	0.952 ± 0.012	0.926–0.994	0.83
Y_2 Redispersibility (inversions)	4.50	4.67 ± 0.58	3.82–5.18	3.78
Y_3 Viscosity (cP)	90.0	92.3 ± 2.52	85.1–94.9	2.56
Y_4 Release at 30 min (%)	96.0	95.1 ± 1.05	92.5–97.7	0.94

3.4 Quality of the Optimised Suspension

The optimised batch satisfied every CQA (Table 6). The suspension was homogeneous on redispersion without caking, with F = 0.95 at 14 days and redispersion in 5 inversions. Rheological characterisation confirmed pseudoplasticity and thixotropy (91.2 cP; thixotropic index 1.56), with a median particle size (D50) of 2.84 µm and D90 of 6.72 µm, and zeta potential of −28.6 mV. Assay yielded 98.7% (oleanolic acid) and 99.1% (achyranthine) of label, with content uniformity RSD of 2.14%. Cumulative release reached 95.4% at 30 min. Among the kinetic models evaluated, the Higuchi model provided the best fit (k_h = 17.84%·min$^{-1/2}$; R^2 = 0.9846), followed by first-order (R^2 = 0.9728) and Korsmeyer–Peppas (n = 0.61; R^2 = 0.9783 over the initial 60% of release), collectively indicating diffusion-dominated, anomalous (non-Fickian) transport through the hydrated xanthan network. Palatability scored 4.1 of 5, and both microbiological quality and preservative efficacy met USP criteria.

Table 6. Evaluation of the optimised suspension against predefined CQAs (mean ± SD, n = 3).

Attribute	Acceptance criterion	Result	Complies
pH	6.0–7.5	6.82 ± 0.04	Yes
Sedimentation volume at 14 days (F)	≥0.90	0.95 ± 0.01	Yes
Redispersibility (inversions)	≤10	5.0 ± 0.58	Yes
Apparent viscosity (cP)	50–150	91.2 ± 2.1	Yes
Thixotropic index	1.2–2.0	1.56 ± 0.05	Yes
D50 (µm)	< 5	2.84 ± 0.16	Yes
D90 (µm)	< 10	6.72 ± 0.31	Yes
Zeta potential (mV)	Report	−28.6 ± 1.4	—
Oleanolic acid content (% label)	90–110	98.7 ± 1.2	Yes
Achyranthine content (% label)	90–110	99.1 ± 1.0	Yes
Content uniformity RSD (%)	≤5	2.14 ± 0.36	Yes
Drug release at 30 min (%)	≥80	95.4 ± 1.8	Yes
Palatability (of 5)	≥3	4.1 ± 0.4	Yes
Microbial quality / preservative efficacy	USP <61>, <62>, <51>	Complies	Yes

3.5 Stability

Over 12 months at 25°C/60% RH, assay declined from 99.1% to 96.7% for oleanolic acid and from 99.3% to 97.0% for achyranthine; total degradation products increased from 0.22%

to 0.96%; apparent viscosity fell from 91.2 to 85.3 cP; sedimentation volume decreased from 0.95 to 0.92; and redispersibility rose from 5 to 6 inversions. All attributes remained within their predefined acceptance limits throughout the study (Figure 2). Accelerated conditions and photostability results collectively supported a provisional storage condition of below 25°C, protected from light.

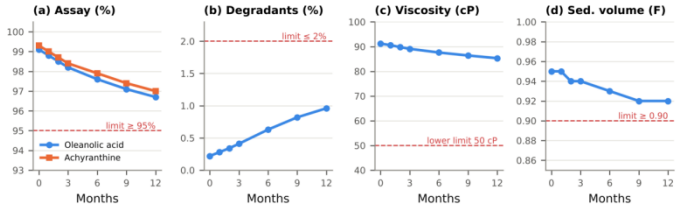


Figure 2. *Long-term stability profiles of the optimised suspension at 25°C/60% RH over 12 months: (a) assay of oleanolic acid and achyranthine, (b) total degradation products, (c) apparent viscosity and (d) sedimentation volume. Dashed lines indicate predefined acceptance limits.*

3.6 Cytotoxicity and Cytoprotection in HepG2 Cells

None of the three investigational articles was cytotoxic within the evaluated concentration range: cell viability remained ≥88.6% (formulation), ≥85.9% (API control) and ≥93.1% (blank matrix) at 100 µg/mL marker equivalent, and IC_{50} exceeded 100 µg/mL for all three; doxorubicin yielded an IC_{50} of 0.42 µM (Figure 3a). The working window for cytoprotection experiments was therefore established at 10–50 µg/mL, over which viability of unchallenged cells exceeded 93%. t-BHP at 500 µM for 6 h reduced viability to 56.4 ± 3.5% and was selected as the insult; CCl_4 reduced viability to only 72.8% at 20 mM for 24 h, consistent with the attenuated CYP2E1 activity of HepG2 cells, and was not used further in vitro.

t-BHP provoked a reduction in viability to 56.8%, an approximately four-fold increase in LDH release, a 56% depletion of GSH and a near-tripling of MDA (Table 7). Formulation pre-treatment attenuated every perturbation in a concentration-dependent manner (one-way ANOVA, $F(7,16) = 69–90.5$, all $p < 0.0001$; linear trend $p \leq 0.031$ for every endpoint). At 50 µg/mL, viability recovered to 87.9%, ROS fell to 55.9% of the insult level, GSH recovered to 86% of control and MDA declined by 49% relative to t-BHP alone, performance closely approximating silymarin. The API control yielded viability of 82.4%, intermediate between the mid and high formulation responses, confirming that the excipient matrix neither potentiated nor diminished marker activity. Critically, the blank matrix afforded no cytoprotection (viability 58.9%; ROS 96.4% of insult level), attributing the observed protection unambiguously to oleanolic acid and achyranthine (Figure 3b–e).

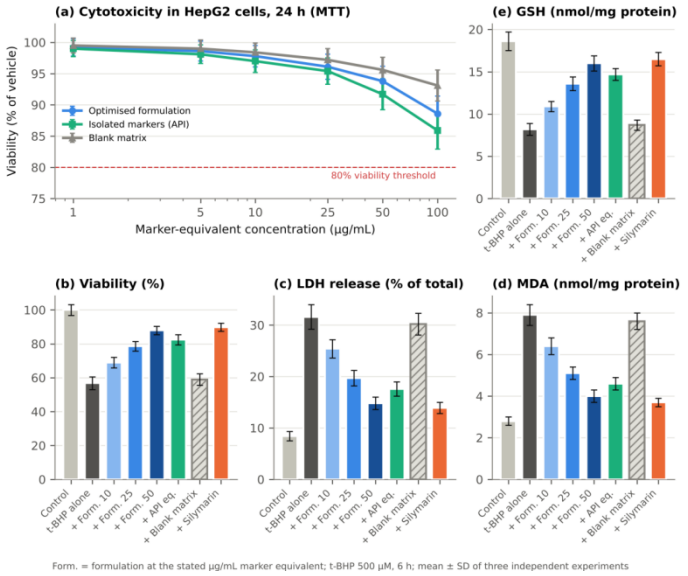


Figure 3. *In vitro evaluation in HepG2 cells. (a) Cell viability after 24 h exposure (MTT); dashed line = 80% non-cytotoxicity threshold. (b–e) Effect of 2 h pre-treatment on t-BHP-induced (500 µg/mL, 6 h) changes in (b) viability, (c) LDH release, (d) MDA and (e) GSH. Mean ± SD of three independent experiments.*

Table 7. Cytoprotection against t-BHP (500 µM, 6 h) in HepG2 cells (mean ± SD of three independent experiments). ROS expressed relative to the t-BHP-alone group.

Group	Viability (% control)	LDH release (% total)	ROS (% insult)	GSH (nmol/mg protein)	MDA (nmol/mg protein)
Untreated control	100.0 ± 3.2	8.4 ± 0.9	—	18.6 ± 1.1	2.8 ± 0.2

Group	Viability (% control)	LDH release (% total)	ROS (% insult)	GSH (nmol/mg protein)	MDA (nmol/mg protein)
t-BHP alone	56.8 ± 3.7	31.6 ± 2.4	100.0 ± 5.3	8.2 ± 0.7	7.9 ± 0.5
+ Formulation 10 µg/mL	68.9 ± 3.1	25.4 ± 1.8	82.7 ± 4.2	10.9 ± 0.6	6.4 ± 0.4
+ Formulation 25 µg/mL	78.6 ± 2.8	19.7 ± 1.5	68.5 ± 3.6	13.6 ± 0.8	5.1 ± 0.3
+ Formulation 50 µg/mL	87.9 ± 2.5	14.8 ± 1.2	55.9 ± 3.1	16.0 ± 0.9	4.0 ± 0.3
+ API, matched concentration	82.4 ± 3.0	17.6 ± 1.4	63.7 ± 3.5	14.7 ± 0.7	4.6 ± 0.3
+ Blank matrix	58.9 ± 3.4	30.2 ± 2.1	96.4 ± 4.8	8.7 ± 0.6	7.6 ± 0.4
+ Silymarin (reference)	89.8 ± 2.3	13.9 ± 1.1	52.8 ± 2.9	16.5 ± 0.8	3.7 ± 0.2

3.7 Acute Oral Toxicity

No mortality occurred at any dose step up to the 2000 mg/kg limit dose, and no treatment-related clinical signs were observed at 2000 mg/kg. Mild, transient lethargy was observed in one of three animals at the 300 mg/kg step and resolved spontaneously within 4 h. Body weight increased progressively at every step (+3.7% to +5.0% over 14 days), and gross necropsy revealed no abnormality of the liver, kidney, spleen, heart or lung. The formulation therefore required no classification under OECD 423.

3.8 Serum Biochemistry

CCl_4 administration elevated ALT four-fold (168.7 vs. 42.6 U/L) and AST 3.7-fold (286.4 vs. 78.4 U/L) relative to normal controls, with concurrent rises in ALP and bilirubin and falls in total protein and albumin (Table 8; Figure 4). The toxin control differed from every other group (Tukey, $p < 0.001$ for both transaminases). Silymarin significantly attenuated ALT and AST elevations ($p < 0.001$ vs. toxin control), confirming the model's sensitivity to detect hepatoprotection. The formulation attenuated every serum parameter dose-dependently: ALT declined by 25.1%, 45.6% and 60.9% at low, mid and high doses respectively relative to the toxin control, with a corresponding AST reduction of 23.6%, 44.8% and 60.6%. The linear dose contrast was highly significant for every parameter (ALT $t(30) = -10.82$; AST −11.84; ALP −6.57; bilirubin −7.28; total protein 4.54; albumin 4.82; all $p < 0.0001$). At the high dose, ALT (65.9 U/L) and AST (112.8 U/L) did not differ significantly from silymarin (68.5 and 116.3 U/L; $p = 0.9$).

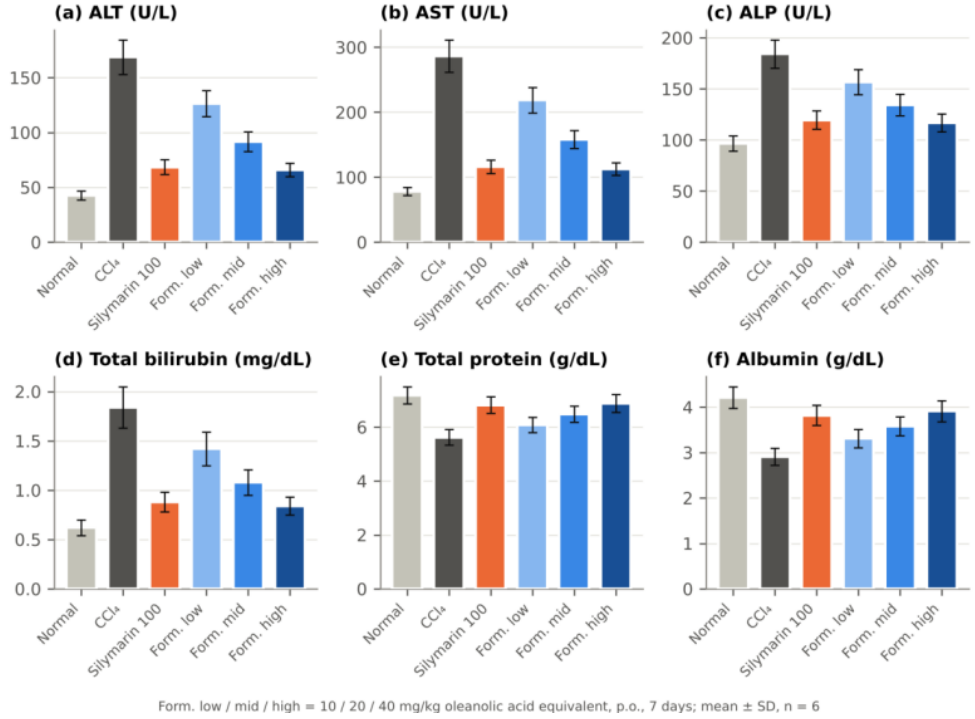


Figure 4. *Serum biochemical parameters 24 h after CCl_4 challenge: (a) ALT, (b) AST, (c) ALP, (d) total bilirubin, (e) total protein and (f) albumin. Formulation low/mid/high = 10/20/40 mg/kg oleanolic acid equivalent. Mean ± SD, n = 6.*

Table 8. Serum biochemical parameters 24 h after CCl_4 challenge (mean ± SD, n = 6). Formulation doses expressed as oleanolic acid equivalent.

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	Total bilirubin (mg/dL)	Total protein (g/dL)	Albumin (g/dL)
I. Normal control	42.6 ± 4.1	78.4 ± 6.2	96.8 ± 7.5	0.62 ± 0.08	7.18 ± 0.32	4.21 ± 0.24
II. Toxin control (CCl_4)	168.7 ± 15.6	286.4 ± 24.8	184.2 ± 13.7	1.84 ± 0.21	5.62 ± 0.29	2.91 ± 0.19
III. Silymarin 100 mg/kg	68.5 ± 6.8	116.3 ± 10.5	119.6 ± 9.1	0.88 ± 0.10	6.82 ± 0.31	3.82 ± 0.22
IV. Formulation low (10 mg/kg)	126.4 ± 11.8	218.7 ± 19.6	156.8 ± 12.4	1.42 ± 0.17	6.08 ± 0.28	3.31 ± 0.20
V. Formulation mid (20 mg/kg)	91.7 ± 8.9	158.2 ± 14.1	134.5 ± 10.6	1.08 ± 0.13	6.48 ± 0.30	3.58 ± 0.21
VI. Formulation high (40 mg/kg)	65.9 ± 6.2	112.8 ± 9.7	116.7 ± 8.8	0.84 ± 0.09	6.88 ± 0.33	3.91 ± 0.23

3.9 Hepatic Oxidative Stress

CCl_4 more than tripled hepatic MDA (5.94 vs. 1.82 nmol/mg protein) and reduced GSH by 52%, SOD by 50% and catalase by 47% (Table 9; Figure 5). The formulation reversed each perturbation dose-dependently (linear contrast: MDA $t(30) = -9.45$; GSH 7.14; SOD 6.37; catalase 6.12; all $p < 0.0001$). At the high dose, MDA fell by 58% relative to the toxin control and GSH recovered to 89% of the normal value; no oxidative-stress parameter differed significantly from silymarin ($p = 0.9$). The direction and dose ordering of the oxidative-stress results precisely paralleled the serum biochemical findings across all dose levels.

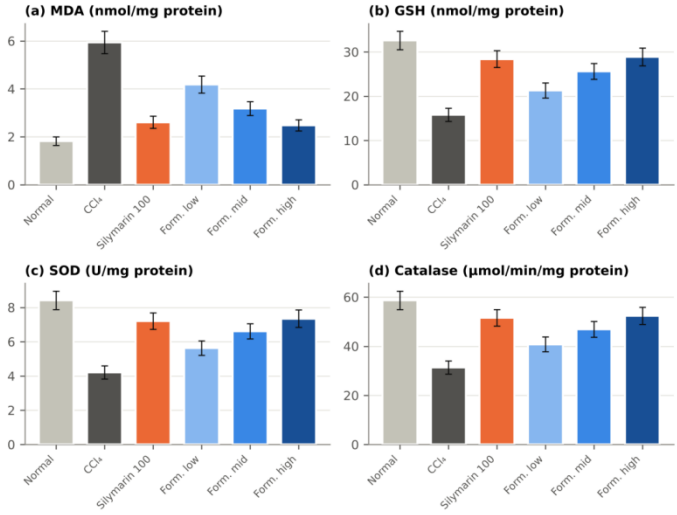


Figure 5. *Hepatic oxidative stress markers 24 h after CCl_4 challenge: (a) MDA, (b) GSH, (c) SOD and (d) catalase, all normalised to protein. Mean ± SD, n = 6.*

Table 9. Hepatic oxidative stress markers 24 h after CCl_4 challenge (mean ± SD, n = 6; normalised to protein).

Group	MDA (nmol/mg)	GSH (nmol/mg)	SOD (U/mg)	Catalase (µmol/min/mg)
I. Normal control	1.82 ± 0.18	32.6 ± 2.1	8.42 ± 0.54	58.7 ± 3.8
II. Toxin control (CCl_4)	5.94 ± 0.47	15.8 ± 1.5	4.21 ± 0.39	31.4 ± 2.7
III. Silymarin 100 mg/kg	2.61 ± 0.25	28.4 ± 1.9	7.21 ± 0.48	51.6 ± 3.4
IV. Formulation low	4.18 ± 0.36	21.3 ± 1.7	5.63 ± 0.43	40.8 ± 3.0
V. Formulation mid	3.18 ± 0.29	25.6 ± 1.8	6.61 ± 0.45	46.9 ± 3.2
VI. Formulation high	2.48 ± 0.23	28.9 ± 2.0	7.35 ± 0.50	52.4 ± 3.5

3.10 Histopathology

Normal-control livers exhibited intact lobular architecture (total score 0). The toxin-control group displayed confluent centrilobular necrosis, dense inflammatory infiltration, prominent steatosis and limited regeneration (total median score 10). Lesions were centrilobular in all affected animals, consistent with zone-3 CYP2E1 bioactivation, and neither bridging nor panlobular necrosis was observed. Scores differed significantly across groups (Kruskal–Wallis $H = 27.8$, $p < 0.0001$), and the toxin control differed from every other group (Dunn, $p < 0.01$). Total histopathological scores declined progressively with increasing formulation dose: 7 (low), 5 (mid) and 4 (high), with commensurate reductions in necrosis, inflammatory infiltration and steatosis and progressive enhancement of regeneration; the high-dose group exhibited near-normal lobular architecture. Silymarin scored 5 and did not differ from the mid- or high-dose formulation groups (Table 10; Figure 6).

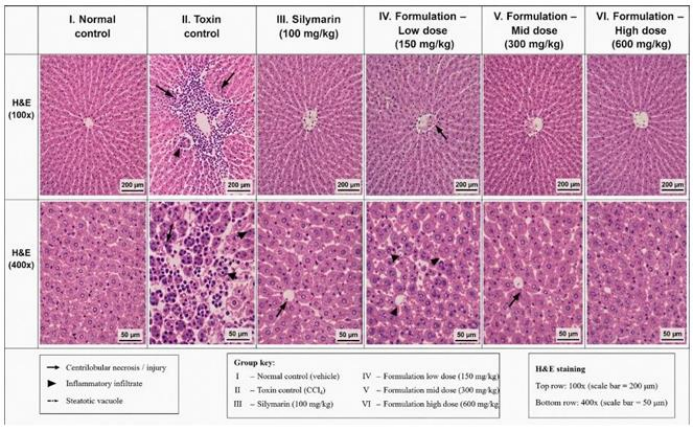


Figure 6. *Representative H&E-stained liver sections (100× and 400×) from (I) normal control, (II) CCl_4 toxin control, (III) silymarin 100 mg/kg, and (IV–VI) the formulation at 10, 20 and 40 mg/kg oleanolic acid equivalent. Arrows denote centrilobular necrosis; arrowheads denote inflammatory infiltrate.*

Table 10. Semiquantitative histopathological scores (median [IQR], n = 6).

Group	Necrosis (0–4)	Inflammation (0–4)	Steatosis (0–3)	Regeneration (0–3)	Total
I. Normal control	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0
II. Toxin control (CCl_4)	3 (3–4)	3 (3–4)	3 (2–3)	1 (1–2)	10
III. Silymarin 100 mg/kg	1 (1–2)	1 (1–2)	1 (0–1)	2 (1–2)	5
IV. Formulation low	2 (2–3)	2 (2–3)	2 (1–2)	1 (1–2)	7
V. Formulation mid	1 (1–2)	1 (1–2)	1 (1–2)	2 (1–2)	5
VI. Formulation high	1 (0–1)	1 (0–1)	0 (0–1)	2 (2–3)	4

3.11 Tolerability

No animal in any group died or reached a humane endpoint. Pre-challenge body weight did not differ between groups (ANOVA, $p = 0.73$). CCl_4 reduced terminal body weight (201.6 ± 7.2 vs. 212.9 ± 6.8 g) and feed intake, and elevated relative liver weight (3.56 ± 0.19 vs. 2.73 ± 0.13%; $F(5,30) = 20.6$, $p < 0.0001$), reflecting hepatic oedema and inflammatory swelling. Relative liver weight declined dose-dependently with formulation treatment (3.26%, 3.09% and 2.94%), and body weight and feed intake recovered proportionally. No gross abnormality of the kidneys, spleen, heart or lungs was detected in any treatment group.

4. DISCUSSION

This investigation carried a standardised, two-marker *A. aspera* drug substance through QbD-directed formulation development and preclinical biological evaluation, yielding a suspension that satisfied all pre-specified quality attributes, whose optimum performance was accurately predictable from fitted response-surface models, and whose hepatoprotective activity in both an in vitro and an in vivo system was dose-dependent and attributable to the phytochemical markers. To the authors' knowledge, this constitutes the first report of *A. aspera* hepatoprotection in which the administered dose is expressible as a quantified quantity of identified, isolated constituents delivered in a characterised pharmaceutical dosage form.

4.1 Formulation Design

The principal formulation challenge resided in the antithetical physicochemical behaviour of the two markers. With oleanolic acid solubility more than 400-fold below its target strength even in the combined surfactant vehicle, a true solution was not feasible, necessitating a suspension in which oleanolic acid particle size, wetting efficiency and physical stability collectively govern dose accuracy and bioavailability potential. The CCD captured the competing compositional effects that render one-factor-at-a-time optimisation unreliable in such complex systems: xanthan gum simultaneously elevated sedimentation volume and apparent viscosity—the latter constraining release at elevated concentrations—whilst polysorbate 80 improved wetting, reduced sediment compaction and enhanced drug release. The small negative X_1X_2 interaction term for viscosity is consistent with polysorbate 80 partially disrupting the xanthan polymer network at the particle interface at high surfactant concentrations. The close agreement between model predictions and independently observed values (≤3.8% error) demonstrates that the fitted equations describe genuine formulation behaviour rather than merely interpolating the design points. Critically, the fitted models also delineate the composition boundaries within which every CQA is satisfied, constituting—together with the proven acceptable ranges for the critical process parameters—the design space and control strategy required for botanical pharmaceutical development [7, 36].

The Higuchi and Korsmeyer–Peppas kinetic profiles, with an anomalous transport exponent of 0.61, indicate that release is governed by combined diffusion of dissolved achyranthine and dissolution-rate-limited transport of the micronised oleanolic acid through the hydrated xanthan gel layer. The near-complete release at 30 min under sink conditions is important because it demonstrates that the suspension vehicle does not sequester the poorly soluble marker, a concern with viscous hydrophilic polymer systems. The zeta potential of approximately −29 mV, in conjunction with steric stabilisation afforded by poloxamer 188 and the three-dimensional xanthan network, is consistent

with the electrostatic and non-electrostatic barriers to particle aggregation and compact sedimentation that underpin the maintenance of redispersibility over 12 months.

4.2 Direct Cytoprotection and Attribution to the Markers

The in vitro experiments served as an interpretive control for the in vivo results. Because CCl_4 hepatotoxicity requires bioactivation by CYP2E1—an enzyme expressed at attenuated levels in HepG2 cells [35]—an agent that merely suppresses CYP2E1 expression would appear hepatoprotective in vivo without directly shielding hepatocytes from oxidative injury; oleanolic acid has been reported to inhibit CYP2E1 expression [13]. The employment of t-BHP, which causes oxidative injury through direct glutathione depletion and reactive oxygen species generation without requiring metabolic activation [2], circumvents this interpretive ambiguity. Concentration-dependent cytoprotection against t-BHP injury across all five endpoints, at concentrations that did not perturb unchallenged cell viability, demonstrates that the formulation acts directly on hepatocytes independently of CYP2E1 suppression. The blank matrix's complete failure to protect—despite containing polysorbate 80 and poloxamer 188, both membrane-active excipients—and the isolated markers' reproduction of the formulation's effect together place the protective activity unambiguously at oleanolic acid and achyranthine rather than the excipient system.

4.3 In Vivo Hepatoprotection and Comparison with the Literature

Three attributes collectively strengthen the internal validity of the in vivo result. First, the concordance across three independent outcome domains: a reduction in serum transaminases alone would indicate attenuated enzyme leakage, which could theoretically arise from membrane stabilisation without true hepatocellular rescue; the parallel improvements in hepatic MDA, GSH, SOD and catalase and in blinded histological scoring are not subject to this constraint. Second, the monotonic dose–response relationship maintained over a four-fold dose range across every domain argues against a saturating effect or dose-idiosyncratic phenomenon. Third, the high-dose formulation consistently matched silymarin across all domains; because the study was powered to detect protection rather than to detect marginal differences between active treatments, this consistency is interpreted as indicative of comparable efficacy in this model, without constituting formal therapeutic equivalence.

The magnitude and direction of protection are broadly consistent with prior reports for crude *A. aspera* extracts [6, 14, 15, 20]. The material distinction is that those investigations tested extracts at 100–400 mg/kg without quantifying constituent content, rendering dose–response analysis in terms of chemical entities impossible, whereas the present dose–response relationship is referenced to 10–40 mg/kg of quantified oleanolic acid delivered from a batch of known composition and characterised physical performance. This transforms the result from a pharmacognostic observation into a reproducible preclinical datum capable of supporting an exposure–response relationship once pharmacokinetic data are available.

4.4 Mechanistic Interpretation

The dose-dependent attenuation of hepatic lipid peroxidation concurrent with restoration of GSH and of SOD and catalase activities in vivo, and the parallel reduction in intracellular ROS and MDA together with GSH recovery in HepG2 cells, collectively place the protective effect at the oxidative and membrane-damaging stage of CCl_4 toxicity [26, 34]. The duration of treatment (7 days pre-challenge) and the 2 h in vitro pre-incubation period were chosen to permit induction of antioxidant enzymes, a pattern consistent with the Nrf2-mediated upregulation of phase II defences reported for oleanolic acid [27, 33], possibly augmented by in vivo CYP2E1 inhibition [13]. However, because Nrf2 activation and its target gene expression, as well as direct CYP2E1 activity, were not measured and because the experimental design incorporated no separate treatment arms for individual markers, the mechanism is attributed to the standardised two-marker preparation acting on the oxidative-stress pathway as an integrated unit, not to either constituent individually.

4.5 Limitations

Several limitations require acknowledgement. No pharmacokinetic data were obtained; the dose–response accordingly describes administered dose rather than systemic exposure, preventing inter-species extrapolation. The CCl_4 model, whilst the most widely accepted hepatotoxicity model for screening purposes, replicates acute chemical injury rather than the metabolic, inflammatory and fibrotic processes characteristic of chronic liver disease. The relative contributions of oleanolic acid and achyranthine were not separated, as no single-marker treatment arms were included. The safety evidence is limited to acute toxicity and short-term tolerability; repeat-dose toxicology, genotoxicity and reproductive toxicity assessments remain outstanding. Stability data derive from a single laboratory-scale batch and are indicative only, without formal shelf-life assignment. Finally, the drug substance originated from a single collection site, and batch-to-batch reproducibility at manufacturing scale has not yet been evaluated.

4.6 Future Work

The priority research agenda, in approximate order of urgency, comprises: (i) development and validation of a bioanalytical method for both markers in rat and human plasma; (ii) a pharmacokinetic study to convert the current dose–response to an exposure–response relationship; (iii) repeat-dose toxicology under Good Laboratory Practice; (iv) efficacy evaluation in a chronic liver injury model such as diet-induced metabolic dysfunction-associated steatotic liver disease or repeated-dose CCl_4-induced fibrosis; (v) a factorial study separating the contributions of oleanolic acid and achyranthine, with measurement of Nrf2 target gene expression and CYP2E1 activity; and (vi) formal ICH stability assessment on primary manufacturing-scale batches under Good Manufacturing Practice conditions.

5. CONCLUSION

A QbD-engineered oral suspension standardised to dual biomarkers of *Achyranthes aspera*—oleanolic acid and achyranthine—was developed, characterised and demonstrated to meet all predefined critical quality attributes. The formulation performed at the model-predicted optimum (prediction error ≤3.8%) and retained marker content ≥96.7% over 12 months at 25°C/60% RH. In vitro cytoprotection against t-BHP-induced oxidative insult in HepG2 cells was concentration-dependent and attributable to the phytochemical markers rather than the excipient matrix. In CCl_4-challenged

Wistar rats, the formulation produced no mortality, no organ toxicity up to 2000 mg/kg, and dose-dependent attenuation of hepatocellular injury across serum biochemical, hepatic oxidative-stress and histopathological endpoints, with the highest dose achieving performance comparable to silymarin. Because every dose is expressed as a quantified marker equivalent delivered from a well-characterised product, these results furnish a reproducible preclinical foundation for the pharmacokinetic, toxicological and chronic-disease studies required prior to first-in-human evaluation of standardised *A. aspera* phytomedicines.

DECLARATIONS

Conflict of interest: The authors declare no conflict of interest.

Funding: No external funding was received for this research.

Data availability: The data underlying the findings of this study are available from the corresponding author upon reasonable request.

Author contributions: Alok Kumar Singh: conceptualisation, methodology, investigation, formal analysis, writing—original draft. Amit Kumar Sharma and Anil Ahuja: supervision, writing—review and editing.

Ethics approval: Institutional Animal Ethics Committee, CCSEA, Government of India (IAEC/2025/February/A01/025).

ABBREVIATIONS

ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, analysis of variance; API, active pharmaceutical ingredient (isolated markers); AST, aspartate aminotransferase; CCD, central composite design; CCl_4, carbon tetrachloride; CQA, critical quality attribute; CYP2E1, cytochrome P450 2E1; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; FMEA, failure mode and effects analysis; GSH, reduced glutathione; ICH, International Council for Harmonisation; LDH, lactate dehydrogenase; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nrf2, nuclear factor erythroid 2-related factor 2; QbD, Quality by Design; QTPP, quality target product profile; ROS, reactive oxygen species; RPN, risk priority number; SOD, superoxide dismutase; t-BHP, tert-butyl hydroperoxide.

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