

Safety Evaluation of Spray-Dried *Curcuma longa* L. Extract for Pharmacopuncture: acute toxicity and neurobehavioral assessment in zebrafish

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Objectives: *Curcuma longa* L. (CL) exhibits potent anti-inflammatory and analgesic properties suitable for pharmacopuncture; however, the clinical application of CL is severely hindered by poor aqueous solubility. Moreover, traditional ethanol-based extracts are unsuitable for safe parenteral administration due to the risk of tissue necrosis and neurotoxicity at sensitive acupoint sites. Thus, no systematic safety evaluation of water-soluble CL extract (CLE) formulations for injection has yet been conducted.

Methods: Spray-dried CLE (SDCLE) was prepared by spray-drying CLE with lactose (1:9 w/w) as the microencapsulation agent. The curcuminoid composition was quantified using a validated high-performance thin-layer chromatography (HP-TLC) method. The solubility of SDCLE was compared with that of native CLE (NCLE). Acute toxicity (LC₅₀), survival, and locomotor behavior were determined using probit regression and automated EthoVision XT tracking following a 96-hour exposure of adult zebrafish to SDCLE (0-500 mg/L).

Results: SDCLE, formulated to contain 10% (w/w) NCLE, exhibited a curcumin content of 0.148 ± 0.02 mg/g. This formulation showed markedly enhanced aqueous solubility (91.30% at a 1:30 dilution) compared with NCLE alone (55.78% at the same dilution), yielding clear, homogeneous solutions suitable for parenteral administration. Post-exposure acute toxicity assessment in zebrafish revealed a time-dependent decrease in LC₅₀ values, from 469.96 mg/L at 24 h to 288.13 mg/L at 96 h. Acute toxicity analysis identified a no-observed-effect concentration (NOEC) of 144.07 mg/L and a lowest-observed-effect concentration (LOEC) of 187.28 mg/L. Exposure to concentrations ≤ 200 mg/L resulted in > 95% survival and preserved normal locomotor activity, whereas concentrations ≥ 300 mg/L induced pronounced neurobehavioral suppression, characterized by a 35% reduction in swimming distance and a six-fold increase in inactivity.

Conclusion: Spray-drying overcame the solubility limitations of CLE, enabling aqueous reconstitution for pharmacopuncture applications. A 96-hour LC₅₀ of 288.13 mg/L was employed to define mortality, while neurobehavioral endpoints indicated toxicity at sublethal concentrations, supporting the safety of concentrations ≤ 200 mg/L for rational SDCLE dose selection.

Keywords: *Curcuma longa*, pharmacopuncture, spray-drying, zebrafish, acute toxicity, neurobehavioral safety

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INTRODUCTION

Pharmacopuncture involves the direct injection of herbal extracts into acupuncture points, combining mechanical stimulation with localized pharmacological effects [1, 2]. Pharmacopuncture was widely adopted in South Korea between 2010 and 2014, during which nearly all inpatients and most outpatients received this treatment. Meanwhile, mandatory accreditation was introduced in 2018, incorporating 165 criteria consistent with the Korean Good Manufacturing Practice (KGMP) standards [3, 4]. Direct acupoint injection bypasses gastrointestinal and hepatic metabolism, resulting in 100-1,000-fold higher tissue concentrations and prolonged activity through depot formation [1, 5, 6], synergistically combining meridian regulation with localized herbal effects [1, 7, 8]. Nonetheless, clinical translation requires pharmaceutical development to address solubility, sterility, tissue compatibility, and preclinical toxicological evaluations [4, 9, 10].

Curcuma longa L. (CL) contains 2-9% curcuminoids (primarily curcumin) and has been used as an anti-inflammatory agent for more than 5,000 years [11-13]. Curcumin modulates multiple molecular targets, including NF- κ B, COX-2, and TNF- α , thereby offering therapeutic potential for inflammatory, neurodegenerative, and metabolic disorders [11, 14, 15]. However, the clinical utility of curcumin is limited by the associated extreme lipophilicity (11 ng/mL aqueous solubility), P-glycoprotein-mediated efflux, and rapid degradation at physiological pH [14, 16, 17]. Extensive hepatic first-pass metabolism yields less active conjugates, resulting in only nanomolar plasma levels even after 10-12 g oral doses, far below the 5-50 μ M efficacy threshold [7, 11, 14]. Therefore, these pharmacokinetic constraints necessitate injectable delivery to bypass gastrointestinal barriers and achieve therapeutic drug concentrations at target sites [1, 4, 7].

Spray-drying technology can address the solubility barrier by converting semisolid CL extract (CLE) into free-flowing powders using lactose as a microencapsulation carrier [18, 19]. Consequently, spray-drying produces spray-dried CLE (SDCLE) with enhanced aqueous dispersibility, making CLE suitable for reconstitution as a clear injectable solution. However, evaluating pharmaceutical improvements requires a systematic comparison with non-spray-dried CLE or native CLE (NCLE) as control formulations. Nonetheless, whether spray-drying genuinely enhances solubility and safety profiles or simply enables the formulation of an otherwise unusable ex-

tract will remain unclear without direct comparison. Therefore, this comparative framework is essential for demonstrating the translational value of pharmacopuncture applications.

Prior toxicological studies have focused exclusively on isolated curcumin, with no investigations examining whole-extract formulations that include curcuminoids, turmerones, polysaccharides, and minor alkaloids combined with pharmaceutical carriers [4, 20, 21]. More importantly, no comparative safety evaluations have been conducted between spray-dried and non-spray-dried formulations. Pharmacopuncture requires regulatory oversight comparable to that of pharmaceutical injections, necessitating comprehensive toxicological data, including acute toxicity thresholds and neurobehavioral safety profiles [3, 9, 22]. The absence of comparative safety data on SDCLE versus NCLE represents a critical gap that hinders standardization and regulatory approval of spray-dried herbal extracts for acupoint injection [18, 23].

Zebrafish (*Danio rerio*) are an effective vertebrate model for toxicology screening, exhibiting approximately 70% genomic homology with humans and the conservation of disease-related genes [24-26]. Moreover, the rapid development, real-time monitoring transparency, and the small size of zebrafish enable high-throughput screening consistent with the 3Rs principles (Replacement, Reduction, Refinement) [3, 24, 25]. OECD Test Guideline 203 establishes standardized protocols for determining LC₅₀ values and assessing acute toxicity [18, 23]. Neurobehavioral endpoints can detect toxic effects at concentrations below lethal thresholds, providing sensitive indicators for safety evaluations [9, 18, 27]. Therefore, by utilizing these sensitive methodologies, toxic effects can be identified at concentrations much lower than those required to induce lethality [25, 26].

This study compared SDCLE with NCLE to establish pharmaceutical and toxicological profiles for the development of pharmacopuncture. The specific objectives were to (1) quantify the solubility improvement achieved by spray-drying, (2) verify curcuminoid preservation during processing [11, 12], (3) determine comparative acute toxicity (LC₅₀ values) over 96-hour exposure in adult zebrafish [9, 18, 19, 28], (4) assess neurobehavioral safety using automated locomotor tracking [23, 27, 29], and (5) establish no-observed-effect concentrations (NOECs) to define safe concentration ranges for mammalian studies and future clinical translation [2, 10].

MATERIALS AND METHODS

1. Plant materials

Fresh CL rhizomes were collected from certified organic suppliers in Batu, East Java, Indonesia, in December 2022. The plant was authenticated at Universitas Airlangga with a voucher specimen (no. CL-2022-12) deposited. The rhizomes were washed, sliced, dried at 50°C, ground (mesh 80), and stored until extraction.

2. Preparation of NCLEs and SDCLEs

Powdered rhizomes (2.5 kg) were extracted using 25 L of 70% ethanol (1:10 w/v) via continuous percolation at 50°C for 6 h. For SDCLE preparation, spray-dried lactose was added to the extract (1:9, w/w) before concentration. The mixture was then concentrated under reduced pressure at 45°C and processed using a spray dryer (inlet 120°C, outlet 80°C, aspirator 35 m³/h, and feed 3 mL/min). For NCLE preparation, the extract was concentrated without the addition of lactose and dried in an oven at 45°C. Both standardized products were stored in light-resistant, airtight containers at 4°C to ensure stability. Yields were calculated as (final product weight / initial rhizome weight) × 100%. For SDCLE, recovery yield was calculated as (final SDCLE weight / theoretical total solids) × 100%.

3. Curcuminoid quantification by high-performance thin-layer chromatography

1) Standard preparation

Five-point calibration curves were prepared for curcumin (500.0-1,000.0 ng/spot), DMC (1,000.0-2,000.0 ng/spot), and BDMC (1,500.0-3,000.0 ng/spot) in ethanol pro-analysis applied per spot. SDCLE (16.0 mg/mL) and NCLE (1.6 mg/mL) samples were sonicated in ethanol pro-analysis for 15 min and filtered through a 0.45 µm filter.

2) High-performance thin-layer chromatography analysis

Quantitative high-performance thin-layer chromatography (HP-TLC) analysis was performed on silica gel 60F254 plates using toluene:acetic acid (4:1, v/v) as the mobile phase. NCLE (1.0 µL) and SDCLE (10.0 µL) samples were applied using the CAMAG® Automatic TLC Sampler 4, and elution was performed using the CAMAG® Automatic Developing Chamber

2 (ADC 2). The plates were scanned at 254 and 365 nm using a CAMAG® TLC Scanner 4 densitometer and a CAMAG® TLC Visualizer 2 image profiler, and curcuminoids were identified based on the R_f values and ultraviolet (UV) spectra. The peak areas were measured using the VisionCats software. Validation was performed in accordance with the International Council for Harmonisation (ICH) guidelines for pharmaceuticals for human use.

4. Solubility test

Solubility classification followed the United States Pharmacopeia (USP) criteria based on solvent-to-solute ratios [30]. The solubilities of the SDCLE and NCLE were evaluated using a modified shake-flask method. Both formulations were prepared with equivalent extract content (10% w/w NCLE) to enable direct comparison. Samples (250.0 mg) were dispersed in varying volumes of distilled water (1:5 to 1:30) to determine the minimum volume required for complete dissolution. All tests were performed in triplicate. The mixtures were sonicated (2 × 5 min), centrifuged at 3,000 rpm for 1 min, and the supernatants were collected. Dissolved solids in the supernatants were quantified using a moisture analyzer and expressed as a percentage of the initial dry weight. The solubility limit was defined as the lowest volume at which complete dissolution occurred without precipitation. Results are expressed as the mean ± standard deviation (SD).

5. Experimental animals

Wild-type adult zebrafish (*Danio rerio*; 120 days post-fertilization) were obtained from the NaturMeds Laboratory, Universiti Putra Malaysia. Fish were housed in a recirculating aquaculture system under standardized conditions (28 ± 1°C; pH 7.0-7.5; dissolved oxygen > 6 mg/L; 14:10 h light-dark cycle) and were fed a commercial diet twice daily. Water quality parameters (ammonia, nitrite, nitrate, and hardness) were monitored weekly.

6. Acute toxicity test

The acute toxicity study was conducted in accordance with the OECD Test Guideline 203, with minor modifications. After 14 days of acclimatization, 120 adult zebrafish were randomly allocated to treatment and control groups (10 fish per 1 L tank,

three replicates per concentration). For SDCLE, preliminary range-finding tests (50–2,000 mg/L) guided the selection of definitive test concentrations of 100, 200, 300, 400, and 500 mg/L. For NCLE, preliminary range-finding tests (5–200 mg/L) were conducted, and definitive concentrations were set at 10% of the SDCLE doses (10, 20, 30, 40, and 50 mg/L). Fish were not fed during the 96 h exposure period. Control groups received dechlorinated tap water without samples. Fish were monitored for mortality, abnormal behavior, and visible changes throughout the exposure period.

7. LC₅₀ determination

Mortality was recorded after 24, 48, 72, and 96 h of exposure. Fish were considered dead when opercular movement had ceased, and no response was observed to gentle prodding. Median lethal concentration (LC₅₀) values with 95% confidence intervals (CIs) were calculated using probit analysis of the relationship between the percentage mortality and log-transformed concentrations. The NOEC and lowest-observed-effect concentration (LOEC) values were derived from probit regression curves, with the NOEC defined as the highest concentration with no statistically significant increase in mortality compared to controls, and the LOEC defined as the lowest concentration at which a statistically significant increase in mortality was detected.

8. Behavioral analysis

Fish behavior was recorded at 24, 48, 72, and 96 h post-exposure using a digital camera positioned above the observation tank. After a 2 min acclimatization period, each fish was recorded for 5 min under standard lighting (1,000 lx). Locomotor activity (total distance traveled, mean velocity, activity duration, and movement categories) was quantified using EthoVision XT 15. Trajectory plots were generated to visualize swimming patterns.

9. Statistical analysis

Data are presented as the mean $\pm$ SD. Normality was as-

sessed using the Shapiro–Wilk test. LC₅₀ values were obtained using probit regression. Behavioral data were analyzed by one-way analysis of variance (ANOVA) with Dunnett's post hoc test, and time-course data were analyzed by two-way repeated-measures ANOVA. All analyses were performed using Graph-Pad Prism v9.0, with p-value < 0.05 considered statistically significant.

10. Ethical approval

All experimental procedures were approved by the Animal Care and Use Committee (ACUC) of Universitas Airlangga (approval number: 2.KEH.135.08.2025) and the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (approval No.: UPM/IACUC/aUP-R026/2021). Animal welfare was ensured by adherence to humane endpoints and international guidelines.

RESULTS

1. SDCLE preparation

The NCLE yield was 12.83% (w/w) based on the dry weight of the starting rhizomes, which yielded a semisolid extract (Table 1). SDCLE was produced as a free-flowing powder with a 64.30% recovery yield, calculated relative to the theoretical total solids (NCLE and lactose, 1:9 ratio). The final SDCLE contained 10% (w/w) NCLE, consistent with the theoretical 1:9 ratio.

2. HP-TLC analysis of curcuminoid standards, NCLE and SDCLE

The HP-TLC chromatographic profile exhibited distinct fluorescent bands for curcumin, DMC, and BDMC under UV light at 366 nm. The R_f values for both NCLE and SDCLE matched those of the analytical standards, with overlapping peaks indicating consistent relative proportions between the formulations. No degradation peaks were observed (Fig. 1).

Curcuminoid quantification by HP-TLC, as summarized in

Table 1. Recovery yield

Formulation	Starting material	Recovery yield	Physical form	Composition
NCLE	Rhizome Powder	12.83%	Viscous Resin	100% Extract
SDCLE	NCLE : Lactose (1:9)	64.30%	Free-flowing Powder	10% Extract

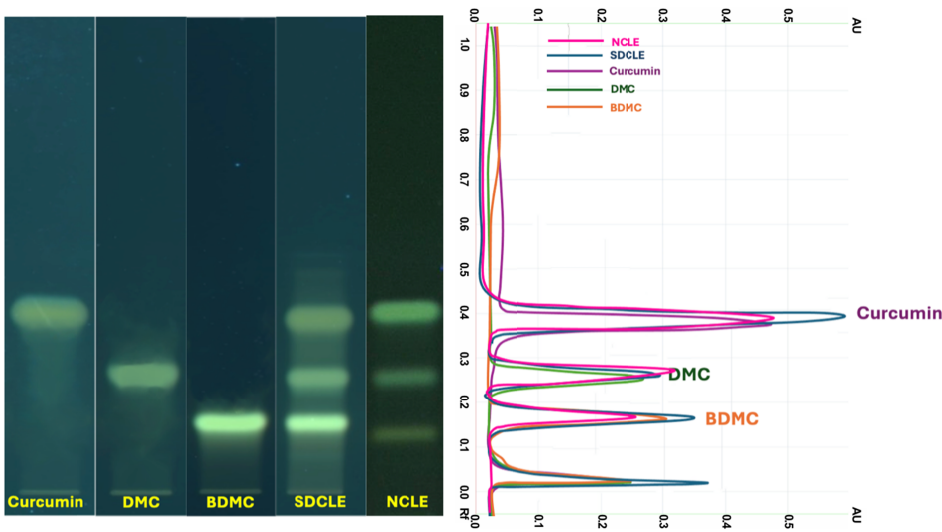


Figure 1. TLC profiles of curcuminoids, native *Curcuma longa* L. extract (NCLE), and spray-dried *Curcuma longa* L. extract (SDCLE).

Table 2. Curcuminoid content in NCLE and SDCLE as determined by HP-TLC

Formulation	Analyte	Correlation coefficient (peak identity)	Peak purity		r	Calibration curve	Content (mg/g ± SD), n = 3
			r (s,m)	r (m,e)			
SDCLE	Curcumin	0.989783	0.999855	0.997864	0.9981	y = 0.0000259x + 0.00319	0.148 ± 0.02
	DMC	0.995723	0.999758	0.998908	0.9982	y = 0.0000209x + 0.00222	0.067 ± 0.01
	BDMC	0.996858	0.999405	0.995862	0.9993	y = 0.000162x + 0.00344	0.066 ± 0.01
NCLE	Curcumin	0.991205	0.999842	0.998012	0.9983	y = 0.0000262x + 0.00324	1.46 ± 0.03
	DMC	0.996218	0.999763	0.998874	0.9985	y = 0.0000211x + 0.00215	0.70 ± 0.02
	BDMC	0.997041	0.999398	0.996045	0.9991	y = 0.0000160x + 0.00338	0.64 ± 0.01

SD, standard deviation.

Table 3. Solubility NCLE vs. SDCLE

Category	Ratio	Water : sampel (v/w)	NCLE	SDCLE
Freely soluble	1:1-10 g/L	1:5	28.92% ± 1.44	65.80% ± 0.66
		1:10	37.39% ± 1.02	72.20% ± 0.44
Soluble	1:10-30 g/L	1:15	40.75% ± 0.46	81.10% ± 1.55
		1:20	48.68% ± 0.39	86.60% ± 1.38
		1:25	53.34% ± 0.23	88% ± 1.04
		1:30	55.78% ± 0.39	91.30% ± 1.08

n = 3. SDCLE, spray-dried *Curcuma longa* extract.

Table 2, established the chemical stability and precision of the spray-drying process. The results demonstrated that the SDCLE formulation achieved proportional mass recovery of the active constituents, yielding curcumin, DMC, and BDMC concentrations corresponding to a precise 10% (w/w) loading relative to the NCLE. The robustness of the analytical method was confirmed by high peak-purity indices (> 0.995) and correlation coefficients for peak identity (> 0.989). Furthermore, the excep-

tional linearity of the calibration curves (r > 0.99) validated the standardization of the analytical method.

3. Solubility test

Table 3 indicates that neither formulation fulfills the USP criteria for being “freely soluble” or “soluble” at lower solvent-to-sample ratios, as the dissolved fractions remain significantly

below 100%. At the 1:5 and 1:10 ratios, the SDCLE achieved solubility values of only $65.80\% \pm 0.66$ and $72.20\% \pm 0.44$, respectively. Although these values are higher than those of the NCLE ($28.92\% \pm 1.44$ and $37.39\% \pm 1.02$), these values confirm the presence of substantial undissolved residue. Only at the maximum dilution of 1:30 did SDCLE approach the 100% threshold required for classification as “soluble,” reaching $91.30\% \pm 1.08$, whereas the raw NCLE remained “poorly soluble” at $55.78\% \pm 0.39$.

Visual examination showed that the NCLE formed sediment after centrifugation (Fig. 2), indicating incomplete dissolution. SDL remained clear and free of sediment throughout testing. SDCLE yielded a clear, homogeneous solution with surface foam that remained stable over the entire observation period, demonstrating complete dissolution with no visible precipitation.

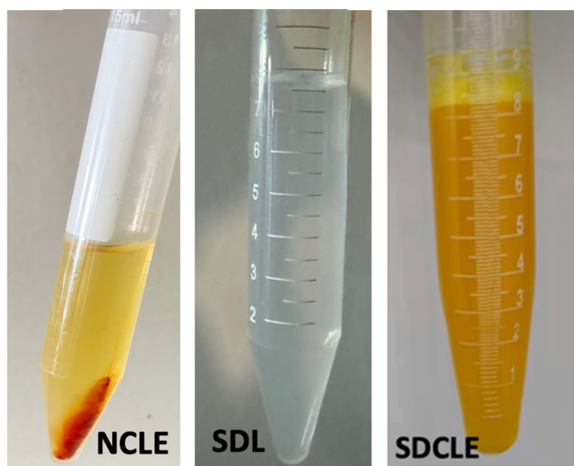


Figure 2. Comparative solubility (1:30) of NCLE, SDL, and SDCLE.

4. Survival rate of adult zebrafish

Preliminary range-finding studies revealed that the NCLE was substantially more toxic than the SDCLE. Although the initial study design proposed testing NCLE at 10% of the SDCLE concentrations (10, 20, 30, 40, and 50 mg/L) to maintain proportional curcuminoid exposure, preliminary data showed 100% mortality at concentrations above 30 mg/L within 48 h. To adequately characterize the dose–response relationship and establish behaviorally relevant safety thresholds, the definitive NCLE concentrations were adjusted to 10, 15, 20, 25, and 30 mg/L, providing finer resolution within the critical toxicity range.

The acute toxicity profiles of the SDCLE and NCLE showed significant differences in dose-dependent mortality over the 96-hour exposure period (Fig. 3). In contrast, the NCLE exhibited high toxicity, with 100% mortality at concentrations as low as 25–30 mg/L (Fig. 3B); the SDCLE maintained a survival rate of approximately 20% even at a significantly higher concentration of 400 mg/L (Fig. 3A).

5. LC₅₀ of SDCLE

The visual and statistical data in Fig. 4 provide a comparative assessment of the acute toxicity profiles of both formulations. Fig. 4A shows the physical appearance of the experimental media, with a sequence of transparent tanks exhibiting increasing yellow turbidity, corresponding to the concentration gradient from 0 to 2,500 mg/L. Fig. 4B presents lateral morphological views of adult zebrafish, documenting physical integrity, fin structure, and scale pigmentation across the specified dosage range.

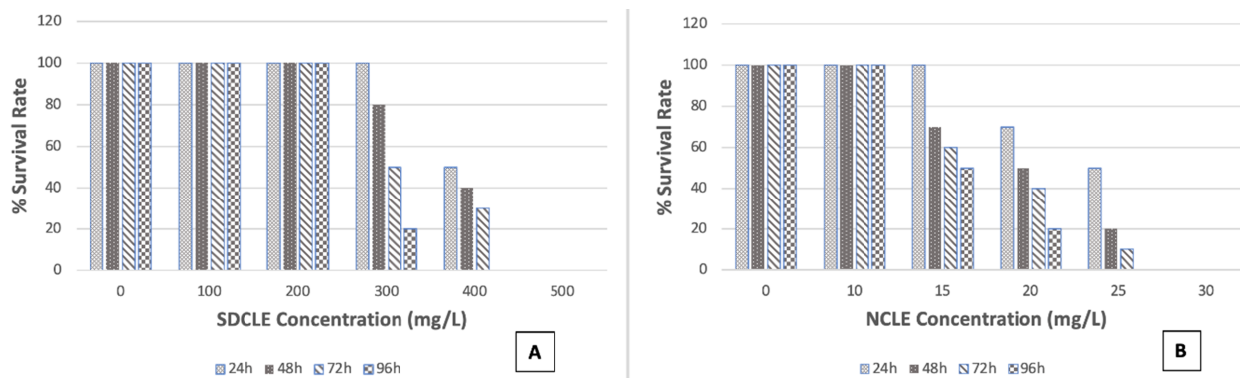


Figure 3. Zebrafish survival rates after acute exposure to SDCLE (A) and NCLE (B).

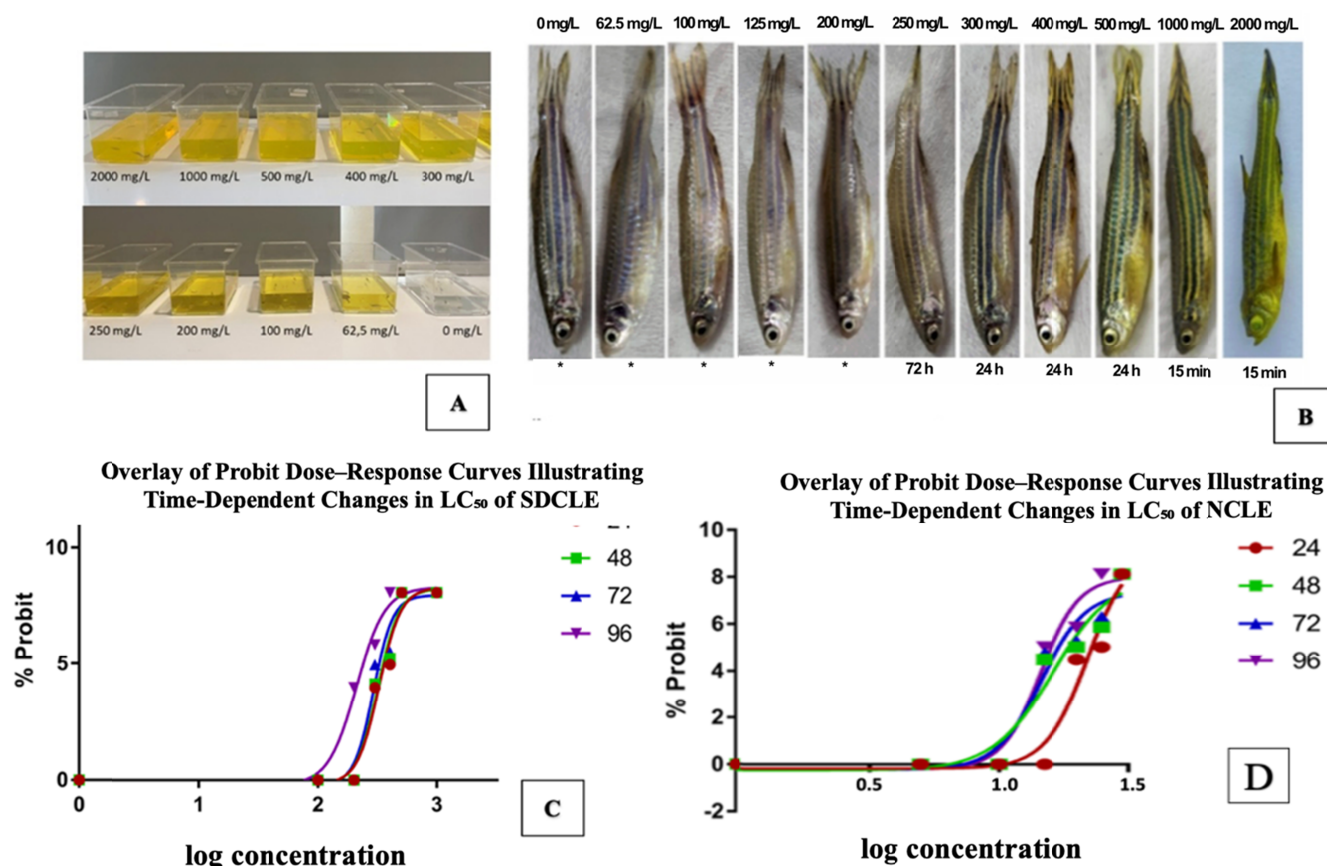


Figure 4. Visual and statistical analyses of acute toxicity. (A) Experimental setup with SDCLE at various concentrations. (B) Zebrafish with concentration-dependent yellow pigmentation. (C) Probit-analysis mortality curves for SDCLE across 24-96 hours. (D) Probit analysis mortality curves for NCLE across 24-96 hours.

Concentration-dependent yellow pigmentation of the fish body and scales was observed as a visible indicator of curcuminoid accumulation in tissues. During preliminary range-finding tests at 2,000 mg/L, pigmentation appeared within 15 min of exposure. In contrast, at the definitive test concentration of 300 mg/L, yellow pigmentation became visually apparent after 72 h, suggesting dose-dependent tissue accumulation kinetics.

The statistical relationship between log concentration and mortality rate is depicted using probit analysis in Fig. 4C and Fig. 4D. In Fig. 4C, the SDCLE curves are shifted toward the higher end of the log concentration axis for the 24-, 48-, 72-, and 96-hour intervals. In contrast, the NCLE curves in Fig. 4D appear at lower log concentrations and exhibit a steeper trajectory toward maximum mortality over the observation period.

The SDCLE formulation demonstrated LC_{50} values that decreased from 469.96 mg/L at 24 hours to 288.13 mg/L at 96 hours, with corresponding NOEC values declining from 234.98 mg/L to 144.07 mg/L. Over the same temporal intervals, the

LOEC for the SDCLE ranged from 305.47 to 187.28 mg/L. In contrast, the NCLE formulation yielded substantially lower values, with the LC_{50} value decreasing from 28.59 mg/L at 24 h to 22.11 mg/L at 96 h. The NOEC and LOEC for the NCLE followed a similar downward trend, with values of 11.05 mg/L and 14.36 mg/L, respectively, at the final 96-hour measurement; calibration curves for both formulations were linear.

Supplementary testing of spray-dried lactose alone yielded an LC_{50} value of 123,787.05 mg/L (95% CI: 92,829.46-155,701.33) at 96 hours, confirming the negligible toxicity of the carrier matrix. This value was approximately 430-fold higher than that of the SDCLE (288.13 mg/L), indicating that the observed toxicity was attributable to the extract rather than the excipient.

6. Locomotor behavior of zebrafish

The behavioral parameters showed concentration-dependent changes (Fig. 5). For the SDCLE, the inactivity duration at 96

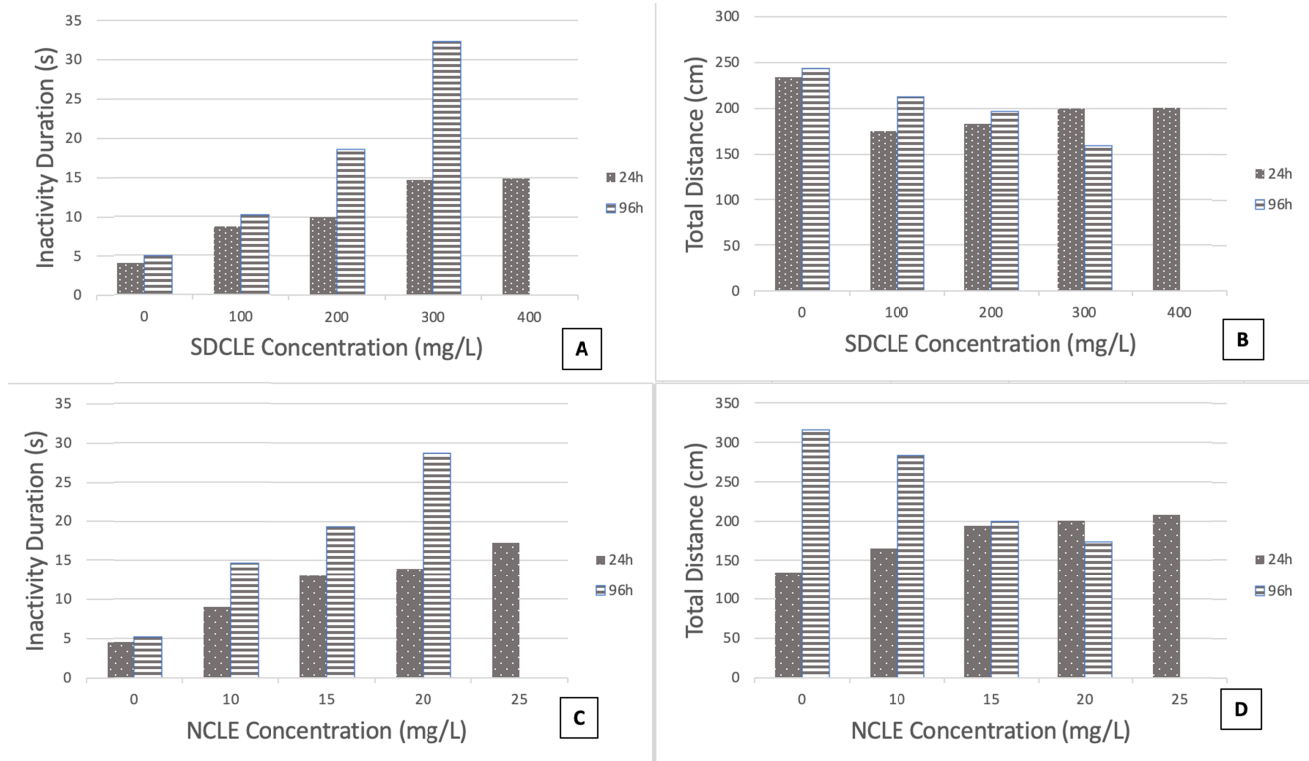


Figure 5. Effects of NCLE and SDCLE on zebrafish activity parameters. (A) Inactivity duration for SDCLE groups. (B) Total distance traveled for SDCLE groups. (C) Inactivity duration for NCLE groups. (D) Total distance traveled for NCLE groups. Data are presented as the mean $\pm$ standard deviation (SD).

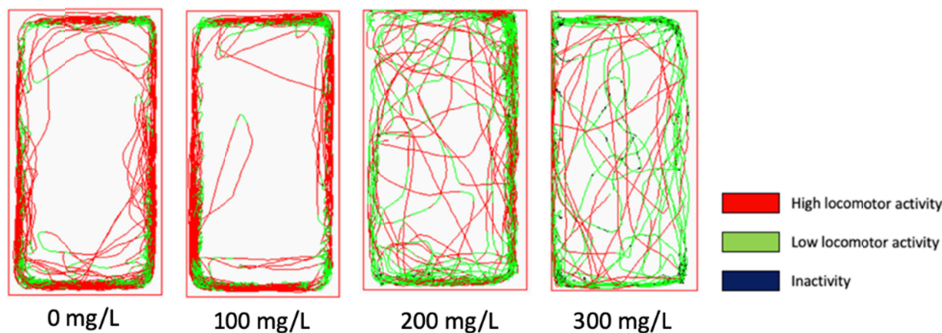


Figure 6. Effect of SDCLE concentration on zebrafish trajectory distance. Representative swimming paths for the transition from high locomotor activity (red) to low locomotor activity (green) and inactivity (black) as the concentration increased from 0 to 300 mg/L. Statistical significance was set at $p < 0.05$.

h increased progressively from 5 s in the controls to 32 s at 300 mg/L (Fig. 5A), representing a six-fold increase. Total distance traveled decreased from 240 cm in the control group to 155 cm at 300 mg/L (Fig. 5B), corresponding to a 35% reduction in swimming activity. Notably, similar patterns were observed at lower concentrations for the NCLE (Fig. 5C, D), with behavioral changes observable at 15 mg/L, a lower threshold than for the SDCLE, indicating an earlier onset of neurobehavioral effects.

Trajectory mapping showed that control fish exhibited extensive red zones (high activity) distributed throughout the

arena (Fig. 6). At 300 mg/L, the trajectories of the SDCLE group shifted predominantly to green zones (low activity) and black zones (inactivity), with swimming paths restricted to approximately 40% of the arena area. This spatial restriction corresponded with the quantitative data, which showed a six-fold increase in inactivity and a 35% decrease in swimming distance at 300 mg/L. Both concentration- and time-dependent patterns were evident, with effects intensifying from 24 to 96 h across all tested concentrations.

DISCUSSION

The use of CLE for pharmacopuncture is limited by the associated poor aqueous solubility and the lack of safety data for whole-extract injectable formulations [4, 9, 14]. Previous research has focused exclusively on isolated curcumin; however, this study evaluated SDCLEs combined with microencapsulation agents [11, 14]. Spray drying resolves the reconstitution barrier while maintaining a safety profile compatible with neurobehavioral thresholds in zebrafish models [9, 22, 25]. To our knowledge, this integrated pharmaceutical and toxicological characterization provides the first comprehensive safety evaluation of SDCLE, establishing a regulatory foundation for pharmacopuncture dose selection [2, 10].

Spray-drying technology preserved curcuminoid integrity, as demonstrated by HP-TLC analysis (Fig. 1, Table 2). SDCLE (0.148 ± 0.02 mg/g) and NCLE (1.46 ± 0.03 mg/g) exhibited proportional curcuminoid concentrations at a 1:10 ratio, with curcumin remaining the predominant constituent. Method validation showed linearity ($r > 0.99$) and peak purity (> 0.995), with no degradation peaks, confirming that the lactose matrix did not interfere with curcuminoid stability [31]. Chromatographic fingerprinting enables batch-to-batch consistency required for regulatory compliance [4, 20, 31]. This technology produces stable powders that are compatible with terminal sterilization and reconstitution in variable volumes for clinical use [5, 32, 33]. The 64.30% recovery yield maintained a consistent active extract content (10% w/w) within pharmaceutical standards (Table 1) [34], supporting GMP-compliant production of sterile injectables [4, 35].

Spray-drying technology overcomes the solubility barrier by converting CLE from a semisolid extract (NCLE) into a free-flowing powder (SDCLE). Lactose microencapsulation (1:9 w/w)

produced microparticles with enhanced aqueous dispersion. SDCLE achieved 91.30% dissolution at a 1:30 dilution compared with 55.78% for NCLE, representing a 63.68% increase in solubility. Although most of the dissolved mass originated from the lactose matrix rather than the extract, the formulation enabled clear reconstitution without precipitation, as required for injection (Table 3, Fig. 2). Visual examination confirmed this improvement: SDCLE solutions remained clear with surface foam, whereas NCLE preparations showed heavy sedimentation. This approach eliminates the need for ethanol-based vehicles, which can cause tissue necrosis at acupoint sites [1, 5, 32].

Acute toxicity assessment showed concentration- and time-dependent responses over 96 h (Table 4, Fig. 3). The LC_{50} values for SDCLE decreased from 469.96 mg/L at 24 h to 288.13 mg/L at 96 h, representing a 38.70% reduction and indicating cumulative toxicity. The 96-hour LC_{50} value of 288.13 mg/L classifies the SDCLE as moderately toxic according to the OECD criteria [27, 36]. SDCLE exhibited lower toxicity than NCLE (96-hour $LC_{50} = 22.11$ mg/L), whereas spray-dried lactose demonstrated negligible toxicity ($LC_{50} = 123,787.05$ mg/L), confirming an associated inertness. Probit regression analysis showed steep dose-response curves with clear mortality thresholds (Fig. 4C, D): SDCLE maintained $> 95\%$ survival at concentrations ≤ 200 mg/L, whereas concentrations ≥ 300 mg/L produced progressively increasing mortality, reaching 80% by 96 h. Concentration-dependent yellow pigmentation appeared within 15 min at 2,000 mg/L but required 72 h at 300 mg/L, likely reflecting curcuminoid tissue accumulation and serving as an early warning indicator of systemic exposure [25, 27].

The toxicity profile reflects the complexity of herbal extracts, where biological effects result from synergistic or antagonistic interactions among curcuminoids, turmerones, polysaccha-

Table 4. Comparative acute toxicity parameters (LC_{50} , NOEC, and LOEC) of SDCLE and NCLE in adult zebrafish

Formulation	Time (hour)	LC_{50} (mg/L)	Calibration curve	95% CI	NOEC (mg/L)	LOEC (mg/L)
SDCLE	24	469.96	$y = 13.594x - 31.324$	352.47-587.45	234.98	305.47
	48	387.61	$y = 9.7740x - 20.299$	290.71-484.51	193.81	252.95
	72	371.12	$y = 9.7633x - 20.087$	278.34-463.90	185.56	241.23
	96	288.13	$y = 13.636x - 28.539$	216.10-360.16	144.07	187.28
NCLE	24	28.59	$y = 14.765x - 16.502$	21.44-35.73	14.29	18.58
	48	25.65	$y = 13.960x - 14.671$	19.23-32.06	12.82	16.66
	72	23.75	$y = 15.733x - 16.644$	17.81-29.68	11.87	15.43
	96	22.11	$y = 17.378x - 18.367$	16.58-27.63	11.05	14.36

rides, and minor alkaloids [14, 20]. Curcumin exhibits dose-dependent biphasic behavior; curcumin is cytoprotective at low concentrations through antioxidant mechanisms but becomes cytotoxic at higher concentrations *via* pro-oxidant activity and mitochondrial dysfunction [14]. The higher LC₅₀ of SDCLE compared to NCLE (288.13 vs. 22.11 mg/L) may reflect altered bioavailability arising from the lactose matrix, which can affect absorption and tissue distribution. This complexity underscores the need to evaluate whole-extract formulations rather than isolated compounds, as pharmacopuncture products encompass the entire phytochemical spectrum and exhibit matrix-mediated release characteristics [4, 20, 31].

Translating mortality data into regulatory safety thresholds required determining the NOEC and LOEC values (Table 4). Probit analysis yielded NOEC values of 144.07 mg/L for SDCLE and 11.05 mg/L for NCLE at 96 h, representing the highest concentrations at which no statistically significant mortality was observed. The corresponding LOEC values were 187.28 mg/L for SDCLE and 14.36 mg/L for NCLE, indicating the lowest concentrations at which significant mortality was observed. Behavioral analysis provides additional safety insights beyond those based solely on mortality thresholds. The narrow margin between the SDCLE (ratio of 0.50) indicates a limited safety buffer [25], requiring strict concentration controls, as concentrations above the NOEC approach lethal thresholds. These mortality-based benchmarks provide the foundation for interspecies extrapolation and for calculating human doses with appropriate safety factors for acupoint injection [3, 9, 22].

Neurobehavioral analysis showed that locomotor deficits emerged at sublethal concentrations, demonstrating that behavioral endpoints detect toxicity more sensitively than mortality endpoint measurements (Figs. 5, 6). The swimming parameters showed concentration- and time-dependent impairments in both formulations. At 96 h, fish exposed to 300 mg/L SDCLE showed a 35% reduction in distance traveled (155 cm vs. 240 cm in controls) and a six-fold increase in inactivity (32 s vs. 5 s) [29, 32]. The NCLE produced similar patterns at lower extract concentrations. Trajectory mapping showed that control fish exhibited extensive high-activity zones (red trajectories) (Fig. 6), whereas fish exposed to 300 mg/L displayed restricted swimming paths with predominantly low-activity (green) and inactive (black) zones. Behavioral suppression at 200-300 mg/L, where survival exceeded 80% at 48 h, indicates that neurobehavioral endpoints can detect toxicity before lethality [25, 26].

Behavioral assay sensitivity enables refinement beyond tra-

ditional mortality metrics. The integration of locomotor data with survival curves established 200 mg/L as a critical safety threshold, at which complete survival and unimpaired swimming activity were maintained. This finding has direct pharmacopuncture safety implications: acupoints are anatomically proximal to peripheral nerves, and the injected solutions are hypothesized to flow along meridians that follow neurovascular pathways [37]. Given this proximity and the potential for direct neurotoxic exposure, concentrations exceeding 200 mg/L pose an unacceptable risk. Although the mortality-based NOEC (144.07 mg/L) defines the primary regulatory threshold, behavioral data at 200 mg/L provide an additional safety margin for neurotoxicity assessment in this anatomically sensitive application.

The zebrafish model was validated for toxicological assessment in accordance with OECD Test Guideline 203 [23, 36]. Zebrafish share approximately 70% genomic homology with mammals and possess conserved neurotransmitter systems and locomotor circuits, enabling translational prediction of neurotoxic effects and facilitating high-throughput screening [24, 25]. However, limitations remain: waterborne exposure differs fundamentally from direct tissue injection in absorption kinetics and local tissue response, and the model cannot replicate acupoint-specific injection dynamics, immune-mediated responses, or the pharmacokinetic profiles associated with parenteral depot formation [25]. These limitations require bridging studies in mammalian models before clinical translation [3, 9, 25].

Translating zebrafish data into clinical applications requires conservative extrapolation, accounting for safety factors to account for interspecies and route-of-administration differences. Based on the mortality-derived NOEC of 144.07 mg/L and the behavioral safety threshold of 200 mg/L, combined with typical injection volumes (0.1-0.5 mL per acupoint), the proposed clinical concentration range is 1-10 mg/mL, with a 10-100-fold safety margin [1, 6].

Mammalian bridging studies remain essential for clinical translation [3, 9, 25]. Regulatory advancement requires GMP-compliant manufacturing, validated analytical methods, sterility testing, and batch consistency verification [4, 31, 34]. Meanwhile, clinical trials should adhere to STRICTA and CONSORT guidelines [1, 38, 39]. This framework, which integrates solubility optimization, preclinical safety characterization, and regulatory compliance, positions SDCLE for pharmacopuncture development pending validation in mammalian models.

CONCLUSION

This study established the pharmaceutical feasibility and preliminary safety of SDCLE for use in pharmacopuncture. Spray-drying enhanced aqueous dispersibility by 63.68% while preserving curcuminoid integrity, enabling sterile reconstitution for injection. Toxicological characterization identified a 96-hour LC_{50} of 288.13 mg/L and a mortality-based NOEC of 144.07 mg/L, with behavioral endpoints providing more sensitive detection of neurotoxicity than lethality at sublethal concentrations. The clinical concentration range of 1-10 mg/mL incorporates appropriate safety margins. Zebrafish screening provides foundational insights, while mammalian bridging studies are essential to confirm tissue tolerance and pharmacokinetic profiles. Nonetheless, with validated analytical methods and GMP-compliant manufacturing, SDCLE represents a viable pharmacopuncture formulation that requires mammalian validation before clinical implementation.

DECLARATION OF AI ASSISTANCE

The authors acknowledge the use of artificial intelligence (AI)-based tools to support language refinement, grammar checking, and paraphrasing during the manuscript preparation. The AI tools did not contribute to the research design, data collection, data analysis or interpretation of the results. The authors take full responsibility for the content, originality, and integrity of this manuscript.

AUTHORS' CONTRIBUTIONS

The authors confirm contribution to the paper as follows: study conception and design: I.K., I.S.S.; data collection: S.R.; analysis and interpretation of results: S.R., D.A.P., D.I.; draft manuscript preparation: S.R., I.K., I.S.S., Admin; technical or material support: A.P.F., R.R.R., M.S.A.B., M.A.N., C.V.P.P. All authors reviewed the results and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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