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Original Research Article

Potent *In Vitro* Anticancer Activity of a Chitosan-Based Nanoemulgel Co-Delivering Cynaropicrin and Salicin: Induction of Apoptosis and Inhibition of Migration in HCT 116 Colon Cancer Cells

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Abstract: The development of effective and targeted therapies for colorectal cancer remains a significant challenge. This study investigates the *in vitro* anticancer efficacy of a novel chitosan-based nanoemulgel co-delivering the natural compounds cynaropicrin and salicin against HCT 116 human colorectal cancer cells. An optimized nanoemulgel formulation (designated F2) was developed by incorporating an oil phase (MCT oil) containing lipophilic cynaropicrin into an aqueous phase containing hydrophilic salicin and chitosan (0.5% w/v), followed by high-shear homogenization, sonication, and incorporation into a Carbopol gel. This F2 formulation exhibited a mean particle size of 85.50 ± 0.44 nm, a low polydispersity index (PDI) of 0.272 ± 0.003, a zeta potential of -35.3 ± 0.2 mV, and high encapsulation efficiencies for cynaropicrin (91.80 ± 0.57%) and salicin (81.40 ± 0.85%), with sustained *in vitro* drug release over 24 hours. Cytotoxicity of F2 against HCT 116 cells, assessed by MTT assay, revealed a potent dose-dependent reduction in cell viability, with an IC_{50} value of approximately 42 µg/mL. Mechanistic studies demonstrated that the observed cytotoxicity was mediated, at least in part, through the induction of apoptosis. Annexin V-FITC/PI staining followed by flow cytometry showed a significant, concentration-dependent increase in both early and late apoptotic cell populations, with an estimated apoptosis IC_{50} of ~55 µg/mL. Furthermore, the nanoemulgel significantly inhibited HCT 116 cell migration in a dose-dependent manner, as determined by the scratch (wound healing) assay. At 48 hours, the highest concentration of F2 (5.00 µg/mL) reduced wound closure to 5.0%, compared to 28.3% in untreated controls. These findings highlight the potent cytotoxic, pro-apoptotic, and anti-migratory effects of the cynaropicrin and salicin co-loaded nanoemulgel, suggesting its promising potential as a multi-modal therapeutic strategy for colorectal cancer.

Keywords: Nanoemulgel, Chitosan, Cynaropicrin, Salicin, Colorectal Cancer, HCT 116, Apoptosis, Cell Migration, Anticancer Activity.

1. INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer-related mortality worldwide, and despite advancements in treatment modalities, significant challenges remain, including systemic toxicity of conventional chemotherapeutics and the development of drug resistance (Yang *et al.*, 2022). Natural compounds offer a rich source of potential anticancer agents with diverse mechanisms of action and potentially lower toxicity profiles (Ding *et al.*, 2021a). Cynaropicrin, a sesquiterpene lactone, and salicin, a glycoside, have individually demonstrated promising anti-inflammatory and anticancer properties (Elsebai *et al.*, 2017; Vekaria &Tirgar, 2021).

The co-delivery of these agents using advanced drug delivery systems could enhance their therapeutic efficacy. Nanoemulgels, which combine nanoemulsions with hydrogels, offer a versatile platform for delivering drugs with differing solubilities while providing sustained release and improved stability (Siddiqui *et al.*, 2020). Chitosan, a natural biopolymer, is well-suited for such systems due to its biocompatibility, mucoadhesion, and gelling properties (Islam *et al.*, 2016).

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This study focused on the development and characterization of a chitosan-based nanoemulgel for the co-delivery of cynaropicrin and salicin, followed by an evaluation of its *in vitro* anticancer efficacy against HCT 116 human colorectal cancer cells. An optimized formulation (designated F2 in developmental stages, and referred to as "nanoemulgel" or "cynaropicrin-salicin nanoemulgel" in this paper) was selected for biological studies based on its favorable physicochemical properties. The investigation focused on assessing its cytotoxic effects, its ability to induce apoptosis, and its impact on cancer cell migration.

2. MATERIALS AND METHODS

2.1. Materials

Cynaropicrin (≥ 98% TLC, Sigma-Aldrich) and Salicin (Pharmacopeial grade, Loba Chemie) were used as active pharmaceutical ingredients. Chitosan (medium-MW, ≥ 85% deacetylation, Bangalore Fine-Chem), Medium-Chain Triglyceride (MCT) Oil (Caprylic/Capric triglycerides, Thermo Fisher Scientific), Tween 80 (Polysorbate 80, Loba Chemie), Propylene Glycol (Bangalore Fine-Chem), Carbopol 940 (Sigma-Aldrich/Lubrizol), glacial acetic acid (Loba Chemie), methanol (HPLC grade, Thermo Fisher Scientific), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (all from Thermo Fisher), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Doxorubicin, Staurosporine, Epidermal Growth Factor (EGF) (all from Sigma-Aldrich), Annexin V-FITC/Propidium Iodide (PI) apoptosis detection kit (Thermo Fisher Invitrogen), and all other reagents were of analytical or cell culture grade and used as received.

2.2. Preparation of Cynaropicrin-Salicin Nanoemulgel

The nanoemulgel investigated in this study was prepared as follows: The aqueous phase consisted of chitosan (0.5% w/v) dissolved in 1% v/v glacial acetic acid, into which salicin (100 mg/100mL) was dissolved. The pH was adjusted to 4.5-5.0. The oil phase comprised cynaropicrin (100 mg/100mL) dissolved in MCT oil (5% v/v), with Tween 80 (2% w/v) and propylene glycol (1% w/v) as surfactant and co-surfactant, respectively. The oil phase was added to the aqueous phase under high-speed homogenization (12,000 rpm, 4 min), followed by probe sonication (40% amplitude, 5 min intermittent cycles). The resulting nanoemulsion was then incorporated into a 0.5% w/v Carbopol 940 gel base (pH adjusted to 6.0-7.0 with triethanolamine) in a 1:1 (w/w) ratio.

2.3. Physicochemical Characterization of the Nanoemulgel

The prepared nanoemulgel (specifically, its nanoemulsion component prior to gel incorporation for size, PDI, zeta, and the final emulgel for EE and release) was characterized for:

- **Particle Size, PDI, and Zeta Potential:** Measured by Dynamic Light Scattering (DLS) (Zetasizer Nano ZS90, Malvern Instruments).
- **Encapsulation Efficiency (EE%):** Determined by separating free drug from encapsulated drug and quantifying free drug using UV-Visible spectrophotometry (IgeneLabserve, IG-2100) against calibration curves. EE% = [(Total drug – Free drug) / Total drug] × 100.
- ***In Vitro* Drug Release:** Studied using Franz diffusion cells (LabSmith, FD-3000) with a dialysis membrane (MWCO 12-14 kDa) and PBS (pH 7.4) as receptor medium at 37°C over 24 hours.

2.4. Cell Culture

HCT 116 human colorectal cancer cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin, maintained at 37°C in a 5% CO_2 humidified incubator.

2.5. MTT Cytotoxicity Assay

HCT 116 cells were seeded (5×10^3 cells/well) in 96-well plates. After 24h adherence, cells were treated with the nanoemulgel (containing total drug concentrations of 12.5, 25, 50, and 100 µg/mL) for 48 hours. Untreated cells (Negative Control, NC) and Doxorubicin-treated cells (5 µg/mL, Positive Control, PC) were included. MTT solution (20 µL, 5 mg/mL) was added for 3-4 hours. Formazan was dissolved in DMSO (100 µL), and absorbance was read at 570 nm. Cell viability (%) = (OD_treated / OD_NC) × 100. IC_{50} was calculated.

2.6. Apoptosis Assay (Annexin V-FITC/PI Staining)

Cells were seeded in 6-well plates, treated with nanoemulgel (25, 50, 100 µg/mL total drug) for 24 hours. NC and Staurosporine-treated cells (1 µM, PC) were included. Cells were harvested, stained with Annexin V-FITC and PI, and analyzed by flow cytometry (BD Accuri C6 Plus). Percentages of live, early/late apoptotic, and necrotic cells were determined.

2.7. Cell Migration (Scratch) Assay

Cells grown to near-confluence in 6-well plates were scratched with a pipette tip. After washing, cells were treated with nanoemulgel (1.25, 2.50, 5.00 µg/mL total drug) in low-serum medium. NC (medium only) and EGF-treated cells (PC) were included. Images were taken at 0, 12, 24, 48 hours. Wound closure (%) = [(Initial Area - Area_t) / Initial Area] × 100.

2.8. Statistical Analysis

Data (mean ± SD, n=3) were analyzed by one-way ANOVA with Tukey's test (GraphPad Prism); $p < 0.05$ was considered significant.

3. RESULTS

3.1. Physicochemical Characterization of the Cynaropicrin-Salicin Nanoemulgel

The cynaropicrin-salicin nanoemulgel selected for biological evaluation exhibited the following key physicochemical properties:

- **Particle Size, PDI, and Zeta Potential:** The nanoemulsion component of the emulgel had a mean particle (droplet) size of 85.50 ± 0.44 nm. The Polydispersity Index (PDI) was 0.272 ± 0.003, indicating a relatively uniform size distribution. The zeta potential was measured to be -35.3 ± 0.2 mV, suggesting good colloidal stability due to electrostatic repulsion.
- **Drug Encapsulation Efficiency (EE%):** The EE% for cynaropicrin was 91.80 ± 0.57%, and for salicin, it was 81.40 ± 0.85%. These values indicate efficient entrapment of both the lipophilic and hydrophilic drugs within the nanoemulgel system.
- ***In Vitro* Drug Release:** The nanoemulgel demonstrated sustained release of both cynaropicrin and salicin over a 24-hour period. Approximately 57% of both cynaropicrin and salicin were released by 6 hours, with nearly 100% cumulative release observed for both drugs by 24 hours (Figure 1A and 1B – *You would insert graphs showing the release profiles here*).

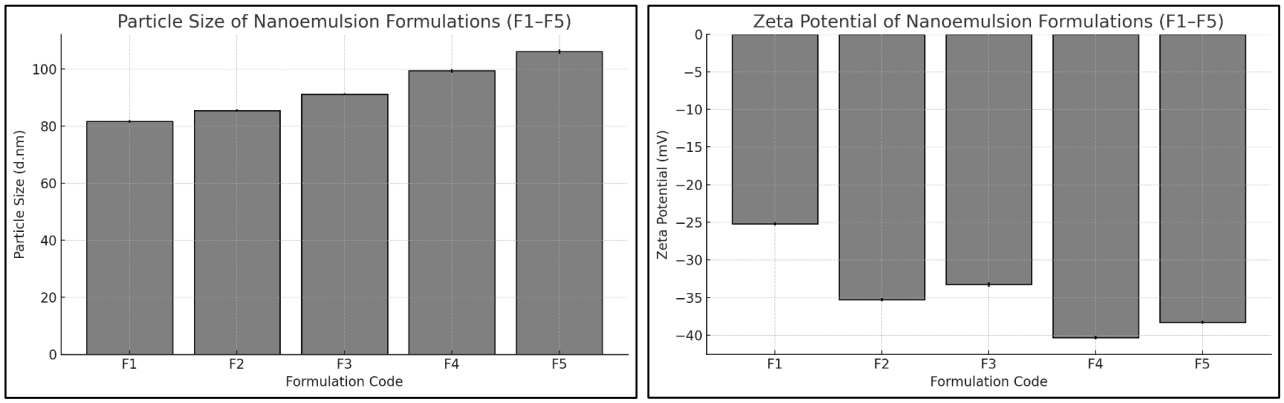

Fig. 1A: Particle size and Zeta potential for F1-F5

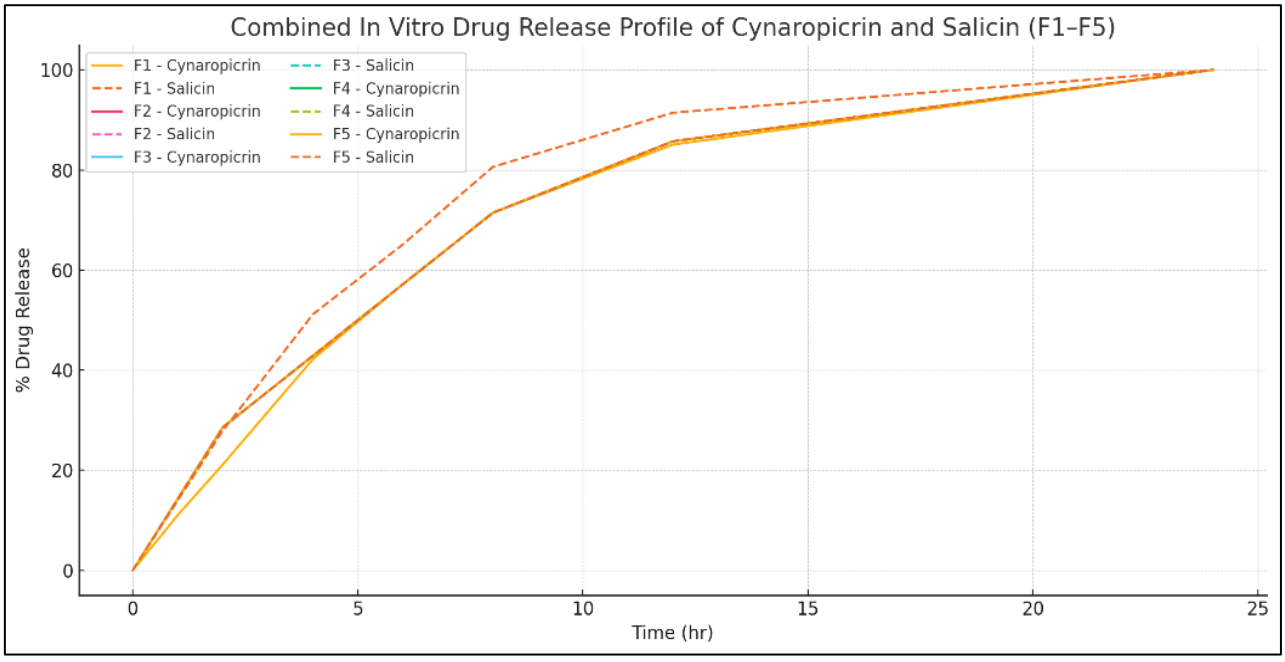

Fig. 1B: *In Vitro* Drug Release for Formulation 1-5

3.2. Cytotoxicity of Nanoemulgel against HCT 116 Cells

The cynaropicrin-salicin nanoemulgel induced a dose-dependent reduction in HCT 116 cell viability after 48 hours of treatment.

Table 1: Cell Viability of HCT 116 Cells after 48h Treatment with Cynaropicrin-Salicin Nanoemulgel (Mean ± SD, n=3) Formulation 2

Treatment Group	Concentration (µg/mL Total Drug)	% Viability ± SD
Negative Control (Untreated)	—	100 ± 1.27
Positive Control (Doxorubicin)	5	30 ± 0.98
Cynaropicrin-Salicin Nanoemulgel	12.5	60 ± 0.43
	25	50 ± 0.66
	50	38 ± 0.62
	100	32 ± 0.77

The IC_{50} value for the nanoemulgel against HCT 116 cells was calculated to be approximately 42 µg/mL (total drug equivalent). ANOVA indicated significant differences between all treatment groups and the negative control ($p < 0.05$).

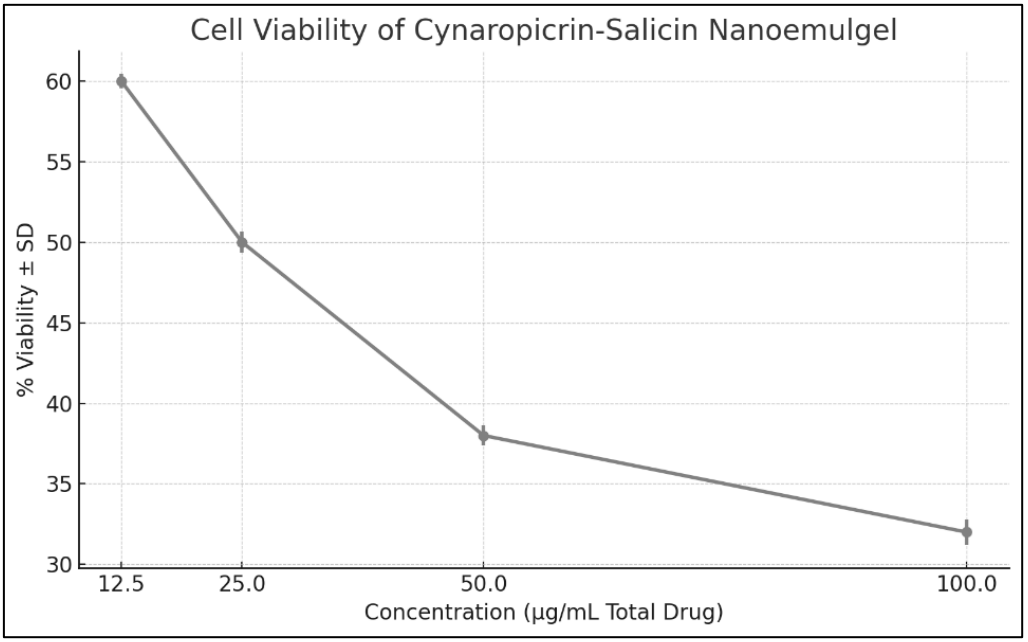

Fig. 2: Cell Viability of HCT 116 Cells after 48h Treatment with Formulation 2

3.3. Induction of Apoptosis by Nanoemulgel

The nanoemulgel induced apoptosis in HCT 116 cells in a concentration-dependent manner.

Table 2: Apoptosis Profile of HCT 116 Cells after 24h Treatment with Cynaropicrin-Salicin Nanoemulgel (Mean ± SD, n=3)

Treatment Group	Concentration (µg/mL Total Drug)	Live Cells (%)	Early Apoptosis (%)	Late Apoptosis/ Necrosis (%)	Total Apoptosis (%)
Negative Control (Untreated)	—	94.0 ± 1.5	3.5 ± 0.5	2.3 ± 0.4	5.8 ± 0.8
Positive Control (Staurosporine)	1 µM	40.0 ± 3.0	20.0 ± 2.0	35.0 ± 3.5	55.0 ± 4.0
Cynaropicrin-Salicin Nanoemulgel	25	85.6 ± 2.5	8.1 ± 1.0	2.0 ± 0.5	10.1 ± 1.2
	50	75.0 ± 3.5	12.6 ± 1.5	10.2 ± 1.2	22.8 ± 2.0
	100	60.0 ± 4.0	15.0 ± 1.8	20.0 ± 2.5	35.0 ± 3.0

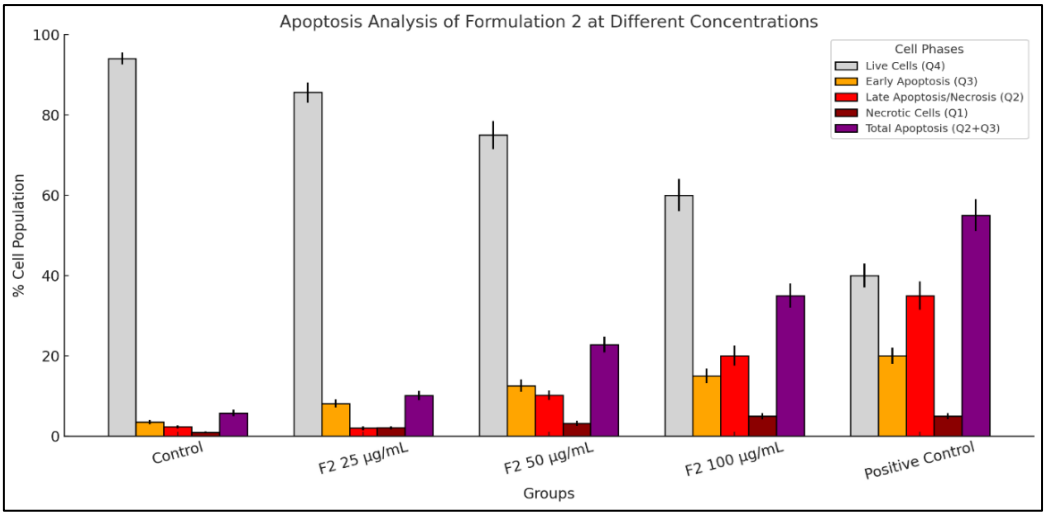

Fig. 3A: Apoptosis Analysis of formulation 2

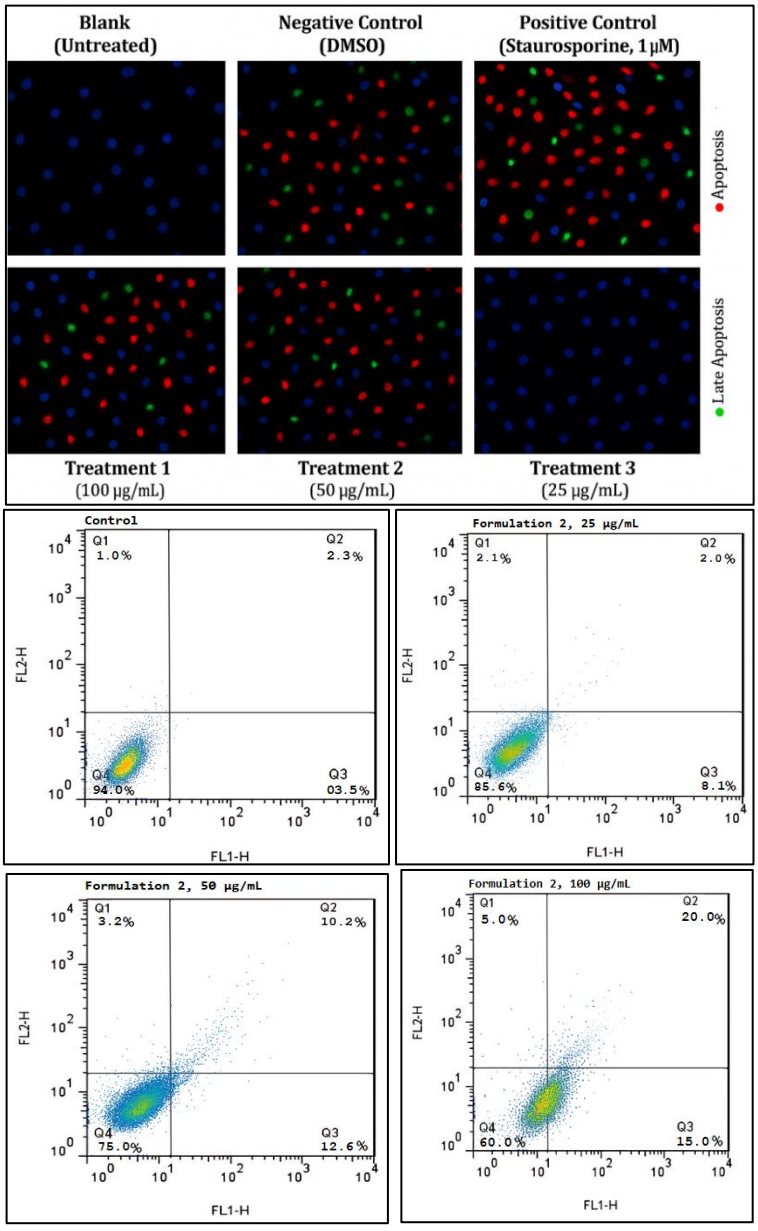

Fig. 3B: Apoptosis Analysis of formulation 2 at different concentrations

Treatment with the nanoemulgel significantly increased the percentage of early and late apoptotic cells compared to the negative control ($p < 0.001$). The estimated IC_{50} for apoptosis induction (50% total apoptotic cells) was approximately 55 µg/mL.

3.4. Inhibition of Cell Migration by Nanoemulgel

The nanoemulgel significantly inhibited HCT 116 cell migration in a dose-dependent manner.

Table 3: Wound Closure (%) in HCT 116 Cells Treated with Cynaropicrin-Salicin Nanoemulgel (Mean ± SD, n=3)

Treatment Group	Concentration (µg/mL Total Drug)	Wound Closure (%) at 12h	Wound Closure (%) at 24h	Wound Closure (%) at 48h
Negative Control (Untreated)	—	9.0 ± 2.0	17.0 ± 3.0	28.3 ± 2.0
Positive Control (EGF)	(Std. Conc.)	24.7 ± 3.5	42.7 ± 5.0	87.3 ± 3.0
Cynaropicrin-Salicin Nanoemulgel	1.25	5.3 ± 0.6	9.0 ± 1.0	17.0 ± 1.0
	2.5	3.3 ± 0.6	5.0 ± 1.0	11.0 ± 1.0
	5	1.3 ± 0.6	2.0 ± 1.0	5.0 ± 1.0

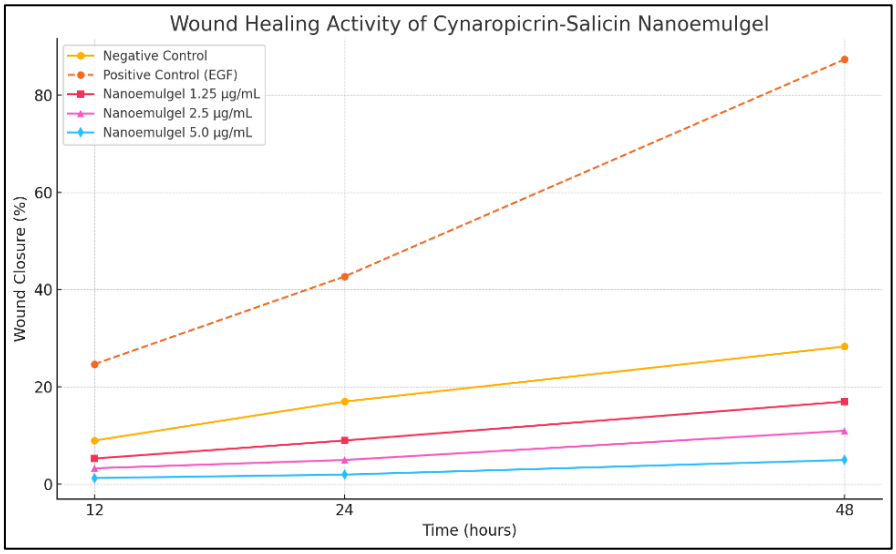

Fig. 4A: Wound healing Activity of the Formulation 2

The nanoemulgel significantly reduced wound closure at all tested concentrations and time points compared to the negative control ($p < 0.05$).

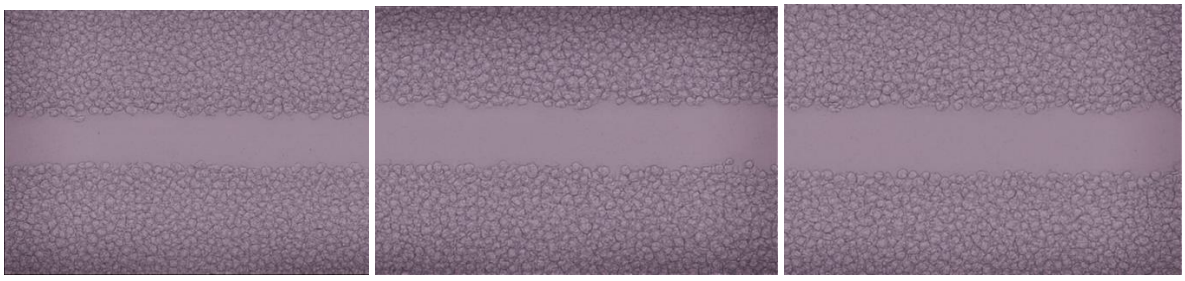

Fig. 4B: Wound healing Activity of Negative Control 12, 24, 48 hours

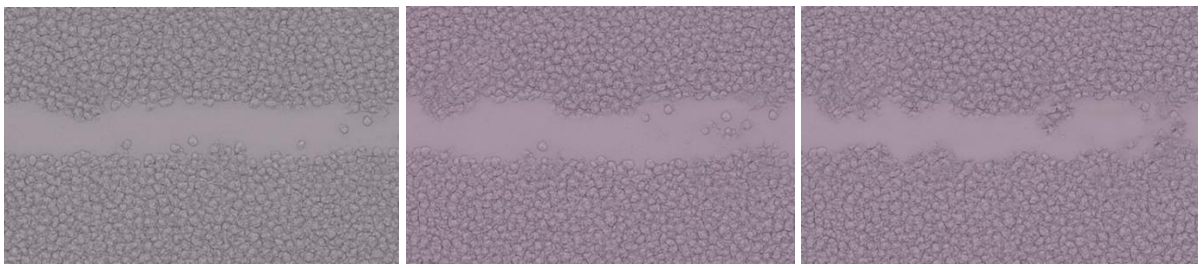

Fig. 4C: Wound healing Activity of Test Control 12, 24, 48 hours

4. DISCUSSION

This study successfully demonstrates the potent *in vitro* anticancer efficacy of a well-characterized chitosan-based nanoemulgel co-delivering cynaropicrin and salicin against HCT 116 colorectal cancer cells. The nanoemulgel formulation exhibited desirable physicochemical properties for drug delivery, including a small particle size (~85 nm), low PDI, good colloidal stability (zeta potential ~-35 mV), high encapsulation efficiency for both drugs, and sustained *in vitro* drug release (Section 3.1). These characteristics are crucial for enhancing the therapeutic potential of the encapsulated compounds.

The nanoemulgel induced significant dose-dependent cytotoxicity in HCT 116 cells, with an IC_{50} of ~42 µg/mL (Table 1). This potent effect is likely attributable to the efficient delivery of the combined active agents, cynaropicrin and salicin, which may act synergistically or additively to inhibit cancer cell proliferation (Ding *et al.,* 2021a; Vekaria &Tirgar, 2021). The nano-carrier system can improve drug solubility, protect drugs from premature degradation, and enhance cellular uptake.

Mechanistic investigations confirmed that apoptosis is a key pathway through which the nanoemulgel exerts its cytotoxic effects (Table 2). The concentration-dependent increase in both early and late apoptotic cells (apoptosis IC_{50} ~55 µg/mL) aligns with the known pro-apoptotic mechanisms of cynaropicrin and the potential contribution of salicin (Srivastava *et al.*, 2013). The ability to effectively trigger programmed cell death is a hallmark of successful anticancer agents.

Furthermore, the nanoemulgel demonstrated a remarkable capacity to inhibit HCT 116 cell migration (Table 3). This anti-migratory activity is of particular importance, as cancer cell migration and invasion are critical steps in metastasis, the primary cause of cancer-related mortality (Dong *et al.,* 2021b). By impeding cell motility, the nanoemulgel shows potential not only to control primary tumor growth but also to limit the spread of cancer cells. The multi-modal therapeutic action observed—cytotoxicity, apoptosis induction, and migration inhibition—positions this nanoemulgel as a promising candidate for CRC therapy. The sustained release profile of the drugs from the chitosan-based system likely contributes to these prolonged biological effects (Islam *et al.,* 2016; Siddiqui *et al.,* 2020).

5. CONCLUSION

The cynaropicrin and salicin co-loaded chitosan-based nanoemulgel exhibited desirable physicochemical characteristics and demonstrated potent *in vitro* anticancer activity against HCT 116 colorectal cancer cells. It effectively induced cytotoxicity and apoptosis, and significantly inhibited cancer cell migration. These findings underscore the potential of this nano-formulation as a multi-modal therapeutic strategy for colorectal cancer, warranting further *in vivo* efficacy and safety evaluations to explore its translational potential.

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