RESEARCH PAPER

Anticancer Activity of Polyherbal Nanoparticles from Curcuma longa, Tinospora cordifolia, and Moringa oleifera (CTM-NPs) against HT-29 Colorectal Cancer Cells: Evaluation of Cytotoxicity, Oxidative Stress, and Mitochondrial Dysfunction.

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Abstract

Background: Colorectal cancer (CRC) has emerged as an important global health issue, thereby requiring the identification of new approaches for its treatment. Natural products such as phytochemicals from Curcuma longa, Tinospora cordifolia, and Moringa oleifera have shown promising results against colon cancer; however, their use has been hampered by low bioavailability. The current study was conducted to prepare and evaluate CTM-NPs against HT-29 colorectal cancer cells.

Methods: Synthesis of nanoparticles of single plants extract and polyherbal formulation (CTM-NPs) was performed by employing chitosan ionic gelation method. Cytotoxicity on HT-29 cells was investigated using MTT assay for 24 h to determine IC_{50} value. Morphological analysis of apoptosis was carried out by AO/PI staining. Intracellular generation of reactive oxygen species (ROS) was performed by using DCFH-DA assay, and disruption of mitochondrial membrane potential ($\Delta\Psi m$) was carried out by using JC-1 fluorescent dye.

Results: The CTM-NPs were highly cytotoxic (IC_{50} = ~40 µg/mL) compared with single nanoparticle forms of Curcuma longa (IC_{50} = ~160 µg/mL), Tinospora cordifolia (IC_{50} = ~80 µg/mL), and Moringa oleifera (IC_{50} = ~160 µg/mL). The CTM-NP-mediated apoptosis was indicated by characteristic morphological features such as chromatin condensation and blebbing of the plasma membrane in AO/PI-stained cells. Moreover, treatment of cells with CTM-NPs resulted in a significant rise in intracellular reactive oxygen species and mitochondrial membrane depolarization.

Conclusion: The newly developed polyherbal CTM-NPs possess excellent antitumor effect on HT-29 colon cancer cells. This is due to the mode of action, which involves the synergistic cytotoxicity effect, oxidative stress, and mitochondrial pathway of apoptosis. This therefore makes CTM-NPs a very promising nanoparticle that can be considered for the treatment of colorectal cancer.

Keywords: Polyherbal nanoparticles; Colorectal cancer; HT-29 cells; Apoptosis; Curcuma longa; Tinospora cordifolia; Moringa oleifera.

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Introduction

Colorectal cancer (CRC) continues to pose a huge health risk all over the world, being the third most common type of cancer and the second major source of cancer-associated fatalities. There are more than 1.9 million incidences of CRC each year, accounting for approximately 935,000 deaths; thus, it is vital to develop innovative treatment methods that are effective and less toxic [1]. Classical methods such as surgery, chemotherapy (5-fluorouracil, oxaliplatin), radiotherapy, and target therapy are limited due to toxicity issues and recurrence rates [2]. These factors have brought about a shift towards integrative oncology with the use of phytochemicals [3,4].

Ayurveda, an ancient Indian form of medicinal practice, has traditionally relied on the use of polyherbal drug formulation (Yoga or Sanyoga) to produce synergistic treatment effects without causing toxicities to individuals [5,6]. Phytochemicals such as curcuminoids, alkaloids, diterpenoids, and flavonoids show great potential in preventing proliferation, promoting apoptosis and inhibiting metastasis in many types of cancers [7,8]; however, they are poorly translatable into clinical practice because of their limited aqueous solubility and fast metabolism [9].

This challenge can be resolved through nanotechnology. Nano-particle based delivery methods, especially those incorporating biodegradable polymers such as chitosan, would improve the solubility, stability and uptake of bioactive phytochemicals [10,11].

Curcuma longa L. (Turmeric; family Zingiberaceae) is well-known for its potential to produce curcumin that can generate oxidative stress, NF-κB inhibition, and apoptosis through mitochondrial apoptosis pathway [12,13]. But because of poor pharmacokinetic properties of curcumin, it becomes necessary to use it in the form of its nanofractionation [14]. Tinospora cordifolia (Willd.) Miers (Giloy; family Menispermaceae), which is considered a “Rasayana” herb as per Ayurveda, exhibits immune-modulating and anticancer activity due to the presence of its diterpenoids (tinocordifolioside) and alkaloids (berberine) [15,16]. T. cordifolia, in the form of its nano-formulation, shows better cell penetration and cytotoxicity [17]. Moringa oleifera Lam. (Drumstick tree; family Moringaceae) contains abundant amounts of flavonoids such as quercetin and kaempferol, as well as specific isothiocyanates known as niazimicin, that have antiproliferative properties via ROS production, cell cycle arrest, and metastasis inhibition [18,19,20]

Considering the complimentary modes of action of the three medicinal plants, it was anticipated that the combination of these three plants in a nanoparticle formulation (CTM-NPs) would have cytotoxic, oxidative, and mitochondrial activity on CRC cells through synergy [21,22]. Hence, the objectives of this research work included: (i) preparation and characterization of CTM-NPs; (ii) investigation of the cytotoxic effects of CTM-NPs on HT-29 human colorectal adenocarcinoma cells, as compared to individual nanoparticles; and (iii) investigation of the mode of action based on ROS production and mitochondrial membrane potential alteration.

2. Materials and Methods

Materials: Hexane, chitosan (medium molecular weight, 85% deacetylated), acetic acid, sodium tripolyphosphate (TPP), and sodium hydroxide were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, L-glutamine, and trypsin-EDTA were obtained from HiMedia Laboratories (India). MTT, acridine orange (AO), propidium iodide (PI), 2’,7’-dichloro-dihydro-flu fluorescein diacetate (DCFH-DA), and JC-1 dye were from Sigma-Aldrich (St. Louis, MO, USA). All reagents were of analytical grade.

Plant Materials and Extraction: Rhizomes of C. longa, stems of T. cordifolia, and leaves of M. oleifera were identified by a botanist (voucher samples). Plant materials were cleaned, shade-dried (10-12 days), and ground to powder form. Extraction was done using Soxhlet extractor with hexane (300-400 ml, 6-8 hours, 60-65°C). Extracts were filtered (Whatman No. 1 filter paper) and concentrated by reduced pressure (rotary evaporator, 40-45°C).

Synthesis of Nanoparticles: Nanoparticles of individual and polyherbal formulations (CTM-NPs) were prepared by ionic gelation method using chitosan and TPP [17].

Preparation of chitosan solution: 0.2% w/v chitosan was dissolved in 1% v/v acetic acid solution with overnight stirring at 600 rpm and pH adjusted to 5.0-5.5 with 1M NaOH.

Nanoparticles loaded with extracts: Hexane extracts (individual or 1:1:1 combination of polyherbs) were suspended in ethanol to make the least amount possible and then mixed in the chitosan solution (final concentration of the extracts was 1 mg/mL). The TPP solution (0.1% w/v) was added gradually (700-800 rpm) until the opalescence was observed, which indicated nanoparticle formation. Further stirring was performed for 30 minutes. After that, the suspension was centrifuged (12,000 rpm, 20 min, 4°C), washed twice with distilled water, and freeze-dried. Nanoparticles were stored at 4°C Table 1.

Table -1. Physicochemical Characterisation of Nanoparticle Formulations (mean ± SD, n = 3)

Formulation	Particle Size (nm)	PDI	Zeta Potential (mV)	EE (%)	pH
C. longa-NPs	187.4 ± 8.2	0.243 ± 0.018	+32.1 ± 2.4	82.3 ± 3.1	11.7 ± 0.9
T. cordifolia-NPs	164.7 ± 6.9	0.221 ± 0.015	+28.7 ± 1.9	78.9 ± 2.8	10.2 ± 0.7
M. oleifera-NPs	192.3 ± 9.1	0.258 ± 0.021	+30.5 ± 2.1	80.1 ± 3.4	12.1 ± 1.1
CTM-NPs	148.6 ± 5.3*	0.198 ± 0.012*	+35.8 ± 2.7*	91.4 ± 2.2*	9.8 ± 0.6*

PDI: Polydispersity Index; EE%: Encapsulation Efficiency; * $p < 0.05$ vs all individual nanoparticle formulations (one-way ANOVA, Tukey's post-hoc). All particles showed spherical morphology on TEM.

Cell Culture: Human colorectal adenocarcinoma cells (HT-29; NCCS, Pune, India) were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin (100 U/mL, 100 µg/mL), and 1% L-glutamine (2 mM) in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were subcultured at 70–80% confluence using 0.25% trypsin-EDTA. Experiments used passages 3–10.

Cytotoxicity assay (MTT): The cells (1 x 10^4/well in 96-well plates) were exposed to NPs (10–200 µg/mL, 24 h). Unexposed cells were served as negative controls. MTT (20 µL, 0.5 mg/mL) was incubated for 4 h followed by dissolution of formazan crystals using DMSO (100 µL). The absorbance was read at 570 nm wavelength (microplate reader). The cell viability (%) = (Absorbance treated / Absorbance control) × 100 IC50 was determined by nonlinear regression (GraphPad Prism9)

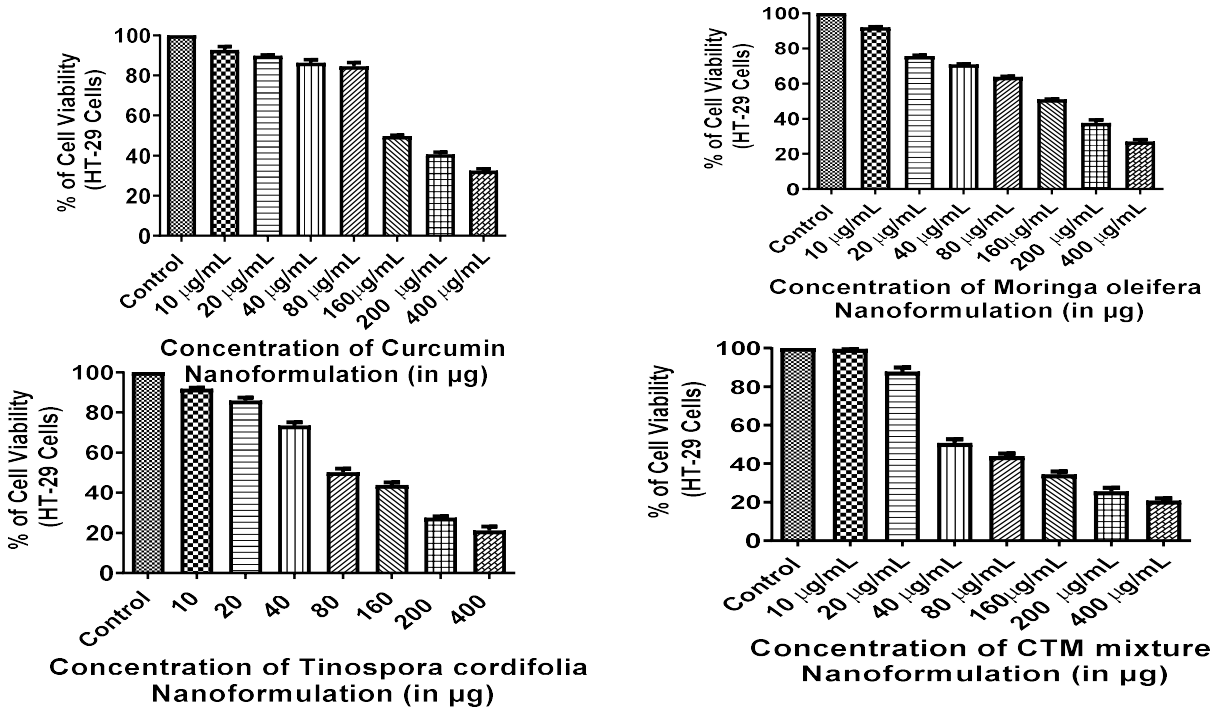
Apoptosis Assay (AO/PI Double Staining)
HT-29 cells (2×10^5 per well in 6-well plates) were subjected to treatment with IC_{50} values of nanoparticles for 24 h. Cells were washed with PBS and stained with AO/PI (10 µg/mL of each, 5 min, dark). Fluorescence microscopy was used to visualize viable (green, intact), early apoptosis (green, condensed chromosomes), late apoptosis (orange/red, condensed), and necrosis (red, intact) cells.

Intracellular ROS Measurement: Intracellular ROS measurement was done by using DCFH-DA. Cells were washed after treatment (IC_{50}, 24 h). The samples were then incubated with 10 µM DCFH-DA (30 min, 37°C, dark), washed, and read for fluorescence (ex/em 485/53 nm). Results expressed as percentage of untreated control.
Mitochondrial Membrane Potential (ΔΨm) Determination: For determination of ΔΨm, JC-1 dye was used. After treatment of cells with IC_{50} dose for 24 hours, cells were stained with 5 µg/mL JC-1 dye at 37°C for 20 minutes in darkness. Cells were washed and fluorescence intensity was determined with microplate reader as red/green ratio.

Statistical Analysis: Experiments were conducted in triplicates (n=3). Mean ± SD values were used for analysis. One-way ANOVA with Tukey's multiple comparison post-hoc test (GraphPad Prism 9) was carried out. *p* < 0.05 was considered significant.

Results
Cytotoxic Effect of the Nanoparticles Against HT-29 Cell Line
All nanoparticles were cytotoxic in a dose-dependent manner (Fig. 1). The IC_{50} values were as follows: C. longa-NPs ≈ 160 µg/mL; M. oleifera-NPs ≈ 160 µg/mL; T. cordifolia-NPs ≈ 80 µg/mL. Notably, CTM-NPs had the lowest IC_{50} (≈40 µg/mL), demonstrating a two- and fourfold increase in potency relative to T. cordifolia-NPs and the rest individual (***p* < 0.001 vs. all individual formulations), indicating strong synergistic interaction (Table 2).

MTT Assay – Bar graph showing % cell viability vs. concentration for all four formulations, with IC_{50} values indicated)



(MTT Assay Figure:1)

Figure 1: MTT Assay – Bar graph showing % cell viability vs. concentration for all four formulations, with IC_{50} values indicated)

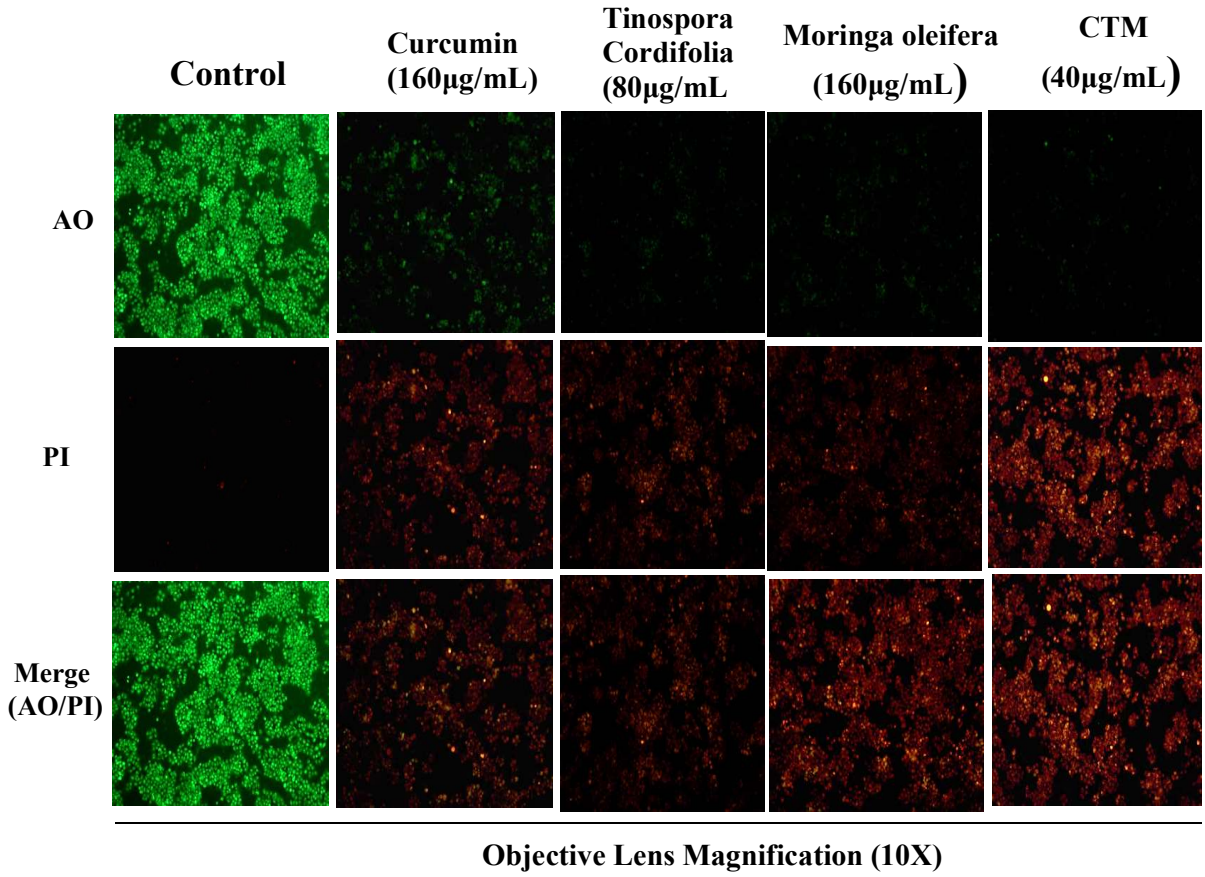
Table-1 Quantitative and Statistical Analysis of IC_{50} Values and Synergistic Activity of Nanoparticle Formulations in HT-29 Cells

Formulation	IC_{50} (µg/mL, mean ± SD)	Fold Difference vs CTM-NPs	Combination Index (CI)	Statistical Significance
C. longa-NPs	160.2 ± 9.4	4.0×	—	$p < 0.001$
T. cordifolia-NPs	79.8 ± 6.1	2.0×	—	$p < 0.001$
M. oleifera-NPs	158.6 ± 8.7	3.97×	—	$p < 0.001$
CTM-NPs	40.3 ± 3.2*	1.0× (ref)	Synergistic (CI < 1)	—
5-FU (positive control)	22.7 ± 2.1	—	—	$p < 0.001$ vs CTM-NPs

5-FU: 5-Fluorouracil (positive control); CI: Combination Index (Chou-Talalay method; CI < 1: synergism); * $p < 0.001$ vs all individual formulations (ANOVA, Tukey). Data represent mean ± SD from three independent experiments.

Apoptosis Induced Using AO/PI Double Staining

Untreated control cells displayed uniform green coloration of their intact nuclei (indicating viability). Individual nanoparticle treatments were found to induce early apoptosis (green coloration and condensation of the chromatin), while CTM-NPs induced more prominent effects—namely, an increased number of cells displaying the late apoptosis/necrosis characteristics (strongly coloured orange-red nucleus, membrane blebbing, and apoptotic bodies) (Figure 2). Apoptotic index of the CTM-NP group was found to be higher compared to controls and individual NPs groups (**p* < 0.01).



(Figure 2: AO/PI Assay – (A) Fluorescence micrographs showing staining patterns;

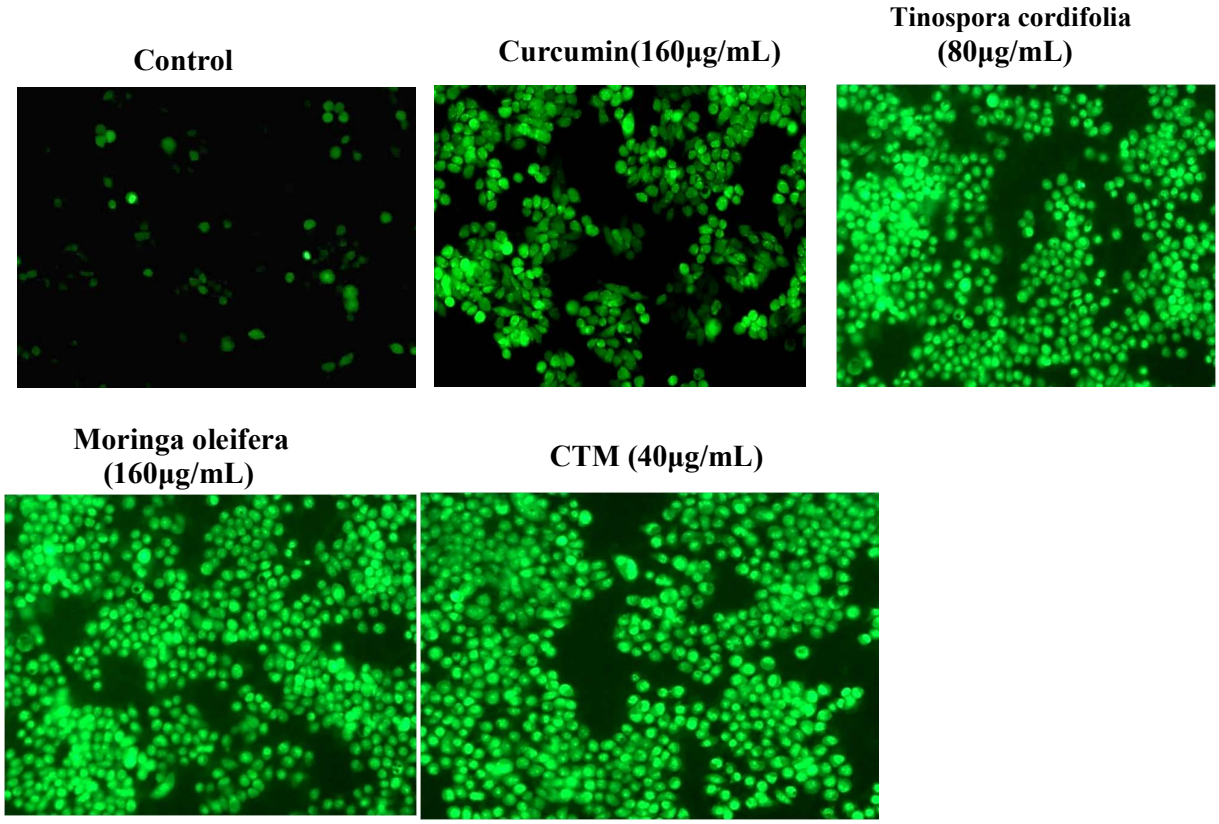
Table-3: Quantitative and Statistical Analysis of AO/PI-Based Apoptosis in HT-29 Cells

Treatment	Viable (%)	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis (%)	Total Apoptotic Index (%)
Control	91.3 ± 2.1	5.2 ± 1.0	2.8 ± 0.6	0.7 ± 0.2	8.7 ± 1.4
C. longa-NPs	68.4 ± 3.5	18.7 ± 2.3	9.6 ± 1.4	3.3 ± 0.7	31.6 ± 3.5 ($p<0.01$)
T. cordifolia-NPs	60.2 ± 4.1	22.4 ± 3.1	13.1 ± 1.9	4.3 ± 0.9	39.8 ± 4.2 ($p<0.001$)
M. oleifera-NPs	65.9 ± 3.8	19.8 ± 2.7	11.2 ± 1.6	3.1 ± 0.8	34.1 ± 3.9 ($p<0.01$)
CTM-NPs	38.7 ± 3.2*	27.1 ± 2.9*	26.8 ± 2.4*	7.4 ± 1.1*	61.3 ± 4.8* ($p<0.001$)

AO: Acridine Orange; PI: Propidium Iodide; Apoptotic Index = Early + Late Apoptotic cells (%). * $p < 0.001$ vs. control; $p < 0.05$ vs. all individual nanoparticle groups (ANOVA, Tukey's). Cells counted under 10× objective magnification.

Intracellular Production of ROS: Oxidative stress was examined as a possible factor responsible for the mode of action of the nanoparticles by quantifying the intracellular ROS using DCFH-DA test. All treatments caused a

significant increase in ROS when compared to the control (Fig. 3). C. longa-NPs and M. oleifera-NPs caused moderate elevation; T. cordifolia-NPs showed high responsiveness. As expected, CTM-NPs showed highest ROS production, showing significantly higher intensity when compared to other groups (***p* < 0.001).



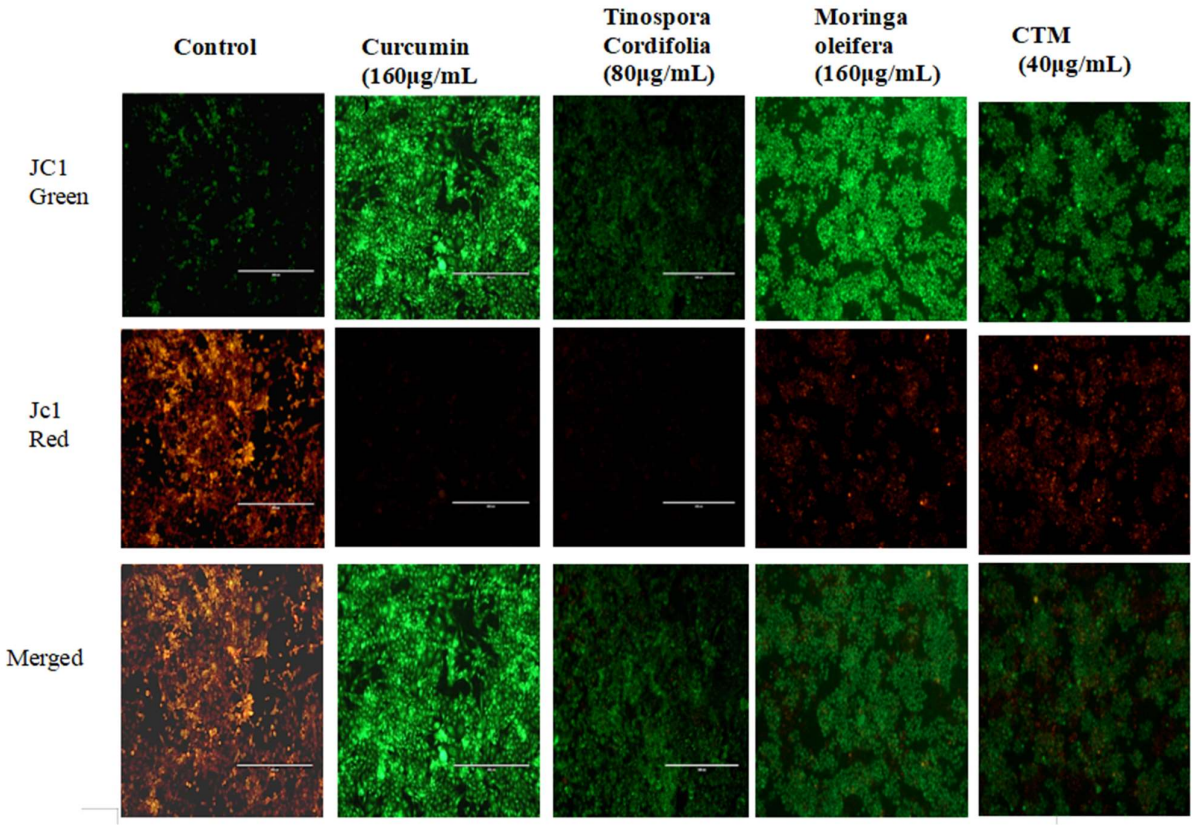
Objective Lens Magnification (20X) Ros Figure:3

Disruption of Mitochondrial Membrane Potential (ΔΨm)

Activation of the intrinsic apoptosis pathway was evaluated by determining mitochondrial membrane potential (ΔΨm) through the use of JC-1 fluorescent dye.

Healthy control cells showed bright red JC-1 aggregates (intact ΔΨm). The effect of each nanoparticle resulted in a partial disruption of ΔΨm (diminished red, increased green). CTM-NPs elicited the highest ΔΨm disruption as indicated by an intense red to green fluorescence shift (Fig. 4A). Quantitative analysis of red/green ratio demonstrated statistical significance of reduction in CTM-NP group in comparison to other treatment groups (***p* < 0.001) (Fig. 4) (Table 4).

JC-1 Assay – (A) Fluorescence micrographs (red and green channels);



Jc1 Assay Figure:4

Table-4 Quantitative and Statistical Analysis of Intracellular ROS Levels and Mitochondrial Membrane Potential (ΔΨm) in HT-29 Cells

Treatment Group	ROS Level (% Relative Fluorescence)	MMP Red/Green Ratio (JC-1)	p-value
Control	100.0 ± 5.3 (ref)	1.00 ± 0.05 (ref)	—
C. longa-NPs (160 µg/mL)	218.4 ± 14.7	0.68 ± 0.04	$p < 0.01$ vs control
T. cordifolia-NPs (80 µg/mL)	284.1 ± 19.2	0.52 ± 0.03	$p < 0.001$ vs control
M. oleifera-NPs (160 µg/mL)	231.7 ± 16.8	0.61 ± 0.04	$p < 0.01$ vs control
CTM-NPs (40 µg/mL)	412.6 ± 22.4*†	0.29 ± 0.02*†	$p < 0.001$ vs all groups

ROS: Reactive Oxygen Species measured by DCFH-DA fluorescence (% vs. control ± SD, n = 3). MMP: Mitochondrial Membrane Potential measured by JC-1 red/green fluorescence ratio (mean ± SD, n = 3). * $p < 0.001$ vs. control; † $p < 0.001$ vs. all individual nanoparticle formulations (ANOVA, Tukey's). H_2O_2 (200 µM) and CCCP (10 µM) served as positive controls for ROS and MMP assays, respectively.

Discussion

The current investigation confirms that the polyherbal nanoparticles CTM-NPs (C. longa, T. cordifolia, M. oleifera) have significantly better anticancer activity against HT-29 CRC cancer cells when compared with each component alone. This improved activity is a result of synergistic generation of oxidative stress and apoptosis.

The marked synergistic cytotoxicity of CTM-NPs (IC_{50} ≈40 µg/mL)—four-fold lower than C. longa/ M. oleifera-NPs and two-fold lower than T. cordifolia-NPs—aligns with the Ayurvedic principle of Yoga or Sanyoga (synergistic combination), where

rationally combined herbs produce amplified therapeutic effects with potentially lower individual doses, thereby reducing toxicity [5,6,23]. This is because of the multi-component and multi-targeting characteristics of the drug. The curcumin from C. longa targets the NF-κB and STAT3 pathways [12,24], the berberine and other alkaloids from T. cordifolia generate ROS and target the topoisomerases [15,25], and the quercetin and isothiocyanates from M. oleifera target the PI3K/Akt and p53 signaling [18,26]. These two approaches, delivered simultaneously at the nano-level, enable the blockade of both pathways, bypassing the compensatory mechanism that is a hallmark of CRC [27,28]. The use of the chitosan nanoparticle itself is vital, as it increases the solubility, stability, and delivery of these phytoconstituents to the cells [14,29,30].

The AO/PI staining method was used to prove that apoptosis was the major form of cell death brought about by CTM-NPs, characterized by condensation of the chromatin, nuclear fragments and plasma membrane blebs [31]. Apoptosis is preferred over necrosis for cancer treatment since it is not associated with inflammation and is regulated [32]. The close relationship of CTM-NP-induced apoptosis and high ROS levels in the cancer cells is of great significance in the mechanism of cell death. Cancer cells have high basal ROS. When ROS exceeds their tolerance limit, oxidative damage takes place leading to apoptosis. The high production of ROS by the CTM-NPs implies that these phytochemicals have synergy in disrupting the redox balance more effectively than when used singly, through their possible inhibition of antioxidant activities such as Nrf2 and glutathione. Mitochondrial malfunction is another hallmark phenomenon of excess ROS generation. JC-1 assay results have proven beyond doubt that CTM-NPs induce dramatic ΔΨm dissipation, a signature process of intrinsic apoptosis [38,39]. Disruption of the mitochondrial membrane potential results in mitochondrial outer membrane permeabilization, release of cytochrome c, apoptosome formation and caspase-9/3 activation [40]. Superior effectiveness of CTM-NPs to induce such a signaling cascade suggests a strong impact of the combination on the mitochondria, a well-known target in CRC cells having frequent mutations in Bcl-2 protein family [41,42]. This sequence mechanistic cascade—ROS upregulation → ΔΨm dissipation → apoptosis execution—is an effective, step-by-step process. This synergy is perhaps achieved by synergistic effects: the sensitizer effect of curcumin through Bcl-2 inhibition [13,24], ROS induction by T. cordifolia alkaloids leading to DNA damage [16,25], and the role of M. oleifera flavonoids as p53 upregulators [19,26]. There have been recent successes with polyherbs nanomedicines in CRC treatment, involving curcumin-based polyherbs [43] and T. cordifolia-based polyherbs [44,45], but our CTM-NPs represent a novel triple combination with demonstrated mechanistic depth.

Limitations and Future Directions: The current study has been restricted to in vitro testing only. It is essential that future studies confirm the results of the present study in vivo CRC models such as xenografts and/or chemical induction of tumors for testing purposes of pharmacokinetics, biodistribution, and systemic toxicity [46]. Additionally, future studies can elucidate on molecular mechanisms through various assays such as caspase activity, Bcl-2/Bax protein by Western blotting, cytochrome c release, and NF-κB inhibition.

Conclusion

In this research, a new polyherbal nanoparticle formulation (CTM-NPs) of C. longa, T. cordifolia, and M. oleifera has been successfully formulated. CTM-NPs exhibited remarkably improved and synergistic anticancer activities against HT-29 CRC cells in comparison to individual plant nanoparticles. It was found that the superior activity of CTM-NPs was based on their remarkable ability to induce oxidative stress, which causes significant reduction in mitochondrial membrane potential and ultimately results in activation of the intrinsic apoptotic pathway. These compelling in vitro findings position CTM-NPs as a promising multi-targeted nanotherapeutic candidate for colorectal cancer, warranting further pre-clinical in vivo investigation.

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