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Anticancer activity of dark chocolate mediated zinc oxide nanoparticles in HT-29 colon cancer cell lines – An in vitro study

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Abstract
Background: Colorectal cancer is a prevalent malignancy characterized by the transformation of normal cells in the colon or rectum into cancerous cells. Dark chocolate-mediated zinc oxide nanoparticles (DC-ZnO NPs) have emerged as a promising therapeutic approach due to their combined antioxidant and cytotoxic properties. This study investigates the anti-cancer activity of DC-ZnO NPs on HT-29 colon cancer cells, focusing on apoptosis induction, cytotoxicity, and morphological alterations.
Methods: DC-ZnO NPs were synthesized using avocado seed extract and characterized by their physicochemical properties. HT-29 colon cancer cells were treated with varying concentrations of DC-ZnO NPs, and cell viability was assessed using cytotoxicity assays. Apoptosis was evaluated through dual staining with ethidium bromide (EtBr) and acridine orange (AO). Morphological changes in the cells were observed using fluorescence microscopy to identify features associated with early apoptosis.
Results: Cytotoxicity assays demonstrated a substantial reduction in cell viability at this concentration. Dose-dependent decrease in the cell viability was observed between concentrations 10-100µg/mL. Morphological analysis at 80µg/mL revealed characteristic apoptotic changes, including cell shrinkage and membrane blebbing. Further, treatment with 80 µg/mL DC-ZnO NPs resulted in significant induction of early apoptosis, as evidenced by the yellowish-green staining of apoptotic cells. These findings indicate that DC-ZnO NPs effectively induce apoptosis in HT-29 cells, enhancing their potential as an anti-cancer therapeutic.
Conclusion: DC-ZnO nanoparticles exhibit potent anti-cancer activity in HT-29 colon cancer cells by inducing apoptosis and causing significant cytotoxic effects. The dual action of dark chocolate and ZnO NPs provides a promising approach for colorectal cancer treatment. Further research is needed to explore the detailed mechanisms and assess in vivo efficacy to fully establish the therapeutic potential of DC-ZnO NPs.

Keywords: Anti-cancer, dark chocolate, zinc oxide, nanoparticles, colon cancer

INTRODUCTION

Colorectal cancer, also referred to as bowel, colon, or rectal cancer, originates from the colon or rectum, which are parts of the large intestine [1]. While inherited genetic disorders such as familial adenomatous polyposis and hereditary non-polyposis colon cancer account for less than 5% of cases, the majority of colorectal cancer cases arise in individuals with minimal or no genetic risk [2]. Typically, the disease begins as a benign polyp that gradually transforms into cancer. Diagnosis is commonly performed through sigmoidoscopy or colonoscopy. Major risk factors include older age, male gender, high consumption of fats, sugars, alcohol, red and processed meats, obesity, smoking, and physical inactivity [3]. Insufficient physical activity is linked to about 10% of cases, while alcohol consumption increases risk significantly when exceeding one drink per day. The APC gene, frequently mutated in colorectal cancer, plays a crucial role in preventing β-catenin accumulation. Without functional APC, β-catenin accumulates and activates the transcription of proto-oncogenes, which are involved in stem cell renewal and differentiation but can contribute to cancer when overexpressed [4].
Despite the availability of various anticancer chemotherapies, these treatments often fall short of achieving a complete response. This is largely due to issues such as drug resistance and the inability to precisely distinguish between healthy and cancerous cells, often necessitating high doses of medication [5]. In recent years, bioactive nanoparticles (NPs) have emerged as a promising area of research in anti-tumor therapy due to their ability to

target tumors effectively and their superior therapeutic effects [6]. Notable examples include silver NPs, copper oxide NPs, and iron oxide NPs [7, 8]. Due to their biocompatibility, zinc oxide nanoparticles (ZnO NPs) are becoming increasingly popular among metal oxide nanoparticles importantly in the field of biomedicine research [9]. Recent studies have demonstrated that ZnO NPs can selectively inhibit tumor growth and may serve as alternatives to conventional chemotherapy. However, research on the impact and mechanisms of ZnO NPs in tumor metastasis remains limited [10].

Dark chocolate, produced from the seeds of the tropical Theobroma cacao tree, is a rich source of essential nutrients including carbohydrates, fats, proteins, and water [11]. It is particularly noted for its high content of dietary minerals such as iron, copper, manganese, magnesium, phosphorus, and zinc [12]. Its antioxidant properties are well-documented, as dark chocolate is known to combat oxidative stress by neutralizing free radicals, which can contribute to cellular damage and cancer risk reduction [13]. This antioxidant activity is primarily attributed to its high levels of flavonoids and polyphenols, which have been shown to mitigate oxidative damage and exert potential anti-cancer effects [14]. Thus, dark chocolate's nutrient profile and antioxidant capacity make it a compelling candidate for exploring its role in cancer prevention and management.

In view of the given background, this study aims to explore the anti-cancer effects of dark chocolate-mediated zinc oxide nanoparticles using the HT-29 colon cancer cell line.

MATERIALS AND METHODS

Chemicals and Reagents

DMEM (Dulbecco's Modified Eagle Medium), Phosphate Buffered Saline (PBS), Trypsin-EDTA, Fetal bovine serum (FBS), were purchased from Gibco, Canada. Acridine orange (AO), ethidium bromide (EtBr), Dimethyl sulfoxide (DMSO), [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), DAPI, AO/EtBr were purchased from Sigma Chemical Pvt Ltd, USA. All other chemicals used were extra pure of molecular grade and were purchased from SRL, India.

Cell line maintenance

HT - 29 human colorectal adenocarcinoma cell lines were obtained from the NCCS, Pune with passage number of 14. The cells were grown in T25 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics. Cells were maintained at 37◦C in a humidified atmosphere containing 5% CO2. Upon reaching confluency, the cells were trypsinized and passaged.

Cell viability (MTT) assay

The cytotoxic evaluation of dark chocolate synthesized from zinc oxide nanoparticles (DC-ZnO NPs) was evaluated by MTT assay. The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. Briefly, $5x10^4$ cells/well were plated in 96 well plates. 24h after plating, the cells were washed twice with 100µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37ºC. After starvation, the cells were treated with different concentrations (10, 20, 40, 60, 80 & 100µL) of the DC-ZnO NPs for 24h. At the end of 20h, the medium from the control and treatment group was discarded and 100µl of MTT containing DMEM (0.5mg/ml) was added to each well. After 4h of incubation at 37ºC in the CO_2 incubator, the MTT containing medium was discarded and the cells were washed with 1x PBS. The formazan crystals formed were dissolved in dimethyl sulfoxide (100µl) and the intensity of the colour developed was measured using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as the percentage of control cells cultured in serum-free medium. Cell viability in the control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells] ×100.

Morphology study

Based on MTT assay we selected optimal doses by linear regression analysis for further studies. Analysis of cell morphology changes was observed using a phase contrast microscope. 2×10^5 cells were seeded in 6-well plates and treated with the highest concentration of 80µL of DC-ZnO nanoparticles for 24h. At the end of the incubation period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase contrast microscope for morphological changes.

Determination of mode of cell death by acridine orange (AO)/ethidium bromide (EtBr) dual staining

The apoptotic effect of DC-ZnO nanoparticles in HT-29 colon cancer cell death was determined by AO/EtBr dual staining assay. The cells were treated with 80µL of the synthesized nanoparticle for 24h and then the cells were harvested, washed with ice-cold PBS. The pellets were resuspended in 5µl of acridine orange (1mg/mL) and 5µl of EtBr (1mg/mL). The induction of apoptotic in the cells were then observed in stained cells using an inverted fluorescence microscope.

Statistical analysis

All data obtained were analyzed by One way ANOVA followed by Student's t-test using SPSS software. Data were represented as mean ± SD for triplicates. The level of statistical significance was set at $p<0.05$.

RESULTS

Effect of DC-ZnO nanoparticles on HT-29 cell viability

To evaluate the impact of dark chocolate-mediated zinc oxide nanoparticles (DC-ZnO NPs) on the viability of HT-29 colon cancer cells, a series of in vitro assays were conducted. HT-29 cells were treated with varying concentrations of DC-ZnO NPs (10-100µg/ml), and cell viability was assessed using the MTT assay.The results demonstrated a concentration-dependent reduction in HT-29 cell viability upon treatment with DC-ZnO NPs (Figure 1). Specifically, at lower concentrations of DC-ZnO NPs, there was a modest decrease in cell viability with a percentage 82%, which became more pronounced at higher concentrations with cell viability showing to be 17.6% at 100µg/ml. This indicates that DC-ZnO NPs exhibit significant cytotoxic effects on HT-29 cells.

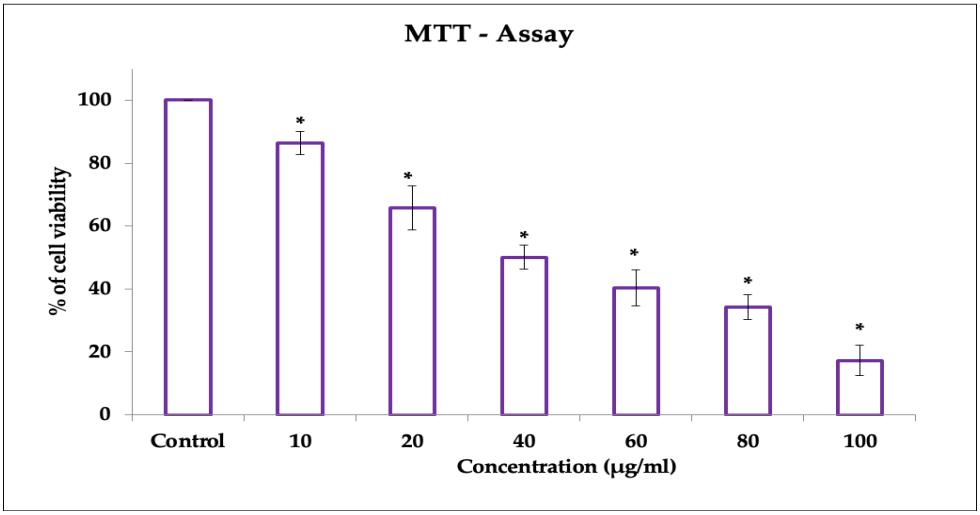

Figure 1: The bar graph showing the cytotoxic effect of dark chocolate mediated zinc oxide nanoparticles in HT-29 colorectal carcinoma cells by MTT assay.

Effect of DC-ZnO nanoparticles on morphological alterations in HT-29 cells

The treatment of HT-29 colon cancer cells with 80µg/ml of DC-ZnOnanoparticles resulted in significant morphological changes, providing insights into the nanoparticles' impact on cellular architecture. Observations under phase contrast microscopy revealed that the HT-29 cells exhibited notable alterations in morphology following exposure to this concentration of DC-ZnO nanoparticles. Specifically, the treated cells demonstrated a marked reduction in cell size and irregular cell shape compared to untreated controls.

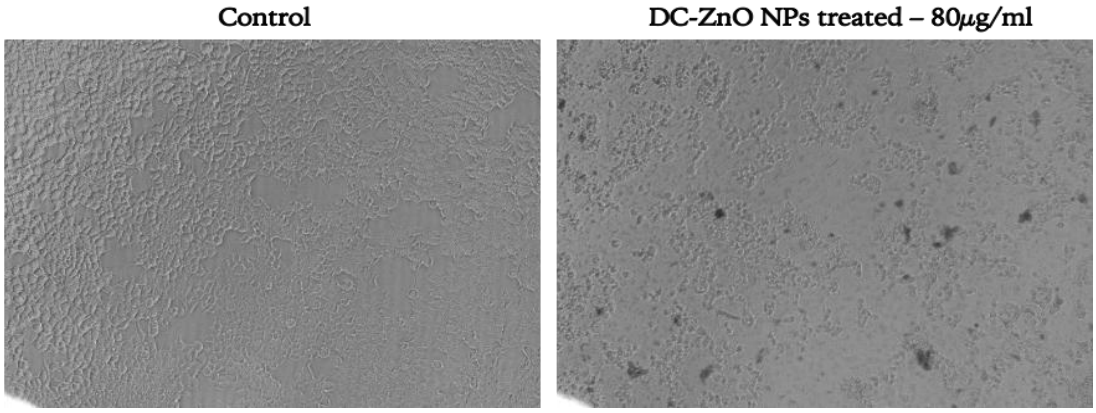

Figure 2: Representative images showing morphological alterations induced by dark chocolate mediated Zinc oxide nanoparticles in HT - 29 colon cancer cells, magnification 10x. Cells were treated with DC-ZnO NPs (80µg/ml) for 24 h along with control group. Images were obtained using an inverted phase contrast microscope.

Effect of DC-ZnO nanoparticles on apoptosis induction by EtBr/AO staining

The evaluation of apoptosis induction by DC-ZnO nanoparticles was conducted using the EtBr/AO (ethidium bromide/acridine orange) staining method. This technique differentiates between live and apoptotic cells based on their staining characteristics. In the fluorescence microscopy images, live cells are indicated by yellow arrows and stained with ethidium bromide, which emits green fluorescence. This green fluorescence signifies that the cells are intact and viable.In contrast, early apoptotic cells are highlighted by red arrows and stained with acridine orange, which results in a yellowish-green coloration. The yellowish-green color indicates the presence of early-stage apoptosis, where cells are undergoing morphological changes but have not yet progressed to late apoptosis or necrosis.At a concentration of 80 µg/mL DC-ZnO NPs, the staining revealed a significant number of early apoptotic cells (yellowish-green), suggesting effective induction of apoptosis.

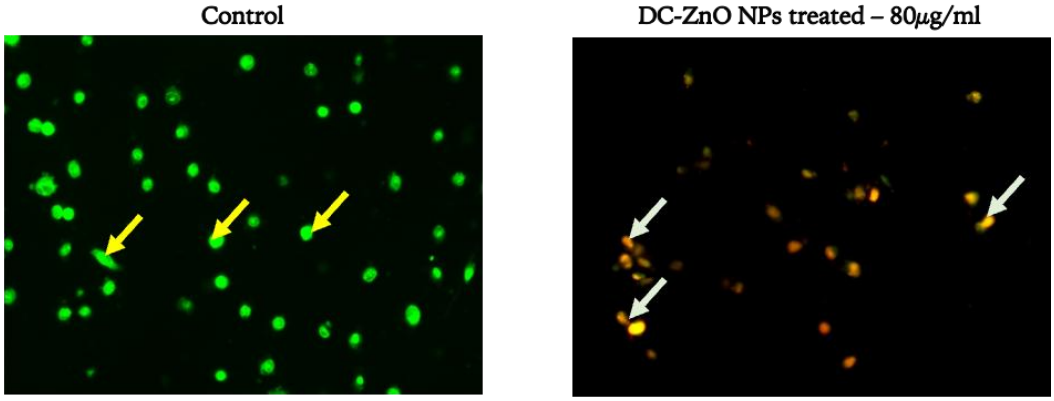

Figure 3: Representative images showing apoptotic effect of dark chocolate mediated nanoparticles in HT-29 colon cancer cells - AO/EtBr dual staining assay, magnification 10x. Cells were treated with dark chocolate mediated ZnO NPs (80µg/ml) for 24 h along with control group. Live cells (yellow arrows) stained with ethidium bromide and appears green in fluorescence. Early apoptotic cells (Red arrows) stained by acridine orange appears yellowish green in colour. Images were obtained using an inverted fluorescent microscope.

DISCUSSION

Due to their high concentration of catechins and procyanidins, bioactive compounds with distinct properties, cocoa and chocolate products may have beneficial health effects against oxidative stress and chronic inflammation, risk factors for cancer and other chronic diseases. The results of this study highlight the significant impact of dark chocolate-mediated zinc oxide nanoparticles (DC-ZnO NPs) on inducing apoptosis in the HT-29 colon cancer cell line. Cytotoxicity assessments provided additional insights into the therapeutic potential of DC-ZnO NPs. The reduced cell viability observed indicates that DC-ZnO NPs exhibit significant cytotoxic effects on HT-29 cells. The observed reduction in cell viability can be attributed to the release of Zn^{2+} ions from the nanoparticles, which may induce oxidative stress and damage cellular components, leading to apoptosis [15].

The EtBr/AO staining results demonstrated that DC-ZnO NPs effectively induce early apoptosis, as evidenced by the yellowish-green staining of early apoptotic cells. This finding aligns with previous research indicating that nanoparticles, including ZnO NPs, have potential anti-cancer properties due to their ability to induce apoptosis in various cancer cell lines [16]. The enhanced apoptosis observed with DC-ZnO NPs suggests that the combination of dark chocolate and ZnO nanoparticles might synergistically enhance their anti-cancer effects, potentially offering a novel therapeutic strategy for colorectal cancer.The observed apoptosis induction at 80µg/mL concentration of DC-ZnO NPs underscores the effectiveness of this formulation in targeting cancer cells. This concentration was found to be optimal for inducing significant apoptosis, as higher or lower concentrations might not yield similar results due to potential variations in nanoparticle activity and cell response [17]. The findings are consistent with reports that nanoparticles can selectively target and kill cancer cells, thus reducing the side effects typically associated with conventional chemotherapy [18-20]. The yellowish-green staining observed in early apoptotic cells further supports the potential of DC-ZnO NPs in triggering cellular pathways associated with apoptosis, which is crucial for effective cancer therapy.

The antioxidant properties of dark chocolate, combined with the targeted effects of ZnO NPs, might contribute to the enhanced anti-cancer activity observed. Dark chocolate's rich polyphenol content is known to combat oxidative stress and prevent cellular damage [19, 20]. When integrated with ZnO NPs, these properties may work synergistically to enhance the nanoparticles' ability to induce apoptosis. This dual action not only targets cancer cells more effectively but also minimize oxidative damage to healthy cells, offering a promising approach to cancer treatment. Future research should explore the underlying mechanisms of this synergy and assess the efficacy of DC-ZnO NPs in vivo to confirm their therapeutic potential and safety profiles.

CONCLUSION

It was concluded that dark chocolate mediated zinc oxide nanoparticles, DC-ZnO nanoparticles possess potent anti-cancer properties against HT-29 colon cancer cells. The incorporation of dark chocolate in the nanoparticle formulation appears to enhance the therapeutic efficacy, potentially through synergistic effects between the nanoparticles and the bioactive compounds in dark chocolate. Further studies are warranted to elucidate the precise mechanisms underlying this cytotoxic effect and to evaluate the potential of DC-ZnO NPs as an alternative therapeutic strategy for colon cancer treatment.

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Conflict Of Interest

The authors declared no conflict of interest pertaining to the study.

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