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Antiproliferative and apoptotic effect of herbal formulation containing black and green tea extract against HCT-116 human colon cancer cells

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ABSTRACT

Background: Due to their safety, greater tolerance, and economics, phytochemical-based dietary interventions have drawn attention as a preventive and therapeutic method against most cancers. The purpose of this study was to investigate the antiproliferative and apoptotic effect of black and green tea formulation.

Materials and Methods: Aqueous extracts of black and tea were prepared. The formulation was prepared by mixing 1:1 part of individual aqueous extracts of black and tea. The prepared formulation was evaluated for in vitro cytotoxicity using HCT-116 colon cancer cells. Morphological changes were observed and the apoptotic effect of the formulation was also assessed.

Results: The results showed that the formulation was able to inhibit the growth of colon cancer cells effectively at all the tested concentrations between the range of 5-50µg/ml. A dose-dependent decrease in the viability of cells was observed. IC50 was calculated by linear regression analysis. Based on the IC50, efficacy concentration of 45µg/ml was chosen for morphology and dual staining assays. Morphological changes were observed after treating the cells at a single concentration of 45µg/ml. The treated cells demonstrated loosely adhered boundaries with spherical morphology indicating the onset of the cell death following the black and green tea formulation treatment. To further confirm the antiproliferative effect, the formulation treated cells were stained with ethidium bromide/acridine orange and assessed for apoptotic effect. The images showed greenish yellow fluorescent cells in addition to few green fluorescent cells when compared to untreated cells that appeared only green in fluorescence.

Conclusion: Hence from the results of the present study it may be concluded that the black and green tea formulation were found to show significant antiproliferative and apoptotic effect in HCT 116 colon cancer cells.

Keywords: *black tea, green tea, polyphenols, colon cancer, antiproliferative, apoptosis*

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INTRODUCTION

Colon cancer is the second most prevalent malignancy and the third leading cause of cancer-related mortalities worldwide, resulting in ~500,000 mortalities every year (1). Approximately, 1 million new cases are diagnosed every year. More than 50% of all cases and mortality can be attributed to preventable risk factors like smoking, eating poorly, drinking excessively, being physically inactive, and carrying too much weight (2- 4). Family history of colorectal cancer, hereditary diseases such polyposis and hereditary nonpolyposis colorectal cancer, as well as personal histories of inflammatory bowel illness, polyps, and other cancers, are additional risk factors (5, 6). The existing treatments for colorectal cancer viz., surgical resection, chemotherapy, radiation therapy and combined regimens have a history of toxic side effects and drug resistance (7, 8). In this context, researchers are working in the search of new compounds that possess negligible or minimal side effects and to improve the effectiveness of treatment for colorectal cancer.

Epidemiology research and meta-analysis studies have demonstrated the ability of various phytochemicals, particularly phenolics, to reduce cancer risk (9, 10). Particularly, the polyphenolic elements in various tea brews have been thoroughly investigated. Among the most polyphenols, tea maintains a promising place among dietary therapies because of its capacity to target many cancer-related cellular locations (9). Tea is made from the Camellia sinensis plant which is categorised into three types. Green tea, which has not undergone any fermentation, black tea, which has had some fermentation, and oolong tea are the three main categories of tea (partially fermented) (11). Green tea contains large amounts of catechins, especially epigallocatechin in it whereas black tea contains high amounts of theaflavins and thearubigins (12). Many pre-clinical and clinical studies have demonstrated the potential health benefits of both black and green tea polyphenols (12, 13). The major catechin, epigallocatechin present in green tea and theaflavins, major components of black tea have been shown to possess significant anti-cancer effects (12 - 14). Although there were many studies carried out on the beneficial effects of black tea and green tea extracts individually (15 -17), studies on their effect as a formulation are seldom reported. Therefore, in the present study the combination of black and green tea extract as a formulation was evaluated for its potential effects against colon cancer using HCT -116 colon cancer cells.

MATERIAL AND METHODS

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), AO/EtBr were purchased from Sigma Chemical Pvt Ltd, USA. All other chemicals used were of molecular grade and were purchased from SRL, India.

Cell line maintenance

HCT-116 human colorectal carcinoma cells were obtained from the NCCS, Pune with passage number of 18. 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% CO_2. Upon reaching confluency, the cells were trypsinized and plated.

Cell viability (MTT) assay

The cytotoxic effect of the black and green formulation was evaluated by an MTT assay (18). 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 of the synthesised nanoparticles 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 4 h 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 Microplate reader at 570 nm. Cell

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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 the MTT assay an optimal efficacy dose of 45µg/ml was selected based on 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 concentration of 45µg/ml of 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 cell death by acridine orange (AO)/ethidium bromide (EtBr) dual staining

The apoptotic effect of black and tea formulation in MCF-7 cell death was determined by AO/EtBr dual staining as described previously (19). The cells were treated with 45µg/ml of the synthesised nanoparticle for 24h and then the cells were harvested, washed with ice-cold PBS. The pellets were resuspended in 5µl of acridine orange (1 mg/mL) and 5µl of EtBr (1 mg/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 analysed by One way ANOVA followed by Student's t-test using SPSS software. Data were represented as Mean±SEM for triplicates. The level of statistical significance was set at $p<0.05$.

RESULTS

Cytotoxicity assay

The cytotoxic effect of black and green tea formulation was evaluated using HCT-116 colon cancer cells. The colon cancer cells were treated with different concentrations (5-50µg/ml) of black and green formulation for 24h. The results of the MTT assay demonstrated dose-dependent decrease in the viability of HCT-116 colon cancer cells after 24 h treatment with the formulation. A maximum inhibition of 80% in the proliferation of cancer cells was observed at 50µg/ml. IC_{50} was calculated by non-linear regression analysis and was found to be 45µg/ml which was used for further functional analysis.

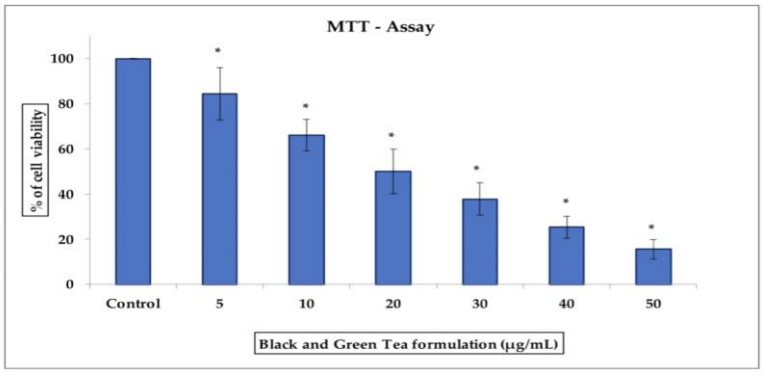

FIGURE 1: Representative bar graph showing the effect of black and green tea formulation on HCT-116 colon cancer cell viability

Morphological study

The cellular morphology of the HCT-116 colon cancer cells was observed following 24h of treatment with black and green formulation. After 24h, the cells treated with the 45µg/ml (IC_{50} value) formulation showed round shape with no clear membrane and ruptured cellular structure whereas the untreated control cells demonstrated spindle shaped morphology indicative of cell viability. From the results, it may be concluded that black and green formulation has exerted cytotoxicity in colon cancer cells.

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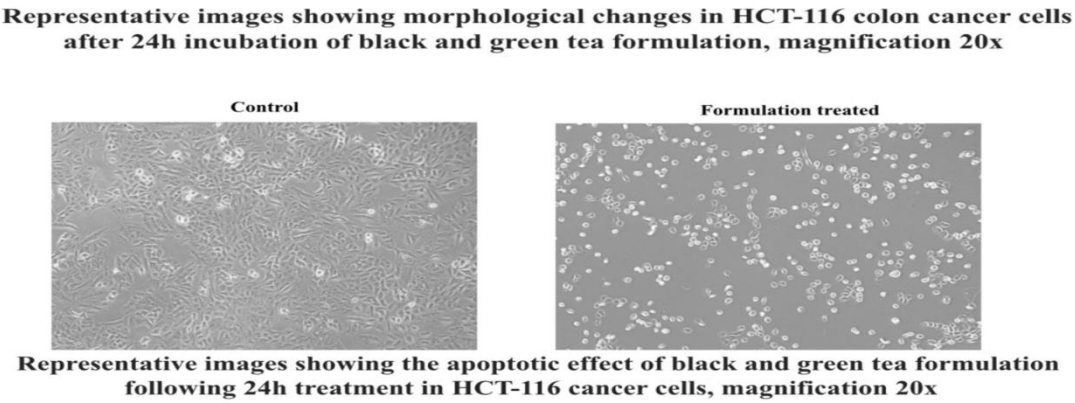

FIGURE 2: Representative images showing morphological changes after 24h incubation with/without black and green tea formulation

Ethidium bromide/acridine orange (EtBr/AO) dual staining - Apoptosis assay

The EtBr/AO dual staining assay was carried out to evaluate whether the cytotoxicity induced by black and green tea formulation was due to apoptosis or necrosis. The results showed that after 24h treatment, the cells treated with 45µg/ml tea formulation demonstrated yellowish green fluorescence emitting cells indicating the onset of apoptosis. Whereas the untreated cells appeared green in fluorescence indicating cell viability.

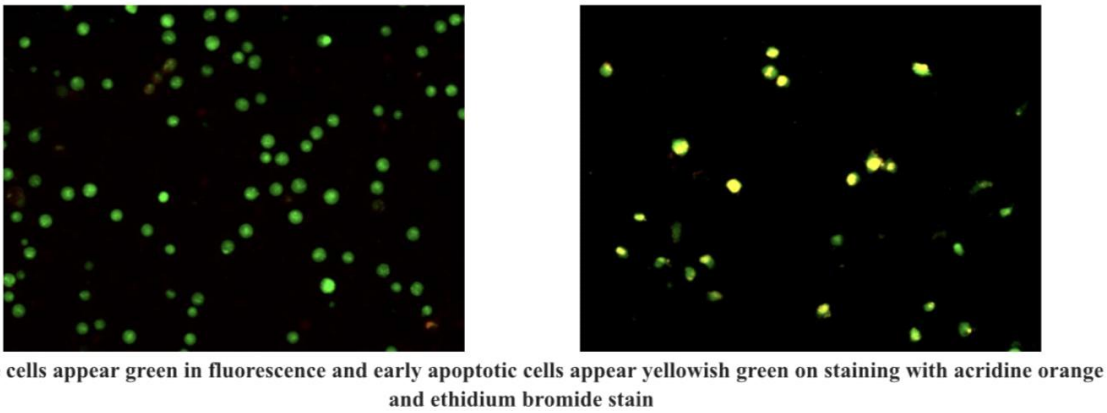

FIGURE 3: Representative images showing induction of apoptosis in HCT-116 colon cancer cells after 24h incubation with/without black and green tea formulation

DISCUSSION

The incidence of colon cancer has been on the rise, primarily attributed to recent lifestyle changes. These changes include reduced consumption of fruits and vegetables, decreased physical activity, excessive alcohol intake, and exposure to potentially harmful chemicals (1, 6). Furthermore, conventional treatments such as chemotherapy and radiation therapy have proven to be costly and associated with adverse effects such as toxicity and drug resistance. As a result, researchers are exploring the use of natural plant products, which are rich in diverse molecular compounds, either as standalone drugs or in formulations, for the treatment of various types of cancer, including colon cancer (20 - 25).

Tea phytochemicals have exhibited notable anti-cancer properties, which can be attributed to the presence of bioactive compounds such as polyphenols, catechins, flavonoids, and alkaloids. These compounds possess antioxidant, anti-inflammatory, and anticancer properties (9, 12, 13). Building upon previous literature documenting the anticancer effects of tea

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polyphenols (26, 27), this study focused on examining the impact of a black and green tea formulation on colon cancer using the HCT-116 colon cancer cell line. Additionally, a previous study demonstrated that HCT-116 colon cancer cells exhibited higher sensitivity compared to HT-29 colon cancer cells (5). Hence, in this study, we also selected the HCT-116 cell line to assess the cytotoxicity and anticancer properties of the black and green tea formulation.

Our findings indicated a significant reduction in the viability of colon cancer cells when treated with a formulation containing black and green tea at the tested concentrations. These results align with previous studies that also reported a considerable decrease in the viability of colon and ovarian cancer cells when exposed to black tea extract and green tea extract. The observed antiproliferative and cytotoxic effects of the tea extracts are attributed to the presence of polyphenol compounds, particularly epigallocatechin (EGCG) and theaflavins, which are abundant in both black and green tea (12 - 14). Moreover, our study demonstrated that the black and green tea formulation induced cell death through apoptosis, as confirmed by distinct changes in cell morphology and staining with EtBr/AO.

Apoptosis, also known as "programmed cell death," is a regulated process by which cancer cells are effectively targeted by various anticancer agents (28 - 30). Previous research has demonstrated that the presence of EGCG in green tea extract and theaflavins in black tea extract can induce apoptosis in cancer cells (11, 14). The tea polyphenols have been shown to trigger apoptosis in cancer cells through different mechanisms (17). Apoptosis mechanisms includes activating intrinsic signalling pathways mediated by mitochondria, reducing the expression of anti-apoptotic markers, and increasing the expression of pro-apoptotic markers (31 - 33). A recent study also found that combining green tea extract with paclitaxel led to synergistic induction of mitochondria-dependent cell death by inhibiting Akt phosphorylation in ovarian cancer cells (25). Similarly, another study demonstrated that EGCG, in combination with sodium butyrate, induced apoptosis in HCT-116 colon cancer cells by suppressing survivin protein expression (22, 34). Based on these previously published findings, it is plausible to suggest that the apoptosis induced by the synergistic effect of black and green tea formulation may also rely on mitochondrial pathways.

CONCLUSION

The black and green tea formulation in combination have exhibited promising anti-carcinogenic effects by inhibiting the cell proliferation, inducing morphological changes ultimately resulting in apoptosis in HCT-116 colon cancer cells.

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