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A Flow Cytometric Analysis of DNA Content and Cell Cycle Distribution in Lipopolysaccharide-induced Caco-2 Cell Model of Inflammatory Bowel Disease Treated with water extract of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.)

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Abstract

Inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, is a chronic inflammation of the digestive tract. Persistent inflammation in IBD can lead to continuous cycles of tissue damage and repair, causing cellular stress and DNA damage over time. This can lead to mutations and increase risk of colorectal cancer. Persistent inflammation promotes genomic instability and abnormal cell growth, providing a supportive environment for mutated cells that can progress to cancer. This study investigates the effects of water extract of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.) on cell cycle phases of Lipopolysaccharide -induced (LPS) Caco- 2 cells, using DNA content analysis, cell cycle distribution using flow cytometry. In the G0/G1 phase, cell percentages were 61.7% in controls, 63.7% in LPS-induced, and 76.3% in treated cells, suggesting cell cycle arrest by the water extract of *Shunthi choorna*. For the S phase, percentages were 12.7% in controls, 17.8% in LPS-induced, and 13.2% in treated cells, indicating that the extract may limit inflammation-induced DNA synthesis. In the G2/M phase, treated cells showed a reduction to 10.3% compared to controls, pointing to reduced mitotic entry. The increase in cellular debris (73% in treated cells) suggests heightened cell death, likely through apoptosis. Overall, these findings position water extract of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.) as a promising natural agent for cell cycle regulation, with potential therapeutic applications in managing inflammation and preventing cancer progression.

Introduction

Inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, is characterized by chronic inflammation of the digestive tract.[1] This persistent inflammation triggers continuous cycles of tissue damage and repair, leading to cellular stress and DNA damage over time. Such damage can result in mutations and an elevated risk of colorectal cancer, as the ongoing inflammation fosters genomic instability and abnormal cell growth, creating a supportive environment for mutated cells that may progress to cancer.[1][2]

Shunthi (Zingiber officinale Rosc.) the highly valuable drug which has been used very commonly in Ayurveda for the diseases related to digestion. *Shunthi (Zingiber officinale* Rosc.) is renowned for its diverse medicinal properties, including anti-inflammatory, antioxidant, and anti-cancer activities.[3] While several studies have highlighted the therapeutic potential of ginger extracts, few have investigated their direct impact on cell cycle progression, particularly in inflammation-induced cellular environments that mimic conditions of chronic diseases and cancer.[4] Cell cycle regulation is fundamental to maintaining normal cellular function and growth, with dysregulation often leading to pathological conditions such as cancer. Treatments that can modulate cell cycle progression, specifically by arresting or delaying cells in certain phases, are of interest in cancer research as they can restrict abnormal cellular proliferation. Flow cytometry provides an efficient and accurate method to analyze DNA content and cell cycle phases, allowing for an in-depth understanding of how therapeutic agents, impact cellular dynamics.

LPS is widely used to simulate inflammation in cell models, as it activates inflammatory pathways and can lead to increased DNA synthesis and cell proliferation. In this context, our study evaluates the effects of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.) on LPS-induced Caco-2 cells, focusing on its potential to induce cell cycle arrest and promote cytotoxic effects. By analyzing the percentage of cells in G0/G1, S, and G2/M phases, along with cellular debris as a marker of cell death, we aim to uncover the therapeutic relevance of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.) in modulating cell cycle progression and enhancing cytotoxic responses. This study seeks to provide insights into the possible anti-inflammatory and anti-cancer properties of *Shunthi choorna*, (powder of dried rhizome of *Zingiber officinale* Rosc.) contributing to the growing interest in natural products as complementary treatments for cancer and inflammatory conditions.

Aim

To study the effect of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.) on DNA content and cell cycle distribution in lipopolysaccharide-induced Caco-2 cells using flow cytometry.

Objective

To evaluate the impact of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.) on DNA content and cell cycle phases (G0/G1, S, and G2/M) in lipopolysaccharide-induced Caco-2 cells through flow cytometric analysis.

Material And Methods

1. Preparation of water extract of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.) for *in vitro* study.

The water extract of *Shunthi choorna* (powdered dried rhizome of *Zingiber officinale* Rosc.) was prepared using the cold percolation method. For this process, 50 g of *Shunthi choorna* was mixed with 500 ml of distilled water and left overnight on a shaker to ensure thorough mixing. The mixture was then filtered using muslin cloth and dried on a petri plate for two days. After drying, the extract was scraped off, yielding a total of 32.2 g. A 1 mg sample was dissolved in 0.1% dimethyl sulfoxide (DMSO) and stored in a 5 ml Eppendorf tube. This water extract was subsequently used in an in vitro study conducted at the Centre for Research in Molecular and Applied Sciences (CRMAS), Thiruvananthapuram.

2. Cell culturing

The Caco-2 cell line, a human colorectal adenocarcinoma cell line, was originally procured from the National Centre for Cell Sciences (NCCS) in Pune, India. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) within a 25 cm^2 tissue culture flask. The medium was supplemented with 10% fetal bovine serum (FBS), L-glutamine, sodium bicarbonate, and an antibiotic mixture consisting of 2.5 µg/ml amphotericin B, 100 µg/ml streptomycin, and 100 U/ml penicillin. The cell cultures were maintained in a humidified incubator at 37°C with 5% carbon dioxide.

3. Analysis of DNA content and cell cycle distribution

DNA analysis and cell cycle distribution are critical for evaluating the anti-cancer activity. It provides insights into cell cycle distribution, proliferation, apoptosis, and cell cycle dysregulation.[5]

a. Principle

In toxicity studies, monitoring a cell's ability to proliferate is crucial for assessing its overall health. The most reliable method for this is the direct measurement of DNA synthesis. The MUSE cell cycle kit employs standard ethanol fixation and detergent permeabilization processes, which are sufficient to access DNA during active cell cycle phases. Among the premixed reagents in the kit is propidium iodide (PI), a nuclear DNA intercalating dye that distinguishes cells at different cell cycle stages (G0/G1, S, and G2/M) by analyzing their DNA content. RNAase is included to enhance the specificity of DNA staining at each phase.[5]

b. Procedure

After achieving sufficient confluency, Caco-2 cells were activated by treating them with 1 µL of lipopolysaccharide (LPS) at a concentration of 1 µg/mL. The LPS-stimulated cells were then treated with a sample (51.2 µg/mL) prepared from a 1 mg/mL stock solution and incubated at 37ºC in a humidified incubator with 5% CO_2 for 24 hours. Untreated control cells were maintained for comparison. Following incubation, the cell

samples were transferred to either a 50 ml conical flask or a 12 × 75 mm polystyrene tube. A minimum of 1×10^6 cells was recommended for fixation in the tube. The samples were centrifuged at 3000 rpm for five minutes, and care was taken not to disturb the pellet while removing the supernatant. After centrifugation, the cell pellet appeared as either a visible pellet or a white film at the bottom of the tube. Phosphate-buffered saline (PBS) was added to each tube at a ratio of 1 ml of PBS per 1×10^6 cells, and the mixture was gently vortexed or pipetted several times to resuspend the cells. The cells were centrifuged again for five minutes at 3000 rpm, and the supernatant was carefully removed, leaving approximately 50 µl of PBS per 1×10^6 cells. The pellet was resuspended in the remaining PBS by gentle vortexing or pipetting. Next, the reconstituted cells were slowly added dropwise to a tube containing 1 ml of ice-cold 70% ethanol while being vortexed at medium speed. The tube was then sealed and stored at -20ºC for freezing.[5][6]

Staining of Cell Cycle

Following overnight incubation, the samples were centrifuged at 3000 rpm for 5 minutes at room temperature. After the supernatant was carefully removed, 250 µl of PBS was added to the pellet, and the mixture was centrifuged again under the same conditions. Once the supernatant was discarded, 250 µl of a light-sensitive cell cycle reagent was added to the pellet. The samples were incubated in the dark for 30 minutes, with an additional 30-minute incubation at a cool, dark temperature to ensure optimal staining. Finally, the samples were analyzed using a flow cytometer. Gating parameters were established based on the untreated control cells for accurate assessment.[6]

Results

Analysis of DNA content and cell cycle distribution

DNA analysis provides insights into genetic changes, mutations, and overall genomic stability, while cell cycle distribution analysis reveals how cells progress through different phases of the cell cycle, it was measured by flow cytometry. Population profile, DNA content profile and cell distribution of control cells (Caco-2 cells), the lipopolysaccharide (LPS) induced Caco-2 cells and lipopolysaccharide (LPS) induced cells treated with water extract of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.) has been given below.

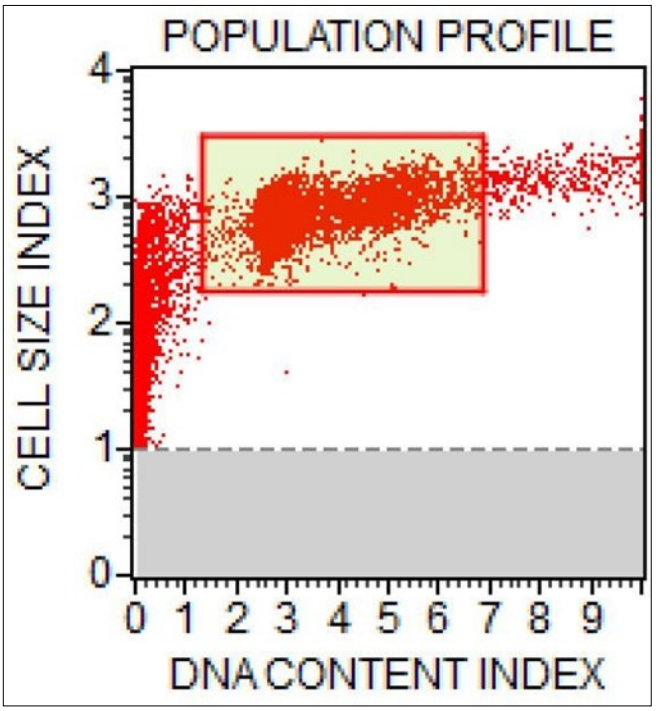

Diagram 1: Population profile of untreated control cells

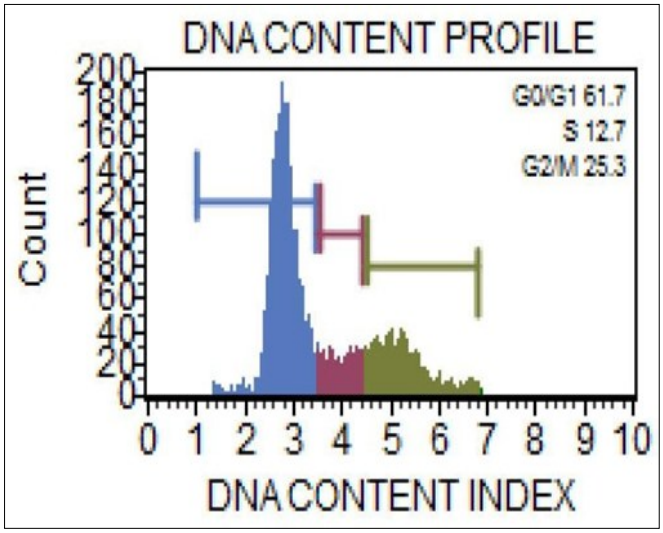

Diagram 2: DNA Content profile of untreated control cells

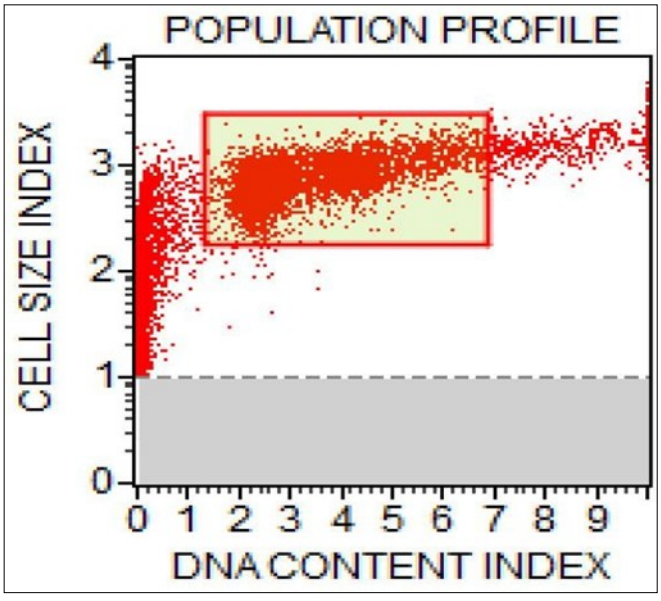

Diagram 3: Population profile of cells treated with Lipopolysaccharide

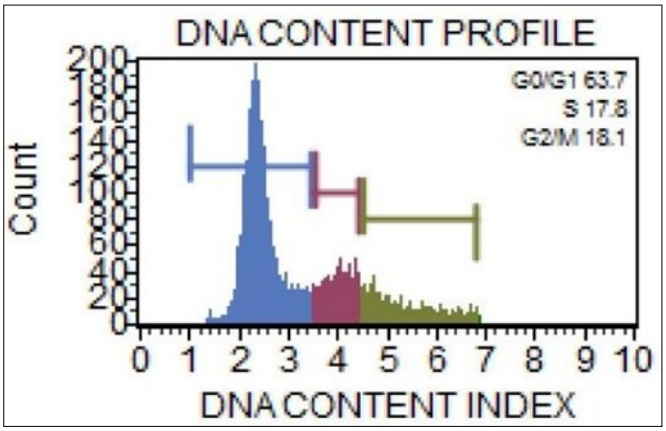

Diagram 4: DNA Content profile of cells treated with Lipopolysaccharide

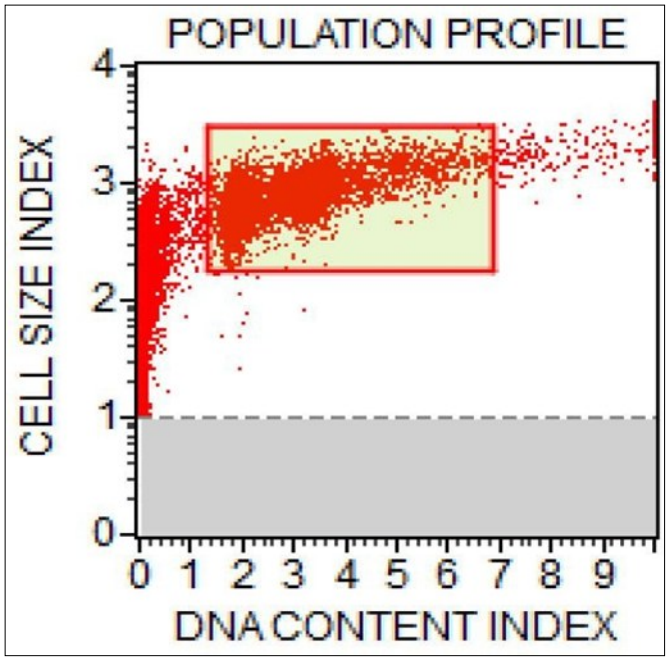

Diagram 5: Population profile of Lipopolysaccharide-induced cells treated with sample

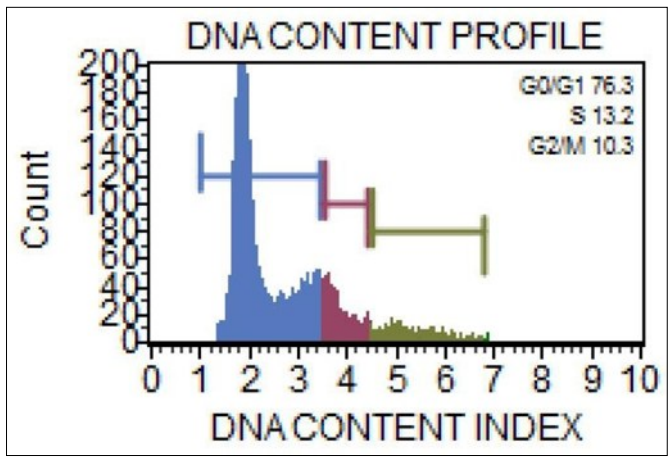

Diagram 6: DNA Content profile of Lipopolysaccharide-induced cells treated with sample

Table 1: Percentage of cells gated in different phases of cell cycle

	G0/G1 phase	S phase	G2/M phase	Debris
	% Gated	% Gated	% Gated	% Gated
Untreated Control cells(Caco-2 cells)	61.7	12.7	25.3	60.7
Lipopolysaccharide-induced Caco-2 cells	63.7	17.8	18.1	66.8
Lipopolysaccharide-induced cells treatedwith sample	76.3	13.2	10.3	73.0

Discussion

DNA content analysis and cell cycle distribution

DNA content analysis and cell cycle distribution was done by flow cytometry and percentage of cells gated in different phases of cell cycle was analysed. The percentage of cells gated in G0/G1 phase was 61.7% in control cells, 63.7% in lipopolysaccharide (LPS)-induced Caco-2 cells and 76.3% in LPS induced cells treated with water extract of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.). An increase in the G0/G1 phase in the sample- treated cells suggests that the drug may induce cell cycle arrest or delay progression into S and G2/M phases. The treatment appears to enhance the number of cells in the resting phase, possibly indicating a response to inflammation and a protective mechanism against uncontrolled proliferation. In S phase, the percentage of cells gated was 12.7% in control cells, 17.8% in lipopolysaccharide (LPS)-induced Caco-2 cells and 13.2% in LPS induced cells treated with water extract of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.). While the S phase increased in LPS-induced cells, suggesting elevated DNA synthesis due to inflammation, the percentage decreased with sample treatment. This reduction indicates that the drug may inhibit the process of DNA replication, which aligns with anti-cancer mechanisms by preventing the progression of potentially cancerous cells through the cell cycle. In G2/M phase, the percentage of cells gated was 25.3% in control cells, 18.1% in lipopolysaccharide (LPS)-induced Caco-2 cells and 10.3% in LPS induced cells treated with water extract of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.). The decrease in G2/M phase cells in both LPS-induced and sample-treated groups suggests that fewer cells are entering mitosis, which may be a desired effect in anti-cancer therapies. The drug's ability to lower the percentage of cells in this phase indicates it may effectively prevent cancer cells from dividing, contributing to its anti-cancer properties. While analysing the debris percentage of cells gated was 60.7% in control cells, 66.8% in lipopolysaccharide (LPS)- induced Caco-2 cells and 73% in LPS induced cells treated with water extract of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.). An increase in debris in treated groups may indicate enhanced cell death, which could be a result of apoptosis or other cytotoxic effects induced by the drug.

Conclusion

DNA analysis and cell cycle distribution has been done by flow cytometry and analysed the percentage of cells gated in different phases of cell cycle. In summary, this study highlights the modulatory effects of the water extract of *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.) on the cell cycle phases of LPS-induced Caco-2 cells, suggesting its potential for both anti-inflammatory and anti-cancer applications. The notable increase in G0/G1 phase cells in the treated group implies that the extract may induce cell cycle arrest or a delay, preventing cells from advancing into DNA synthesis and mitotic stages. This phase-specific arrest aligns with the behavior of compounds that limit uncontrolled cellular proliferation by restricting cell cycle progression, which is crucial in combating cancerous growth.

The observed increase in S phase cells in LPS-induced cells compared to controls, likely due to inflammation-stimulated DNA synthesis, was reduced upon treatment with *Shunthi choorna (*powder of dried rhizome of *Zingiber officinale* Rosc.), indicating the extract's potential to mitigate inflammation-driven DNA replication. Inhibition at the S phase can prevent the accumulation of genetic material necessary for rapid proliferation, thereby slowing the progression of cells that might otherwise contribute to tumorigenesis.

Furthermore, the reduced percentage of cells in the G2/M phase after treatment points to a possible reduction in the number of cells undergoing mitosis, a crucial phase for cell division. By decreasing the population of cells entering mitosis, the extract demonstrates potential in limiting the overall rate of cell division, a desirable effect in anti-cancer strategies. The concurrent rise in cellular debris observed in treated groups, compared to controls, suggests an increase in cell death, possibly through apoptosis or other cytotoxic mechanisms. This heightened cytotoxic response may further contribute to its anti-cancer efficacy by promoting the selective elimination of damaged or cancerous cells. Overall, the findings provide valuable insights into *Shunthi choorna*'s (powder of dried rhizome of *Zingiber officinale* Rosc.). effects on cell cycle regulation, showing promise for future research on its mechanisms and potential therapeutic applications in conditions involving inflammation and cancer. The data suggest that *Shunthi choorna* (powder of dried rhizome of *Zingiber officinale* Rosc.) could act as a cell cycle modulator, inhibiting proliferation and promoting cell death, thus offering a natural alternative that may enhance the efficacy of current anti-cancer therapies. Further studies are warranted to explore the detailed molecular pathways involved and to evaluate the clinical relevance of these findings.

Conflict of interest

None

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