



Research Article

Green Curmin Reduces Pro-inflammatory Cytokines and Fibroblast-Associated Colon Cancer Migration

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Abstract

Background: Green Curmin is a soluble curcumin extract product made in Thailand that has been reported to reduce gastric inflammation. Inflammatory cytokines including IL-1 α , IL-6, and TNF- β play a crucial role in cancer cell migration, which is a major development towards poor prognosis. The aim of this study is to investigate the effects of Green Curmin on colon cancer cell growth and migration, which involve pro-inflammatory cytokines.

Methods: *Curcuma longa* L. were extracted and named Green Curmin. The cytotoxicity of the Green Curmin-treated HCT116-colon cancer cells was obtained by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry. The effect of the extract on cancer migration was investigated with a wound-healing assay. In addition, fibroblast-associated cancer migration was confirmed by using the Transwell migration assay. Pro-inflammatory cytokines were also determined.

Results: Green Curmin had cytotoxicity in the induced-cancer cell apoptosis at concentrations of 1 and 5 mg/ml. However, reduced colon cancer migration and fibroblast associated-cancer migration was found at concentrations of 0.05 and 0.1 mg/ml (non-toxicity dose), respectively. They also suppressed pro-inflammatory cytokines expression including IL-1 α , IL-6, and TNF- β in colon cancer cells and IL-6 in fibroblast cells.

Conclusion: Green Curmin has the potential to suppress pro-inflammatory cytokines and reduce fibroblast associated-cancer migration.

Introduction

Colorectal cancer (CRC) is the most common form of malignant cancer in the gastrointestinal tract. This cancer presents as the third most common type of cancer in oncologic pathology.¹ According to the WHO, cancer causes more death than other diseases.² The accumulation of genetic mutations and environmental factors such as smoking, alcohol consumption, and chronic inflammatory bowel disease has been linked to a high risk of CRC progression.^{3,4} Tumor progression is associated with the hyperactive response of stromal fibroblasts.⁵ One previous study demonstrated that inflammatory cytokines were recognized as a key regulator in fibroblast-activated tumor stromal in cancer migration and invasion.⁶ Several types of pro-inflammatory cytokines, especially IL-1 β , IL-6 and TNF α , are recognized as factors in the tumor microenvironment that play a crucial role in the functions of tumor proliferation, tumor development, tumor angiogenesis and metastasis.⁷ Moreover, they play a critical role in activating the transcription factors that regulate epithelial-mesenchymal transition (EMT) to

promote colon cancer migration.⁸

Chemo-preventive agents such as celecoxib and rofecoxib are used to treat CRC. They can reduce the risk of CRC and retard the progression of colorectal adenomatous polyps to carcinomas. However, prolonged treatment with rofecoxib has the side effect of increasing the risk of cardiovascular disease.⁹ Natural products with minimal toxicity to the normal system are a good choice for cancer-curing agents.¹⁰ Turmeric rhizome (*Curcuma longa*, L.), known locally by Thai people as "Kha Min Chan", belongs to the family Zingiberaceae, which is normally used in folk medicine for the treatment of biliary disorders, anorexia, coughs, diabetic wounds, hepatic disorders, and sinusitis.^{11,12} Curcumin is mainly extracted from turmeric rhizome (*Curcuma longa*, L.) as a yellow-colored hydrophobic polyphenol from the group of phenolic compounds. The hydroxyl group of this compound is related to the ability to inhibit tumor initiation, tumor proliferation, and tumor promotion.¹³ It also has a direct effect on induced-apoptosis and suppressed-multiple signaling pathways such as cancer

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cell proliferation, invasion and metastasis.¹⁴ However, curcumin's low solubility in aqueous solutions leads to low oral bioavailability and therapeutic limitations.¹⁵ Green Curmin is a Thai herbal product that is composed of completely water-soluble turmeric extract. The product is used for the treatment of gastric inflammation and GERD. The effect of Green Curmin on colorectal cancer cell migration associated with inflammation has not yet been elucidated. In this study, we aim to investigate Green Curmin's ability to suppress cancer cell growth and migration associated inflammation.

Materials and Methods

Plant material and extraction

The dried turmeric rhizome (*Curcuma longa*, L.) was obtained from the eastern region of Thailand. First, it was ground roughly using an electric blender to obtain a rough powder. Before extraction, the dried ground turmeric was analyzed for moisture content and total curcumin content.¹⁶ Subsequently, 100 g of turmeric powder was macerated with 500 ml of aqueous ethanol. The sample was stored at room temperature overnight. The extractant was filtered by Whatman no.1 with a vacuum pump, and the residue was re-macerated twice with the same solvent and method. Each of the three extractants was pooled and evaporated to achieve dryness. The weight of the crude turmeric extract was measured and the curcumin content was determined by UV-vis spectrophotometer (PG Instruments Limited, Thailand) at 425 nm. The measured absorbance of the crude extract was compared with the standard curve (Standard curcumin was purchased from Tokyo Chemical Industrial Co., Ltd. Japan.) and expressed as %w/w of curcumin in crude extract. The sample was named Green Curmin.

Sample preparation

Green Curmin was provided by Detox (Thailand) Co, Ltd., Chiangmai, Thailand. The sample was dissolved and diluted with McCoy's 5A medium and filtered with a 0.45 mm filter for sterilization.

Cell culture

Human colon cancer cells, HCT116 (ATCC CCL-247), and human colon fibroblast cells, CCD-18Co (ATCC CRL-1459TM), were purchased from ATCC. These cells were cultured with McCoy's 5A medium (Sigma-Aldrich, USA) supplemented with fetal bovine serum (10% FBS), penicillin (100 µg/ml), streptomycin (100 mg/ml), and 3.7 mg/ml of NaHCO₃. The cells were cultured in an incubator at 37°C with 5% CO₂.

Cell viability assay

The cellular toxicity of Green Curmin was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with some modification.¹⁷ Briefly, the HCT116 cells (2×10⁴ cells/ml) were seeded into 96 culture well plates and incubated at 37°C with 5% CO₂ for 24 hours.

The seeded cells were treated with various concentrations of the sample ranging from 0-5 mg/ml. After 24 hours, the media were discharged and replaced with 100 µl of MTT solution and then incubated for 2 hours to form formazan crystals, after which they were solubilized with dimethyl sulfoxide (DMSO) at 100 µl. An automatic microplate reader (1420 Victor 2, Wallac, USA) at a wavelength of 530 nm was used to measure the soluble formazan crystals. The results were expressed as a percentage of the viable cells.

Apoptotic cell population

The population of the apoptotic cells was obtained by using flow cytometry.¹⁸ The HCT 116 cells were cultured in 6-well plates at 37°C with 5% CO₂. Then, the cells with 1 and 5 mg/ml of the Green Curmin were treated and incubated for 24 hours. The treated cells were detached by 0.25% of trypsin in Hank's balanced salt solution (HBSS) (Gibco1, UK). Then, the cells were stained with an AnnexinV/Propidium Iodine (PI) apoptosis detection kit (Sigma, USA) and incubated at 37°C for 15 minutes under dark condition. The cells were washed with cold Phosphate-buffered saline (PBS) and then the apoptotic populations were measured by using a flow cytometer (FAC Scan, Becton Dickinson). The results were expressed as the percentage of cell apoptosis (% cell apoptosis) compared with the untreated cells.

Cell migration assay

A wound-healing assay was used to investigate cell migration. The assay was modified from that used in a previous study.¹⁹ For the HCT116 cells, 2×10⁵ cells/well were seeded in 6-well plates and then incubated at 37°C for 24 h. The confluent monolayer of the cells was wounded by scratching lines with a scratcher set. The wounds were then washed with PBS pH.7.4. The cells were treated with non-toxic doses (0.05 and 0.1 mg/ml) of Green Curmin and incubated at 37°C with 5% CO₂ for 24 hours. The migration area was obtained by measuring the area of the wounded region lacking cells and then analyzing it with the Image J program. The results were presented as the percentage of cell migration (% cell migration) compared with the untreated cells.

Fibroblast-induced cancer migration

The fibroblast-induced cancer cell migration was investigated by Boyden chamber assay, as modified from a previous study.²⁰ The HCT116 cells (5×10⁴ cells/ml) were seeded in a hanging chamber (Falcon, USA) and incubated at 37°C in a humidified incubator with a 5% CO₂ concentration for 24 hours. The CCD-18Co cells (5×10³ cells/ml) were seeded in a 24-well plate (Falcon, USA) and incubated at 37°C in a humidified incubator with a 5% CO₂ concentration for 24 hours. These cells were co-cultured for 48 hours. The HCT116 migrated cells were fixed with absolute methanol and stained with Hematoxylin & Eosin (H&E). Ten fields of the migrated cells were photographed randomly under a light microscope and the migrated area

was calculated with the Image J program.

Curcumin-reduced fibroblast associated-cancer migration

The method used to investigate cancer cell migration induced by fibroblast was improved from the previous experiment in this study. The HCT116 cells (1×10^5 cells/ml, 500 ml) were seeded in a hanging chamber (Falcon, USA) and incubated at 37°C in a humidified incubator with a 5% CO_2 concentration for 24 hours. The CCD-18Co cells (5×10^3 cells/ml, 2 ml) were seeded in a 24-well plate (Falcon, USA) and incubated at 37°C in a humidified incubator with a 5% concentration of CO_2 for 24 hours. These cells were co-cultured for 72 hours. The CCD-18Co cells were treated with Green Curmin at concentrations of 0.05 and 0.1 mg/ml during co-culturing. The migrated HCT116 cells were fixed with absolute methanol and were stained with H&E. The migrated cells were photographed under a light microscope and the migrated area was calculated with the Image J program.

Pro-inflammatory cytokines expression

Pro-inflammatory cytokines were obtained by the ELISA method, as modified from a previous study.²¹ The HCT116 cells (3×10^4 cells/well) and CCD-18Co cells (1×10^4 cells/well) were seeded in 96-well plates and incubated at 37°C in a humidified incubator with a 5% concentration of CO_2 for 24 hours. These cells were treated with Green Curmin at concentrations of 0.05 and 0.1 mg/ml for 48 hours. The cells were fixed with cold absolute methanol, then washed with cold PBS and blocked with 1% FBS in PBS. The cells were incubated with rabbit-anti IL- 1β , rabbit-anti IL-6, and rabbit-anti TNF α (1:1000) for 30 minutes at RT and washed four times with PBS. Next, the cells were incubated with goat-anti-rabbit-HRP (1:5000) for 30 minutes at RT and washed four times with PBS. The color of the cells was developed with TMB ELISA solution (50 ml) for 5 minutes and the reaction was stopped with 1M HCl (100 ml). The results were measured with a microplate reader at 450 nm.

Statistical analysis

All results were presented as mean \pm standard deviation (mean \pm SD). One-way analysis of variance (ANOVA) and T-test analysis were used to compare the significant differences between the treated and untreated cells. Statistical significance was considered at $p \leq 0.05$ with the SPSS version 25-computer software.

Results

Moisture and curcumin content of Green Curmin

The moisture content and total curcumin content of the dried turmeric were measured and shown to be 9.58% and 7.55%, respectively. In 2018, the Thai Herbal Pharmacopoeia reported that the water content should not be more than 10% and the curcumin content should not be less than 5%. Therefore, the dried turmeric in this study was suitable for further extraction. In this work, the dried turmeric was extracted by the maceration method.

The curcumin content of the crude turmeric extract was determined by UV-vis spectrophotometer, which showed that the level of curcumin in the crude extract was 22.81%, while the yield of the extraction reached 19.64%.

Cytotoxicity of Green Curmin

The colorectal cancer cells (HCT116) were treated with Green Curmin at various concentrations (0.01, 0.05, 0.1, 0.5, 1 and 5 mg/ml) to study the cytotoxicity. The results of the MTT assay showed that the percentage of cell viability decreased in a dose-dependent manner. At 1 and 5 mg/ml of Green Curmin, a significant decrease in cell viability was observed at $69.37 \pm 9.87\%$ ($p \leq 0.005$) and $32.17 \pm 6.83\%$ ($p \leq 0.001$), respectively, when compared with the untreated cells (100%) (Figure 1). The IC_{50} of the Green Curmin was 3.71 ± 0.37 mg/ml. This result suggests that Green Curmin can induce cytotoxicity in a dose-dependent manner.

Green Curmin induced colon cancer apoptosis

High concentrations (1 and 5 mg/ml) were used to study cellular apoptosis. The summations of the early and late apoptotic cells treated with 1 and 5 mg/ml of Green Curmin were $33.46 \pm 6.032\%$ ($p \leq 0.01$) and $52.94 \pm 6.353\%$ ($p \leq 0.01$), respectively. They showed a significant increase in cellular apoptosis when compared with the untreated cells ($17.54 \pm 5.467\%$) in a dose-dependent manner (Figures 2A and 2B). These results indicate that Green Curmin can cause cytotoxicity by inducing cancer cell apoptosis.

Green Curmin reduced colon cancer migration

In the HCT116 cells treated with non-toxic concentrations (0.05 and 0.1 mg/ml) of Green Curmin, there was a significant decrease in cancer cell migration at 48 hours with a $40.73 \pm 7.54\%$ ($p \leq 0.001$) migration area in the cancer cells treated with a 0.1 mg/ml concentration of Green Curmin and a $70.48 \pm 2.03\%$ migration area in the cancer cells treated with a 0.05 mg/ml concentration when compared with the untreated cells ($66.55 \pm 0.32\%$ migration area) (Figures 3A and 3B). These results show that non-

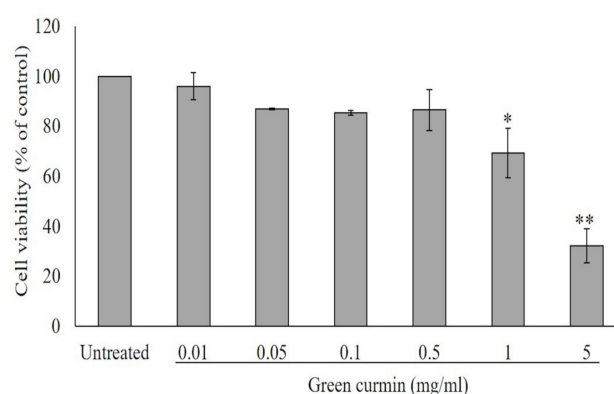


Figure 1. The cytotoxicity of Green Curmin on HCT116 cells. The bar graph represents the percentage of cell viability after treated with various concentrations of samples for 24 h. The untreated cells group was considered 100% of control. Data were expressed as mean \pm SD (n=3). *, ** represent the statistical analysis between the treated and untreated cells at $p \leq 0.05$ and $p \leq 0.01$, respectively.

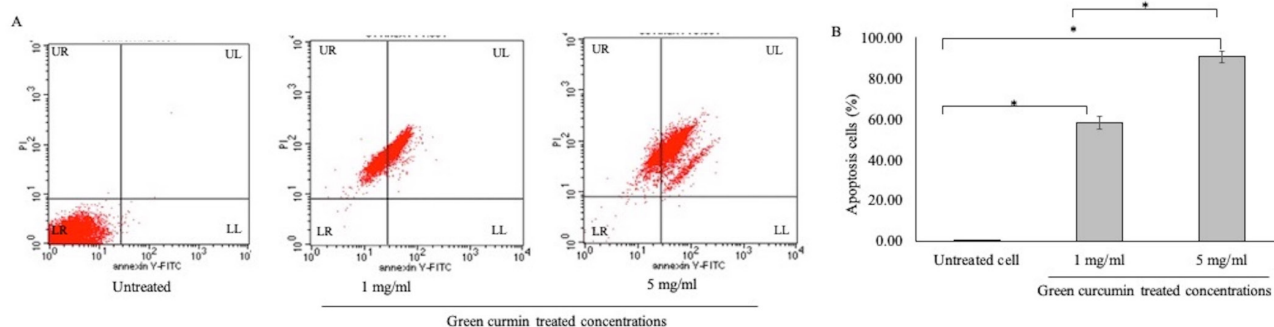


Figure 2. Green Curmin effect on HCT116 apoptosis. LR, LL, UL, and UR represent viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells, respectively (A). The percentage of apoptotic cells was calculated by the summation of early and late apoptotic cells that were expressed as mean±SD (B). *, ** represent the statistical analysis between the treated and untreated cells at $p \leq 0.05$ and $p \leq 0.01$, respectively.

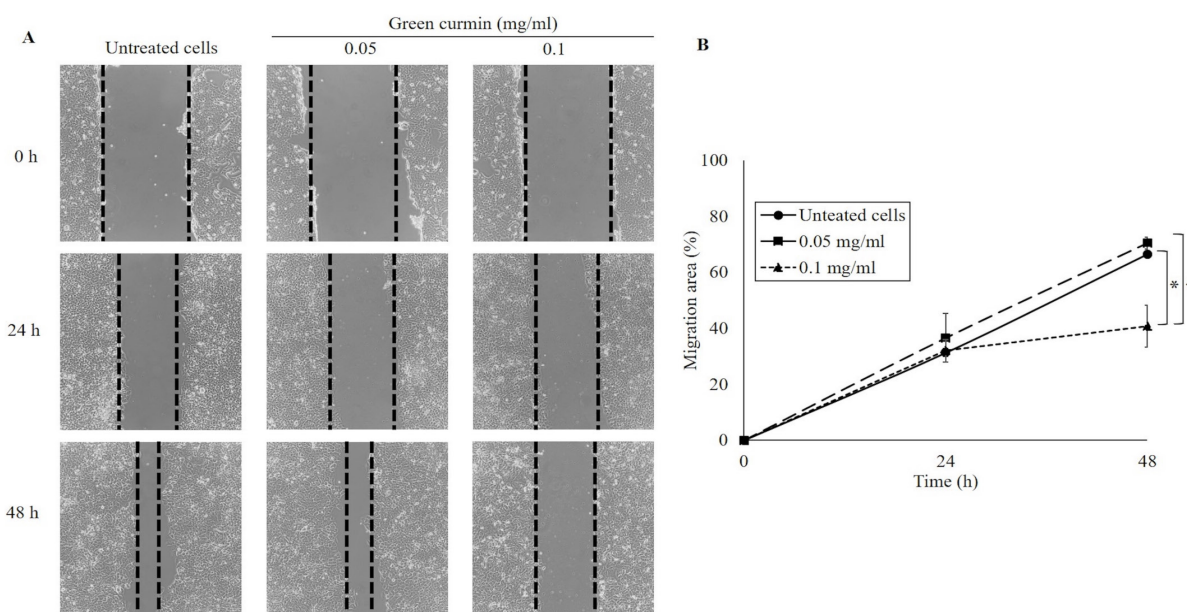


Figure 3. Green Curmin effect on HCT116 migration. Wound-scratched lines were observed under a light microscope (40x) at 24 and 48 h after treated with Green Curmin (A). The percentages of cell migration area (B). ** represents statistical significance at $p \leq 0.01$.

toxic concentrations of Green Curmin can reduce HCT116 cancer cell migration.

Fibroblasts induced colon cancer

The results from the co-culture between the fibroblasts and the colon cancer cells showed that the HCT116 cells had a significantly higher number of migrating cells (82.33 ± 3.25 % per area, $p \leq 0.001$) when compared with the self-cancer migrating cells without fibroblasts (48.51 ± 3.94 % per area) (Figure 4). This result suggests that colon fibroblast can promote colon cancer migration.

Green Curmin reduced fibroblast-associated colon cancer migration

Next, we investigated the effect of Green Curmin on fibroblast-associated cancer migration. The Green Curmin treated-fibroblasts reduced cancer migration (67.21 ± 8.19 % per area ($p \leq 0.005$) and 62.14 ± 5.40 % per area ($p \leq 0.001$) at 0.05 and 0.1 mg/ml, respectively) when compared with the untreated group (84.79 ± 7.58 % per area) (Figure

5). These results suggest that Green Curmin can reduce fibroblast-associated cancer migration.

Green Curmin decreased pro-inflammatory cytokines

The pro-inflammatory cytokines of the colon fibroblasts may be associated with fibroblast-associated cancer migration. The results show that the Green Curmin decreased the IL-6 expression of the colon fibroblasts (Figure 6A). Moreover, the HCT116 cells treated with Green Curmin showed a reduction of IL-1 β , IL-6, and TNF- α in a dose-dependent manner (Figure 6B). These results suggest that Green Curmin can decrease IL-6 expression in colon fibroblasts and also decrease IL-1 β , IL-6 and TNF- α in colon cancer cells.

Discussion

Colorectal cancer (CRC) is the most common form of cancer. The late stage of this cancer has a poor survival prognosis as it is difficult to treat after advancement to this stage.²² Currently, the chemotherapeutic agents used

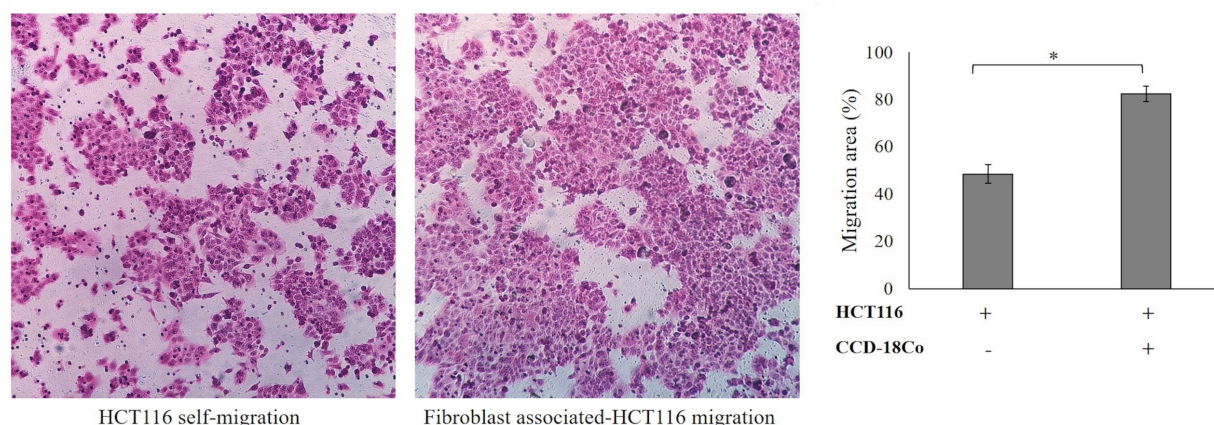


Figure 4. Comparison between HCT116 self-migration and colon fibroblast associated-HCT116 migration. The cancer cell migrations were stained with H&E and observed under a light microscope (40x) (A). The numbers of cancer cell migration were calculated from the area of migrated cells per field area. * represents statistical significance at $p \leq 0.01$.

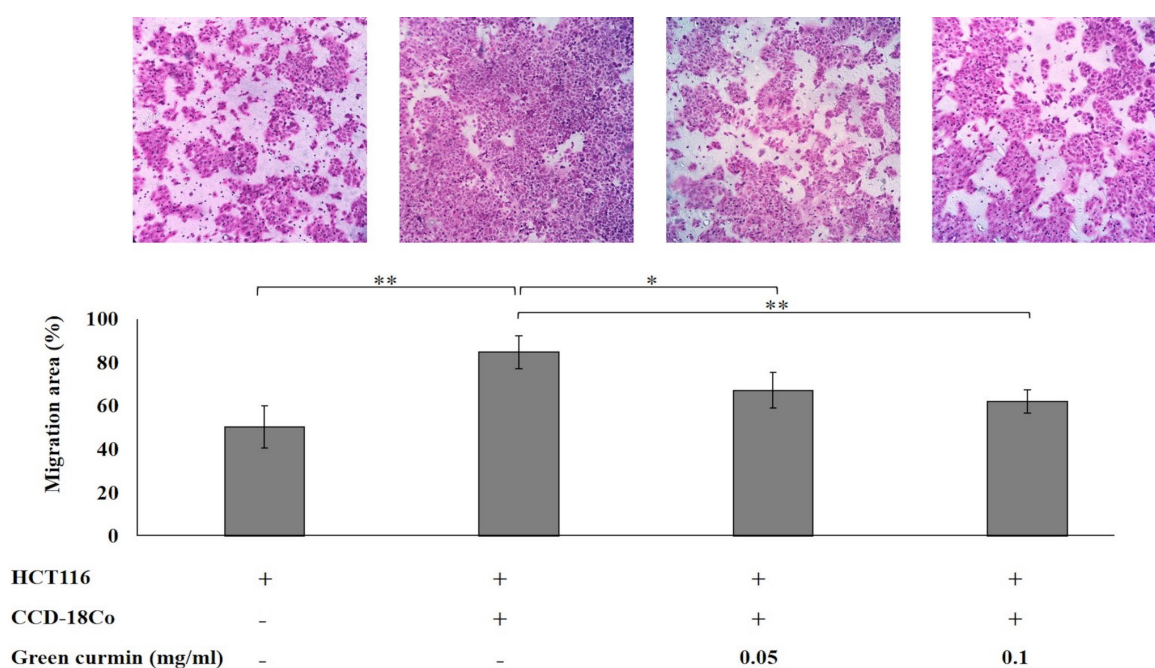


Figure 5. Green Curmin effect on fibroblast induced-HCT116 migration. Various concentrations of Green Curmin were treated on fibroblast and then colon cancer cells were allowed to move to the fibroblast. *, ** represent statistical significance at $p \leq 0.05$ and $p \leq 0.01$, respectively.

for the treatment of CRC are associated with significant side effects. Previous research has suggested that natural products that contain polyphenols may offer potential treatment options due to their anti-proliferation, anti-carcinogenesis, and anti-inflammation properties.²³ In particular, curcumin has been studied for its anti-inflammation and anti-cancer properties.²⁴⁻²⁶ In this study, we tested Green Curmin comprised of soluble turmeric extract, mainly curcumin. According to a recent study that evaluated various extraction methods of curcumin from *C. longa*, the maceration method was shown to produce the highest yield in extraction (20 %) related to the experiment.²⁷

In this study, we hypothesized that Green Curmin might

potentiate the anti-proliferative effect of chemotherapeutic agents in advanced CRC as well as inhibit migration, invasion and metastasis. Therefore, the primary goal of this study was to determine the effect of Green Curmin on HCT116 in terms of colon cancer cell proliferation and apoptosis. The results showed that Green Curmin could suppress cancer cell proliferation and induce cancer cell death through an apoptotic mechanism in a dose-dependent manner. Concurring with the findings of previous research, we observed that curcumin has an effect on cell arrest in the G2/M phase and partially in the G1 phase of the cell cycle to inhibit cancer cell proliferation and promote cellular apoptosis.²⁸

Inflammation is one of the risk factors for cancer as it

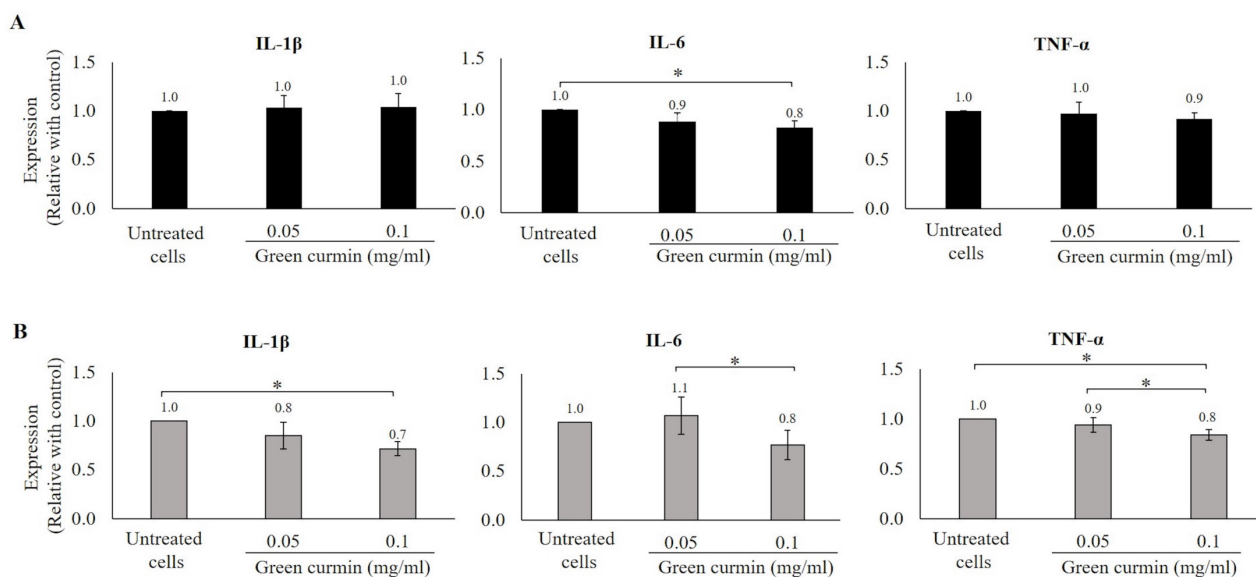


Figure 6. Green Curmin effect on pro-inflammatory cytokines. Pro-inflammatory cytokines in Green Curmin treated colon fibroblast cells (A) and colon cancer cells (B). The expressions were investigated with ELISA assay and calculated by a relative level with untreated cells as control. * represents statistical significance at $p \leq 0.05$.

enables molecules such as cytokine (IL-1 β , IL-6, and TNF- α) to penetrate the tumor microenvironment. A group of cytokines may play a crucial role in supporting the production of cell proliferative and cell survival signals to avoid apoptosis as well as extracellular matrix-modifying enzymes such as metalloproteinases that promote epithelial-mesenchymal transition (EMT) and carcinogenesis. The study of curcumin has shown that it exhibits anti-inflammatory effects via suppressing the production of major pro-inflammatory cytokines.²⁹ As a result, the HCT116 cells in this study were treated with Green Curmin. The treated cells presented a reduction in pro-inflammatory cytokines, including IL-1 β , IL-6, and TNF- α . Based on these results, Green Curmin may play an essential role in suppressing cancer cell proliferation by promoting cellular apoptosis and reducing the production of pro-inflammatory cytokines.

The cancer microenvironment, especially cancer-associated fibroblasts (CAFs), plays an essential role in regulating cancer migration and invasion. Also, CAFs induce cancer inflammation, which increases the severity and causes a high progression of cancer metastasis.³⁰ Therefore, cancer treatment should aim to treat not only cancer itself, but also the tumor microenvironment.³¹ IL-6 is an inflammatory cytokine that regulates cancer progression and cross-talk between active fibroblasts and cancer cells.^{32,33} Green Curmin caused a reduction in the IL-6 of the CAFs. This result may suggest that Green Curmin reduces fibroblast-induced cancer migration through inhibiting fibroblast-IL-6 production. A previous study supported this finding that curcumin can suppress cross-talk between cancer cells and stromal fibroblasts in colon cancer.³⁴

Conclusion

Green Curmin induced HCT116 colon cancer cell

apoptosis and suppressed colon cancer migration by decreasing the pro-inflammatory cytokines, IL-1 β , IL-6, and TNF- α , in colon cancer, while also suppressing the cross-talk mediator, IL-6, in fibroblast cells. Thus, the results show that Green Curmin could be used as a pharmaceutical product in anti-colon cancer and colon inflammation treatments.

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Author Contributions

NP and PS contributed to the conception and design and interpretation data. TM and PA contributed to the experiment and analyzed. NP, TM and PA contributed to write the publication. PS also contributed to critical revision and approval for manuscript publication. All authors have agreed to this published version.

Conflict of Interest

The authors report no conflicts of interest.

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