



# *Clitocybe nebularis* extract and 5-fluorouracil synergistically inhibit the growth of HT-29 colorectal cancer cells by inducing the S phase arrest

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## Abstract

Although 5-fluorouracil (5-FU) is an important anticancer agent for the treatment of colorectal cancer, drug resistance, and dose-related side effects limit the effectiveness of the treatment. Therefore, developing new pharmaceuticals with effective and low toxicity is critically necessary for cancer therapy. This study aimed to investigate the cytotoxic activity of the *Clitocybe nebularis* mushroom extract (CN) on HT-29 human colon cancer cells. A series of in vitro experiments were performed on the HT-29, Caco-2, and HEK-293 cells, which includes cytotoxicity, drug interaction, colony formation, cell cycle, and migration assays. In addition, qRT-PCR experiment was also performed to investigate the potential molecular mechanisms of action of CN on the proliferation of colon cancer cell line. Our results show that CN exhibited selective cytotoxic activity on HT-29 and Caco-2 colon cancer cells, whereas no cytotoxic effect was observed on normal HEK-293 cells. With the combination of CN and 5-FU, their cytotoxic activity on HT-29 cells was significantly increased compared to their use alone. In addition, the combination of CN and 5-FU also showed synergistic anticancer activity through cell cycle arrest in the S phase. The results also show that p21, p27, and p53 expression levels increased as a result of CN treatment. Our in vitro findings show that CN has a synergistic effect with 5-FU by inhibiting cell proliferation of colon cancer cells and inducing cell cycle arrest in the S phase.

**Keywords** Anticancer · Cell-cycle arrest · *Clitocybe nebularis* · Colorectal carcinoma · 5-fluorouracil

## Abbreviations

|      |                             |
|------|-----------------------------|
| CN   | Clitocybe nebularis extract |
| CRC  | Colorectal cancer           |
| 5-FU | 5-Fluorouracil              |
| CI   | Combination index           |
| DRI  | Dose reduction index        |

## Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer type in the world. In 2030, CRC cases are expected to increase by 60%, reaching 2.2 million new cases and 1.1 million deaths (Arnold et al. 2017). Surgery, radiotherapy, and chemotherapy are the still main options for the treatment of CRC. However, 40–50% of patients are losing their lives due to recurrence, metastasis, and drug resistance (Bray et al. 2018). Despite recent therapeutic advances, 5-fluorouracil (5-FU) is still the first-line chemotherapy drug for the treatment of advanced colorectal cancer (Webber et al. 2015). 5-FU, the antimetabolite drug shows its anticancer effects mainly through inhibition of thymidylate synthase, a key enzyme for catalyzing the de novo synthesis of thymine (Parker and Cheng 1990). In the treatment of CRC, 5-FU is used alone or in combination with other drugs such as oxaliplatin, irinotecan, or leucovorin. However, drug resistance remains a major problem limiting the efficacy of 5-FU or its combinations. This drug also has serious side effects such as nausea, fatigue at high doses, which reduces

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the patient's life quality and decreases the number of blood cells (Delval and Klasterky 2002). The drug-related side effects not only worsen the quality of life of patients but can also cause them to refuse treatment. Therefore, the development of novel treatment strategies is required to improve the effectiveness of 5-FU and overcome drug resistance.

There is an increasing interest in biologically active molecules derived from natural compounds for the discovery of new drugs and precursor compounds. More than half of modern pharmaceuticals used today also have a natural origin, confirming this interest (Clardy and Walsh 2004). Recently, several studies have shown that combination therapy of chemotherapeutic agents with natural compounds could enhance the anti-cancer activity of chemotherapeutic agents and reduce the side effects they cause (Riahi-Chebbi et al. 2019; Lin et al. 2020). Therefore, effective natural compounds with low toxicity could be used to develop treatment strategies for CRC. Using existing therapeutic drugs with new natural agents could increase treatment response rates and reduce the toxicity of existing drugs.

Mushrooms are one of the most sources of these functional natural compounds for the scientific world over the last few years. Edible mushrooms have considerable nutritional value and have been used for thousands of years as food all over the world. Besides, some species, also known as medicinal mushrooms, are used as dietary supplements in Asia (Dai et al. 2009). Fungal species have attracted great attention in recent years because of their therapeutic potential due to containing several bioactive compounds, such as lectins, terpenoids, polyphenol, and polysaccharides (Valverde et al. 2015). Extensive studies have confirmed the biological activity of mushrooms, they are promising with their antioxidant, anti-cancer, and anti-inflammatory effects (Shomali et al. 2019; Nowakowski et al. 2021). Mushroom extracts are reported to be used for the prevention and treatment of certain cancers, including gastrointestinal and breast cancers (Opattova et al. 2019; Lee et al. 2021). Therefore, medicinal mushrooms and their synthetic derivatives are expected to play an important role in developing innovative agents for the prevention of human cancers.

The clouded agaric or cloud funnel mushroom, *Clitocybe nebularis* is a saprotrophic species that belong to the Tricholomataceae family. This edible mushroom is common in mixed forests throughout Europe and North America. Despite *Clitocybe* species including *Clitocybe nebularis* not classified as medicinal mushrooms, recent studies showed clearly their pharmaceutical potential based on various extracts and compounds. Important bioactive molecules such as ribonucleosides, lectins, L-amino-acid oxidases, and phenolic compounds have been found in *Clitocybe* species and have been associated with antioxidant, anti-proliferative, anti-viral, and proteinase inhibition properties (Pohleven et al. 2016; Kosanić et al. 2020; Dizeci et al. 2021).

In this study, we investigated the anti-proliferative activity of *Clitocybe nebularis* extract (CN) in HT-29 and Caco-2 human colorectal cancer cell lines as well as potential molecular mechanisms in comparison with normal cells, HEK-293. We also aimed to test CN extract's ability to enhance the effectiveness of 5-FU. As far as we know, this study is the first that anti-cancer properties of *C. nebularis* have been investigated in detail.

## Materials and methods

### Mushroom sample

*C. nebularis* was obtained from the public market in the Black Sea region of Turkey. The mushroom sample was identified by Dr. Ilgaz Akata and collected at the Ankara University Fungarium with CN-2015 (*C. nebularis* (Batsch) P. Kumm.) code number. Ethanolic extraction was performed using the method described by Shomali et al. (2019).

### Cell lines and cell culture

The CRC cell lines used in this study were HT-29 (ATCC HTB-38), Caco-2 (ATCC HTB-37), whereas the normal cell line used was HEK-293 (ATCC CRL-1573) kidney epithelial cells. Cell lines were obtained from the American Type Culture Collection (ATCC). HT-29 cells were cultured in RPMI-1640 medium and Caco-2 and HEK-293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% L-Glutamine, 0.1 mM nonessential amino acid, and 100 U/ml penicillin/streptomycin. Cells were incubated in a humidified incubator at 37 °C with 5% CO<sub>2</sub>. Cells were used 10–20 passage numbers and tested negative for mycoplasma contamination.

### Cell viability assay

For the determination of the cytotoxic activity of the CN,  $1 \times 10^4$  cells were seeded in 96-well tissue culture plates. The cells were incubated for 18 h and then treated with the mushroom extract at various concentrations along with the vehicle (DMSO) and positive (5-FU) controls. After 24 h of incubation, the cell viability was evaluated using the MTT assay as described previously (van Meerloo et al. 2011). Cytotoxic activity of CN was expressed as the IC<sub>50</sub> and calculated based on the dose–response inhibition curve. All

experiments were performed in triplicate with three technical replicates.

### Drug interaction analysis

Drug interactions between CN and 5-FU were calculated according to the median-effect principle of the Chou and Talalay method (Chou 2006). HT-29 cells were treated with various dose combinations of CN and 5-FU (ratios of IC<sub>50</sub> as CN:5-FU; 1:1, 1:2, 2:1, 1:3, 3:1, 1:4, and 4:1) to investigate drug synergism. Analysis of drug synergy was performed by calculating the combination index (CI) using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). Synergism was indicated by CI < 1.0, additivity was indicated by CI = 1.0, and antagonism was indicated by CI > 1.0. In addition, the dose reduction index (DRI) was calculated to evaluate synergism. DRI > 1.0 represents dose reduction to achieve the same inhibitory effect in a combination setting.

### Crystal violet staining assay

As a second method for evaluating the cytotoxic activity, crystal violet staining assay was performed. In this method, HT-29 cells were seeded in 6-well plates ( $1 \times 10^5$  cells/well) and treated for 24 h as CN, 5-FU, and CN + 5-FU using the IC<sub>50</sub> values obtained from the MTT assay. After 24 h of drug exposure or control conditions, the cells were washed and stained with 0.5% crystal violet for 5 min and photographed. Finally, the uptaken crystal violet was solubilized by methanol and quantified by measuring the absorbance at 570 nm in a plate reader (BioTek) (Feoktistova et al. 2016).

### Clonogenic assay

HT-29 cells were plated on 6-well plates (1000 cells/well) to perform the clonogenic assay and treated with CN, 5-FU, and CN + 5-FU for 24 h. Each sample, as well as control, was performed in triplicates. After 24 h of treatment, the medium was replaced with fresh medium. After 14 days, colonies were fixed with ice-cold absolute methanol and stained with 0.5% crystal violet. Colonies of more than 50 cells were counted and percentages of colonies were calculated (Franken et al. 2006).

### Wound healing assay

The migration ability of the HT-29 cells was demonstrated by a wound-healing assay. HT-29 cells were seeded at a density of  $5 \times 10^5$ /well in 6-well plate and were allowed

to attach overnight. A scratch in the cell monolayer was made using a sterile 200  $\mu$ L pipette tip, and then the monolayer was washed with PBS. Cells were exposed to CN and 5-FU alone or in combination for 24 h. After the treatment, images were taken at 0, 24 h, and ImageJ software was used to analyze the wound areas.

### Cell-cycle analysis

HT-29 cells ( $5 \times 10^5$ ) were seeded into six-well plates and grown overnight as monolayers and then exposed for 24 h to testing agents as CN, 5-FU, and CN + 5-FU. Thereafter, cells were harvested by trypsinization and fixed with 70% ice-cold ethanol. After washing twice with 1X ice-cold PBS, cells were resuspended in propidium iodide (PI)/RNase staining solution for 30 min in the dark at 37 °C. The distribution of different cell cycle phases was analyzed using flow cytometry (Acea NovoCyte). The experiment was performed in triplicate.

### Real-time PCR

HT-29 cells were plated at a density of  $0.1 \times 10^6$ /2 ml in 6-well plates and incubated with CN, 5-FU, and CN + 5-FU for 24 h. Total RNA was extracted using the High Pure RNA Isolation Kit (Roche, Switzerland) according to the manufacturer's instructions. The purity and concentration of RNA were determined using ND-2000 Spectrophotometer (Thermo Fischer Scientific, USA). Reverse transcription was carried out using RevertAid First Strand cDNA Synthesis Kit (Invitrogen, USA) according to the manufacturer's protocol using 1  $\mu$ g of total RNA. Real-time PCR was performed using LightCycler 480 SYBR Green I master mix (Roche, Switzerland) in CFX Connect detection system (Bio-Rad,

**Table 1** Real-time PCR gene primer list

| Primer            | Sequence 5' → 3'            |
|-------------------|-----------------------------|
| GAPDH forward     | GAAATCCCATCACCATCTTCCAGG    |
| GAPDH reverse     | GAGCCCCAGCCTTCT CCATG       |
| Cyclin A2 forward | AGACCTACCTCAAAGCACCACAG     |
| Cyclin A2 reverse | GGTTGAGGAGAGAAACACCATGA     |
| CDK1 forward      | TGGATCTGAAGAAATACTTGGATTCTA |
| CDK1 reverse      | CAATCCCCTGTAGGATTTGG        |
| CDK2 forward      | TTCTCATCGGGTCCTCCACC        |
| CDK2 reverse      | TCGGTACCACAGGGTCACCA        |
| p53 forward       | TTGCAATAGGTGTGCGTCAGA       |
| p53 reverse       | AGTGCAGGCCAACTTGTTTCAG      |
| p21 forward       | CGATGGAACCTCGACTTTGTCA      |
| p21 reverse       | GCACAAGGGTACAAGACAGTG       |
| p27 forward       | GGTTAGCGGAGCAATGCG          |
| p27 reverse       | TCCACAGAACCGGCATTG          |

USA), and amplification was performed in triplicate and repeated twice. GAPDH was used as internal control and analysis was done using Livak's  $\Delta\Delta CT$  method (Livak and Schmittgen 2001). The primer sequences used in this study were described in Table 1.

## Statistical analysis

Comparisons between different groups were made using Student's t-tests and One-way ANOVA. The results obtained are expressed as the mean  $\pm$  standard deviation of three repeated experiments. A level of  $P < 0.05$  was considered statistically significant. SPSS 17.0 and GraphPad Prism 8 software were used for statistical analysis and data presentation.

## Results

### CN displayed selective cytotoxic activity on CRC cells

To evaluate the cytotoxic activity of CN, we used HT-29, Caco-2 CRC cell lines, and HEK-293 normal kidney cell line as a normal cell line. We first studied the effect of different doses (7.81–500  $\mu\text{g/mL}$ ) of CN on the viability of HT-29, Caco-2, and HEK-293 cells for 24 h using an MTT assay. The chemotherapeutic agent 5-FU, which is widely used in the treatment of CRC, was used as a positive control during

the study. As shown in Fig. 1, CN treatment resulted in a dose-dependent decrease in cell proliferation in HT-29 and Caco-2 cells albeit at different levels. On the other hand, no similar result was observed in HEK-293 cells after CN treatment. The effect of the CN on cell viability was calculated using the dose–response curves generated for each cell line and expressed as the  $IC_{50}$  value.  $IC_{50}$  values of  $243 \pm 17$  and  $38 \pm 1$  in HT-29 and  $298 \pm 44$  and  $21 \pm 4$   $\mu\text{g/mL}$  in Caco-2 cells were calculated for CN and 5-FU, respectively. Since a 50% decrease in cell viability was not observed in HEK293 cells, the  $IC_{50}$  value could not be calculated. As a result, while CN exhibited cytotoxic activity in both CRC cell lines, it did not decrease cell viability significantly in the normal cell line.

### Synergistic effects of CN and 5-FU

We next investigated whether CN and 5-FU could act synergistically to suppress HT-29 cell viability. Using  $IC_{50}$  values, 7 different combinations were prepared, and cell viability experiments were repeated on HT-29 cells using these combinations (Fig. 2a). Compared with the viability of HT-29 cells treated with CN or 5-FU alone for 24 h, the combination of CN with 5-FU significantly increased the proliferation inhibitory effect of HT-29 cells (Fig. 2b). The combination index for this synergist combination was calculated as  $CI = 0.21867$  (Table 2). Therefore, this combination was also

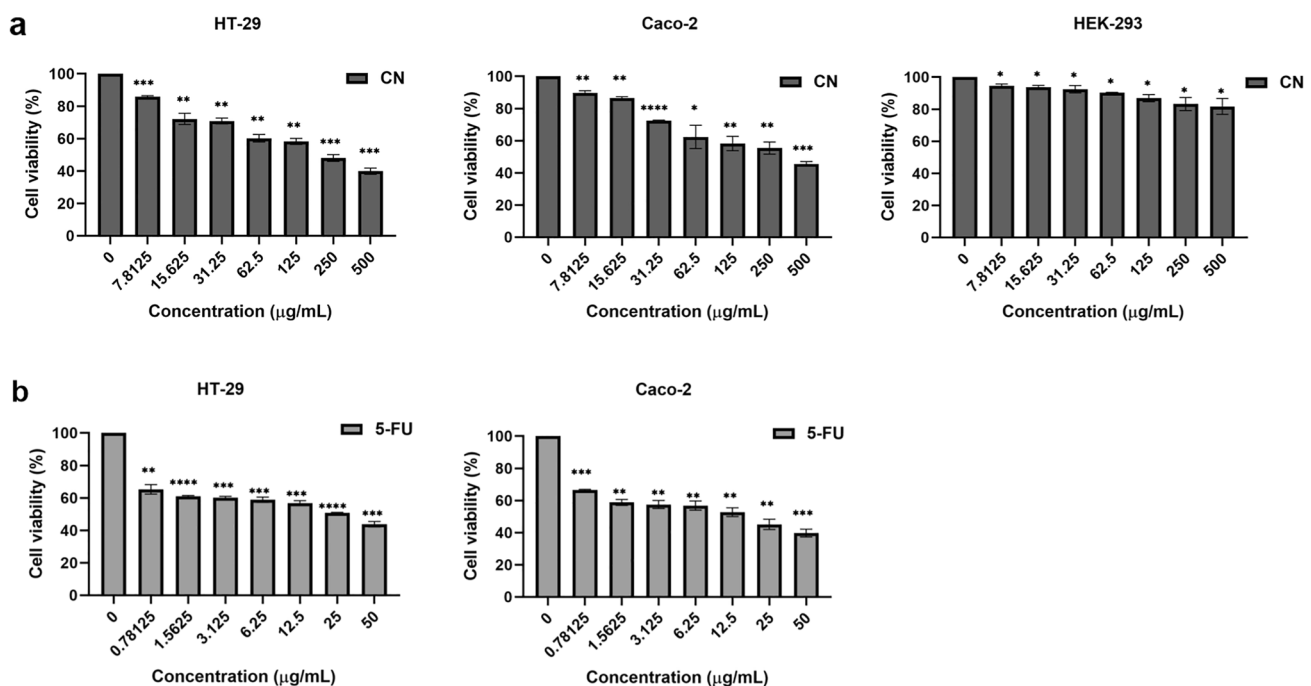


**Fig. 1** The cytotoxic effects of **a** CN (7.81–500  $\mu\text{g/mL}$ ) and **b** 5-FU (0.78–50  $\mu\text{g/mL}$ ) on HT-29, Caco-2, and HEK-293 cells for 24 h. MTT assay was performed to assess cell viability. The results are

given as mean  $\pm$  SD of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$

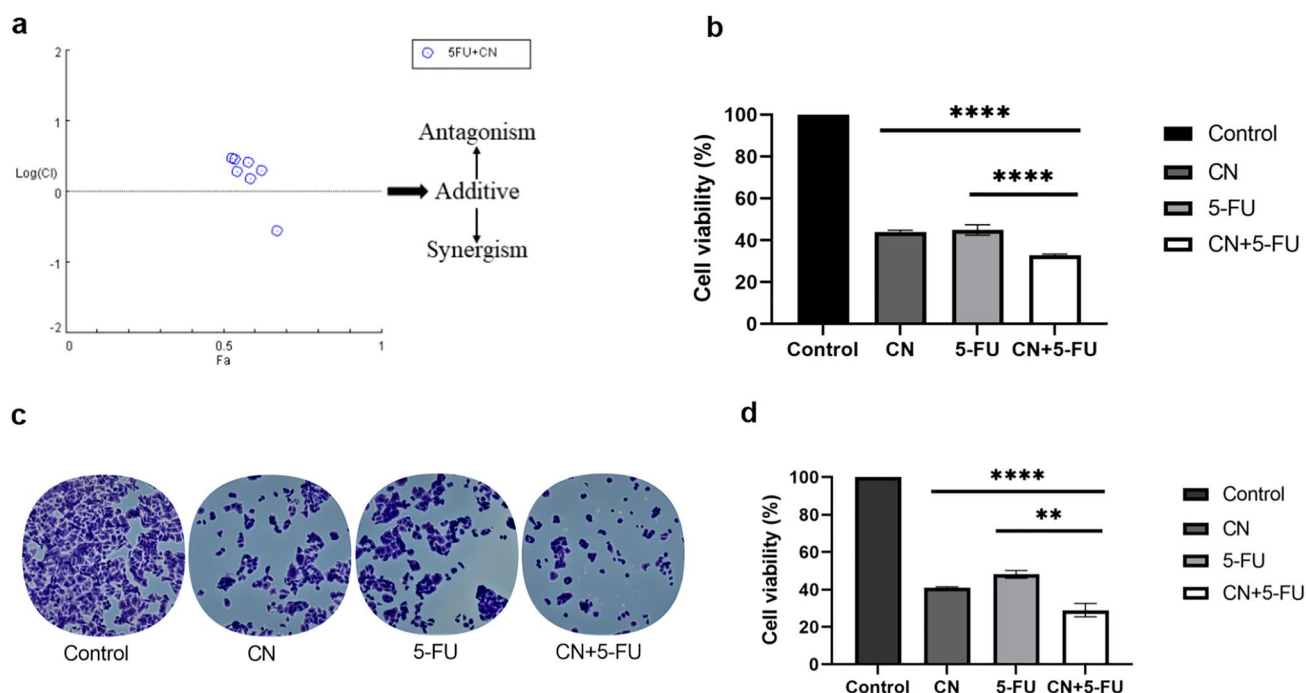


**Fig. 2** Synergistic effects between CN and 5-FU in HT-29 cells. **a** Combination index (CI) analysis. CI=1, additivity; CI>1, antagonism; CI<1, synergy. **b** The cell viability of HT-29 cells treated with synergistic CN+5-FU combination. HT-29 cells treated with CN (243 µg/ml) and 5-FU (38 µg/ml) alone or in a combination for 24 h.

The results are given as mean ± SD of three independent experiments. \*\*\*\* $P$ <0.0001. **c,d** Crystal violet staining of HT-29 cells treated with CN (243 µg/ml) and 5-FU (38 µg/ml) alone or in a combination for 24 h. The results are given as mean ± SD of three independent experiments. \*\* $P$ <0.01, \*\*\*\* $P$ <0.0001

**Table 2** In vitro combination index (CI) values of CN and 5-FU combinations in HT-29 cells

| CN | 5-FU | CI      | Description*     |
|----|------|---------|------------------|
| 1  | 1    | 0.21867 | Strong synergism |
| 1  | 2    | 2.96587 | Antagonism       |
| 2  | 1    | 1.50970 | Antagonism       |
| 1  | 3    | 1.94315 | Antagonism       |
| 3  | 1    | 2.56553 | Antagonism       |
| 1  | 4    | 2.85315 | Antagonism       |
| 4  | 1    | 2.00585 | Antagonism       |

CI values were calculated from the MTT cell viability assays. The data represent the mean of three independent experiments. \*According to the grading system developed by Chou (2006)

investigated in further analyzes in the study. In addition, the dose reduction index (DRI) was calculated to evaluate drug synergism. A DRI value > 1 indicates the fold change in drug dose reduction for the targeted effect level. For the synergistic CN + 5-FU combination, DRI values were calculated as 1.96 for CN and 3.19 for 5-FU. As a result, the IC<sub>50</sub> values of each drug in combination decreased to 123 and 12 µg/ml for CN and 5-FU, respectively.

### CN enhances the effect of 5-FU in HT-29 cells

Crystal violet assay was performed as a second method to investigate the effect of CN or 5-FU combination on the viability of HT-29 cells. After 24 h treatment with CN, 5-FU, or their combination, remaining living cells in the plates were photographed after staining with crystal violet. The viability of HT-29 cells treated with CN was decreased to 40% after 24 h ( $P$ <0.0001). In addition, as shared in Fig. 2d, the cytotoxic effect of 5-FU on HT-29 cells were enhanced by 24 h of co-incubation with CN and the cell viability decreased up to 29%.

### CN and 5-FU combination inhibits colony formation

Clonogenic assay was performed to confirm the anti-proliferative effect of CN on the proliferation of HT-29 cells by further analysis. The colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. As a result of the clonogenic assay, the effects of monotherapy or combination on the proliferation and colony-forming ability of HT-29 cells were determined. As shown in Fig. 3a, b, CN quite effectively inhibited the proliferation and colony-forming ability of HT-29 cells. Also, the results indicated that the colony formation ability

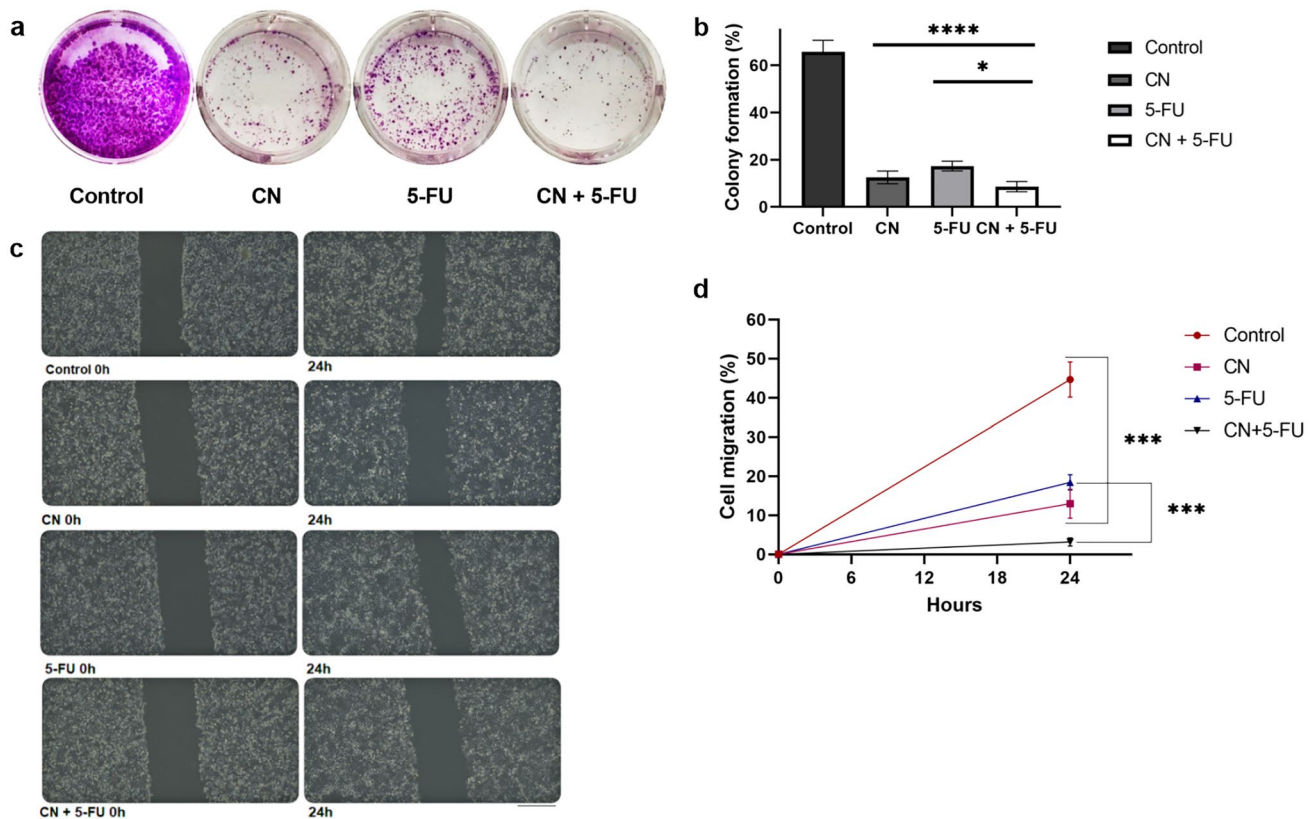


**Fig. 3** The effects of CN and 5-FU combination on colony formation and migration in HT-29 cells. **a** Colony formation image following treatment with CN (243  $\mu\text{g/ml}$ ), 5-FU (38  $\mu\text{g/ml}$ ), and CN+5-FU combination for 24 h. **b** Histogram plots of colony formation percentage. The results are given as mean  $\pm$  SD of three independent

experiments.  $*P < 0.05$ ,  $****P < 0.0001$ . **c,d** In vitro wound healing experiment. HT-29 cells were wounded and treated with CN (243  $\mu\text{g/ml}$ ), 5-FU (38  $\mu\text{g/ml}$ ), and CN+5-FU combination for 24 h. Scale bar (80  $\mu\text{m}$ ). The results are given as mean  $\pm$  SD of three independent experiments.  $***P < 0.001$

of HT-29 cells would be diminished with the CN treatment. In addition, combination treatment inhibited clone formation more than single-drug treatment ( $P < 0.05$ ). All these results show that CN and 5-FU have a significant anti-tumor synergistic effect and effectively inhibit the colony-forming ability of HT-29 cells.

### CN and 5-FU inhibits migration of colon cancer cells in vitro

In vitro wound healing experiments were performed to investigate whether CN or the combination with 5-FU has an inhibitory effect on the migration of HT-29 cells. As shown in Fig. 3c, d, wound repair appears to be delayed in HT-29 cells after administration of CN and 5-FU alone or in combination, compared to control groups. At the end of 24 h, it was observed that the cells in the control group covered approximately 44% of the wound area. The percentage of the area covered after 24 h exposure of cells to CN extract and 5-FU alone was calculated as 12% and 18%, respectively ( $P < 0.001$ ). However, when CN and 5-FU were

applied together, cell migration was effectively inhibited, and the coverage decreased to 3.2% (Fig. 3d). As a result, the combination of CN with 5-FU synergistically inhibited the motility of HT-29 cells ( $P < 0.001$ ).

### CN + 5-FU combination synergistically induced an S-phase cell cycle arrest

To examine whether CN-induced growth inhibition was associated with cell cycle regulation, the cell cycle distribution was analyzed by flow cytometry. After 24 h of treatment, the percentage of cells in the S phase increased after cells were exposed to CN (15% to 25%) and 5-FU (15 to 29%) alone ( $P < 0.01$ ). In connection with this, the percentage of cells in the G2 phase decreased to 23% and 21%, respectively. Besides, the combination treatment led to a greater increase in the percentage of cells in the S phase (from 15 to 36%) compared with either agent. Thus, combined treatment of CN with 5-FU led to a synergistic cell cycle arrest in both S phases on HT-29 cells. As a result, CN and 5-FU kept the cells in the S phase, and

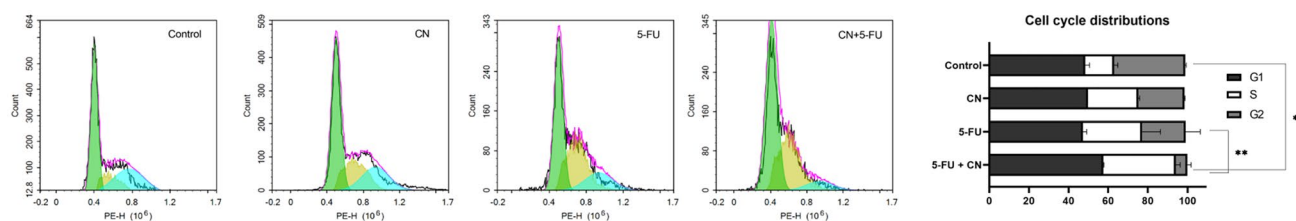


**Fig. 4** The effect of CN and 5-FU on cell cycle progression. HT-29 cells were treated for 24 h with CN (243 µg/ml), 5-FU (38 µg/ml) alone or in combination, and cell cycle distributions were analyzed

by flow cytometry. The results are given as mean  $\pm$  SD of three independent experiments. \*\* $P < 0.01$

a significant increase in this effect was observed when they were applied together. Thus, the combination of CN extract and 5-FU led to cell cycle arrest induced in the S phase in HT-29 cells (Fig. 4).

### CN regulates cell cycle-associated mRNA expression

To elucidate the mechanisms of CN-induced cell cycle arrest, HT-29 cells were treated with CN, 5-FU, or their combination for 24 h, then collected and analyzed by

qRT-PCR. The results showed that CN treatment downregulated mRNA expression levels of key S phase regulators Cyclin A2, CDK1, and CDK2, ( $P < 0.05$ ) (Fig. 5). Moreover, we observed an increase at the mRNA levels of p53, p21, and p27 after the treatment with CN for 24 h (Fig. 5). Therefore, CN regulates mRNA levels of certain cell cycle-related genes to induce the S-phase cell cycle arrest in HT-29 cells.

We also investigated the mechanisms of CN + 5-FU combination-induced cell cycle arrest. After combination treatment, a decrease in mRNA expression of Cyclin A2 gene

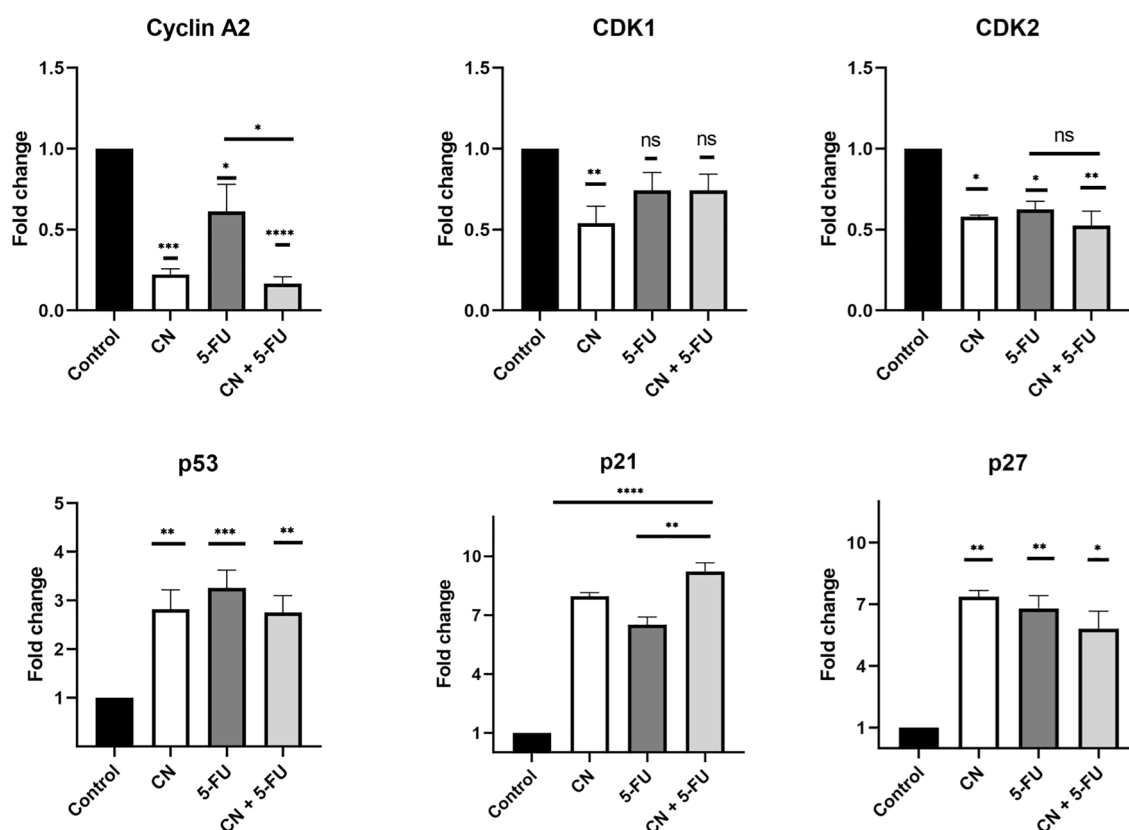


**Fig. 5** The expression levels of Cyclin A2, CDK1, CDK2, p53, p21, and p27 in HT-29 cell line. HT-29 cells were treated with CN (243 µg/ml), 5-FU (38 µg/ml) alone, or in combination for 24 h. qRT-PCR analysis was performed to assess gene expression. GAPDH

was used as the reference gene. Results are the mean  $\pm$  SD of two independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , ns  $P > 0.05$

and an increase in p21 gene was observed compared to cells treated with 5-FU alone ( $P < 0.05$ ) (Fig. 5). Also, a decrease in CDK2 gene expression was observed after combination therapy, but the results were not statistically significant ( $P > 0.05$ ). Although a twofold reduction was observed for CDK1 gene expression after 24 h of CN treatment, the single and combined administration of 5-FU was not significant compared to the control group ( $P > 0.05$ ).

## Discussion

Colorectal cancer is the most common type of cancer in the gastrointestinal tract. It ranks third among cancer-related deaths with an average survival rate of 55% (Sung et al. 2021). The incidence of colon cancer is constantly increasing due to environmental and modifiable lifestyle factors such as a vegetable and fruit-free diet, lack of physical activity/exercise, tobacco products, and excessive alcohol consumption (Ait Ouakrim et al. 2015). In connection with this, it is estimated that the incidence of colorectal cancer will increase to 2.5 million new cases worldwide in 2035 (Arnold et al. 2017). Surgery is the primary treatment option for colorectal cancer, but approximately 33% of patients relapse after surgery, thus it is combined with adjuvant chemotherapy. In addition, lymph node or distant site metastases are observed in approximately 60% of patients diagnosed with colorectal cancer (Wang et al. 2007). Surgery alone is insufficient to provide curative treatment in advanced stages and metastatic cancers. Therefore, chemotherapy is considered the most potent treatment option for improving poor survival rates in colorectal cancer. Fluoropyrimidines such as 5-FU has been the most widely used chemotherapeutic agent clinically for nearly 30 years in the treatment of advanced and metastatic colorectal cancer (Xie et al. 2020). However, as with other chemotherapy drugs, multiple drug resistance remains the primary barrier to appropriate chemotherapy treatment for colon cancer. In addition, the side effects caused by drugs adversely affect the quality of life of patients and limit the effectiveness of the treatment. Therefore, the clinical use of the new drugs or chemotherapy sensitizing combinations has become the focus to increase the effectiveness of chemotherapeutic agents and reduce the side effects they create. Natural compounds have been used for a long time to prevent and treat many diseases, including cancer. In addition, it is thought that natural compounds may play a critical role in the development of new strategies to be used in cancer treatment. Due to their rich structural diversity and complexity, natural products and their derivatives have been invaluable resources in the discovery of primary-line drugs (Clardy and Walsh 2004). Natural compounds and their potential clinical uses have many advantages. Regarding its anticancer properties; they have properties such as

antioxidant (chemoprotective) and even prooxidant (pro-apoptotic) that affect proliferation, angiogenesis, tumor metastasis, or induce tumor cell apoptosis (Baena Ruiz and Salinas Hernández 2016). Therefore, the use of natural compounds with chemotherapeutic agents in colorectal cancer treatment has emerged as a popular approach in recent years to combat the negative effects of chemotherapeutics and increase their cytotoxic activity on cancer cells (Rejhová et al. 2018). It is possible to target multiple signaling pathways with the combined therapy application. So, in addition to increasing the cytotoxic activities or reducing the side effects of drugs, effects can also be observed in the changing cellular pathways responsible for the development of drug resistance. Therefore, it will be possible to develop new strategies in cancer treatment by the interaction of traditional chemotherapy agents with effective natural compounds.

In the previous work, Dizeci et al. (2021) demonstrated that CN contains many biologically active molecules and has a strong antioxidant potential. Moreover, CN extract effectively inhibited the activity of the Glutathione S-transferase enzyme which is very important, especially in cases where heavy drug therapy is required in patients undergoing chemotherapy. Therefore, this study aimed to identify a new, effective and non-toxic natural compound-based anti-cancer activity for the treatment of colon cancer. The effects of CN combined with 5-FU on the proliferation of HT-29 colorectal cancer cells were systematically evaluated and the mechanism of action of this combination was investigated in detail.

Our results showed that CN could reduce the viability of colorectal cancer cells by up to 60% in a concentration-dependent manner (Fig. 1a). CN reduced the viability of both HT-29 and Caco-2 cells and exhibited a highly effective activity profile except at low concentrations. As a result, we confirmed the cytotoxic potential of CN using two different CRC cell lines. On the other hand, CN did not have a significant cytotoxic effect on HEK-293 normal kidney epithelial cells (Fig. 1a). In conclusion, CN showed selective cytotoxicity on human colon cancer cells and low sensitivity to normal cells. Similar to our results Kosanić et al. (2020) showed that acetone extract of *C. nebularis* did not express cytotoxic activity on the MRC-5 normal cell line while exhibiting moderate cytotoxic activity in some cancer cells. Most of the current anticancer drugs used in cancer treatment cause undesirable side effects due to their toxicity to normal cells. Today, studies are carried out to develop new drugs with more selective effects, therefore the result we obtained in HEK-293 cells is very important for further studies. Next, we investigated drug-drug interactions for the combined use of CN with 5-FU on HT-29 cells by the Chou-Talalay method (Fig. 2a). When the CN was co-administered with 5-FU, their anti-cancer activities on HT-29 cells were significantly increased compared to their single-use. By increasing the therapeutic efficacy of conventional agents

in combination, it will be possible to obtain positive results such as maintaining or increasing the same efficacy without causing toxicity as a result of dose reduction, reducing or minimizing the development of drug resistance (Chou 2006). The DRI > 1 indicates a favorable dose reduction of each drug in combined use compared to their use alone. Thus, it is possible to reduce the 5-FU dose up to 3.19 fold despite maintaining its efficacy when combined with CN. In addition to the MTT assay, we further performed a crystal violet staining to confirm the effect of monotherapy and combination on HT-29 cells. Similarly, CN effectively inhibited the proliferation of HT-29 cells, and when co-administered with 5-FU to cells, it increased the activity of 5-FU (Fig. 2c, d).

Abnormal cell proliferation is a typical feature of malignant transformed cells. Cells, which are characterized by invasive growth, metastasize to various tissues and organs and exhibit these properties in distant regions as well (Welch and Hurst 2019). Therefore, the inhibitory effects of CN on the colony formation and motility of HT-29 cells were investigated. The inhibitory effect of CN on the colony-forming ability of HT-29 cells was investigated by the clonogenic assay. This method tests the ability of tumor cells to survive and grow to form colonies after treatment with cytotoxic agents (Franken et al. 2006). Our result revealed that HT-29 cells treated with CN had an irreversible inhibition of cell growth inhibition compared to the control group. Many cancer patients die due to tumor mobilization, which often leads to metastatic foci in distant parts of the body. Tumor metastasis is an important pathological feature of a malignant tumor, which is the main cause of clinical treatment failure and high mortality (Seyfried and Huysentruyt 2013). Therefore, attempts to prevent or slow down the motility and metastasis of cancer cells are very important in cancer treatment. Therefore, an in vitro wound-healing assay was performed to investigate whether CN extract alone or in combination with 5-FU has an inhibitory effect on the migratory ability of HT-29 cells. After application with CN and 5-FU alone or in combination for 24 h, the repair of the wound formed in HT-29 cells was considerably delayed compared to the control group. In conclusion, it has been shown that CN has a synergistic inhibition on the migratory abilities of HT-29 cells, with the number of migrating cells being reduced by both a single agent and the combination. These results show that when compared with various anti-cancer drugs, especially 5-FU, CN extract can inhibit tumor mobilization with the advantage of being less toxic. Because, as shown in previous studies, 5-FU could strongly inhibit non-malignant cells (Li et al. 2015). Therefore, CN can be considered a potential natural anti-cancer compound with research value.

The cell cycle is a very important biological activity that regulates the proliferation of cells. Dysregulation of the cell

cycle is closely associated with malignant transformation therefore, drugs aimed at inhibiting the mitotic process have been a popular area of traditional anti-cancer research, and agents such as 5-FU have been used extensively in the clinic for many years (Williams and Stoeber 2012). To investigate the effects of CN and 5-FU on the cell cycle of HT-29 cells we performed flow cytometry analysis. 5-FU an antimetabolite exerts its cytotoxic effect by limiting the availability of thymidylate and inhibiting DNA synthesis during the S phase of the cell cycle (Longley et al. 2003). Similar to 5-FU, CN treatment also resulted in the S phase arrest (Fig. 4). We also found that combination therapy induces synergistic S phase arrest in HT-29 cell lines. The cell cycle is regulated by many cyclins and cyclin-dependent kinases (CDKs) that are a group of serine/threonine kinases (Ding et al. 2020). The progression of the S phase in the cycle is regulated by the Cyclin A2/CDK2 complex. Cyclin A2 is expressed in S phase and binds to Cdk2, resulting in DNA replication and S-phase progression. The activities of CDKs are regulated by cyclin-dependent kinase inhibitors (CDKI). p21<sup>(WAF1/CIP1)</sup> and p27<sup>(KIP1)</sup> keep cell cycle progression under control by inhibiting the kinase activities of Cyclin A/Cdk2 complex in S phase (Abbas and Dutta 2009; Bertoli et al. 2013). The tumor suppressor gene p53 also plays an important regulatory role in cell cycle progression (Bieging et al. 2014). The downstream targets of p53, p21, and p27, are universal inhibitors that can inhibit the activity of CDKs and induce cell cycle arrest. Up-regulation of p21<sup>(WAF1/CIP1)</sup>, p27<sup>(KIP1)</sup>, and downregulation of Cyclin A2/CDK2 complexes inhibit progress in the S phase, resulting in cell cycle arrest. Our results showed that p21, p27, and p53 mRNA expression was increased in CN-treated cells compared with controls. Furthermore, Cyclin A2/CDK2 mRNA expression was also decreased in cells treated with CN further confirming the flow cytometry results (Fig. 5). p21 represents the main target of p53 activity after DNA damage. Cell-cycle arrest by DNA damage is associated with p21 activity via p53 (Abbas and Dutta 2009). 5-FU targets the S phase of the cell cycle and exerts cytotoxic activity by causing DNA damage and inducing p53 activation (Longley et al. 2003). This is consistent with our results, and similar results were obtained in cells treated with CN. In addition, combination treatment resulted in up-regulation of p21 and downregulation of Cyclin A2 compared to cells treated with CN and 5-FU alone. Therefore, obtained results suggest that the cytotoxic activity of CN extract follows a similar path to that of 5-FU. CN treatment induces the alterations of p53-p27-p21 signaling to arrest the cell cycle in the S phase.

## Conclusions

Our findings from this study show that CN has selective anti-cancer activity on colorectal carcinoma cells. It was also found that CN and 5-FU have synergistic anti-cancer effects when applied to HT-29 cells together. The combination of CN with 5-FU has a synergistic anti-tumor effect on the cell cycle in addition to affecting the proliferation, clonogenicity, and motility of colorectal cancer cells. These results suggest that CN strengthens the anti-cancer activity of 5-FU, so it can be used in the treatment of 5-FU-resistant colon cancer. As a result of co-administration of CN and 5-FU, besides overcoming drug resistance, it can also help reduce the side effects of 5-FU thanks to the reduced dose. In conclusion, our findings indicated that CN potentiated the chemotherapeutic effect of 5-FU in HT-29 colorectal cancer cells via cell cycle arrest.

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## Declarations

**Conflict of interests** The authors declare that they have no conflict of interest in the publication.

**Ethics approval and consent to participate** Not applicable.

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