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ORIGINAL PAPER

Anticancer activity of lycopene in HT-29 colon cancer cell line

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Abstract

An inverse association between serum lycopene levels and the risk of cancers has been pointed out by many prospective and retrospective epidemiological studies which prompted more studies to be performed on animal models and cell cultures in order to test this hypothesis. The aim of the present study was to evaluate the antiproliferative and pro-apoptotic effect of lycopene on colon cancer HT-29 cell line. The effect of lycopene on the viability of HT-29 cell line was investigated using XTT assay. The levels of Bcl-2, cleaved caspase 3, BAX, cleaved PARP, and 8-oxo-dG in lycopene-treated HT-29 cells were measured using ELISA. Gamma-H2AX and cytochrome *c* expression was assessed semi-quantitatively using immunofluorescence staining. Lycopene at doses of 10 and 20 µM produced a significant antiproliferative effect on HT-29 cells compared to the control ($p < 0.05$). The IC_{50} value of lycopene in HT-29 cells was found to be 7.89 µM for 24 h. Lycopene (7.89 µM) significantly elevated cleaved caspase 3 ($p < 0.01$), BAX, and cleaved PARP, 8-oxo-dG levels ($p < 0.05$). The levels of γ-H2AX foci are significantly higher while the levels of cytochrome-*c* are lower ($p < 0.05$) in lycopene-treated HT-29 cells. These results indicate that lycopene has an antiproliferative apoptotic and genotoxic effect on HT-29 colon cancer cell line.

Keywords Lycopene · Colon cancer · Cell proliferation · Apoptosis

Introduction

Colon cancer is one of the main causes of death related to cancer in western countries. It is also the third most common cancer diagnosed in the United States [1]. It represents about 10% of total cancer cases and 9.4% of total cancer deaths in the world in 2020. Overall survival in patients with cancer has increased thanks to the wide variety of therapeutic options produced by recent advances in the knowledge base of colon cancer and research. Nevertheless, there is a continuous increase in the global colon cancer burden which is predicted to be 3.2 million cases in 2040 [2]. This increase mainly affects young people between 20 and 39 years old, possibly due to the widespread western pattern diet. On the other hand, old people, particularly between 50 and 75 years old are reported to have a decrease in the incidence of colon cancer [3].

Even though the causes are not exactly identified, it is agreed that the genetic factors, added to low vegetable, fruit, and fiber intake, besides diets with high levels of red and processed meats and fats, represent the main risk factors. Therefore, it seems necessary to determine dietary ingredients that can prevent or reduce cancer-causing factors [4].

Tomatoes have many advantageous effects, particularly in the prevention of cardiovascular diseases, osteoporosis, and cancer. Over 20% risk reduction of colon cancer has been attributed to the daily consumption of vegetables, including tomatoes. These beneficial effects of tomatoes have been linked to their high content of carotenoids, especially lycopene and b-carotene [5]. Carotenoids are plant-derived pigments. The main carotenoids in plasma are b-carotene, lycopene, lutein, b-cryptoxanthin, and a-carotene [6]. Lycopene is a highly unsaturated acyclic isomer of β-carotene; its hydrocarbon chain contains 11 conjugated and 2 unconjugated bonds [7] (Fig. 1).

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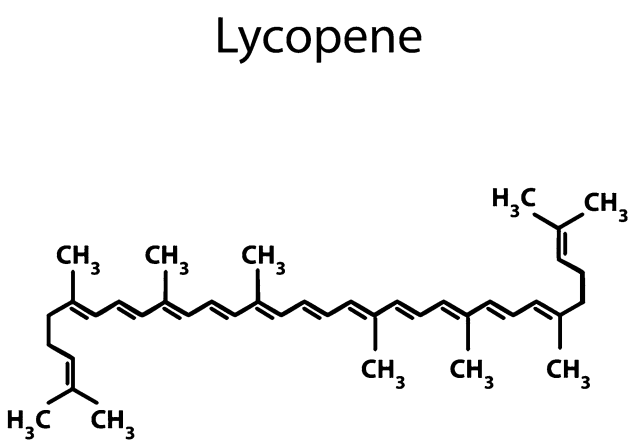

Fig. 1 Chemical structure of lycopene

An inverse association between serum lycopene levels and the risk of cancers has been pointed out by many prospective and retrospective epidemiological studies which prompted more studies to be performed on animal models and cell cultures in order to test this hypothesis [8]. Lycopene has a strong antioxidant activity that is implemented through different mechanisms; trapping singlet molecular oxygen, reacting with free radicals, repairing radicals obtained from vitamins C and E, and protecting cells against lipid peroxidation and oxidative DNA damage. Lycopene also inhibits carcinogenesis via the inhibition of growth factor-mediated antiapoptotic signals by directly blocking growth factor-receptor binding or by inactivating components of the PI3K-AKT signaling pathway. Lycopene can induce apoptosis by alteration of the cell-cycle distribution, down-regulation of cyclin D and Bcl-2, and up-regulation of Bax [9].

The aim of this research was to examine the antiproliferative effect of lycopene on the HT-29 cell line and to investigate the mechanisms underlying this activity.

Materials and methods

Cell culture and cell line

Colon adenocarcinoma cell line HT-29 (ATCC HTB-38) was purchased from American Type Culture Collection. The cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) with 10% fetal bovine serum (Sigma-Aldrich), 1% antibiotic mixtures of penicillin, and streptomycin (Sigma-Aldrich) at 37°C in a 5% CO_2 humidified incubator.

Cell viability test

Using XTT test assay (Roche Diagnostic, MA, USA), the effect of lycopene on the viability of HT-29 cell line was investigated. These cells were cultured at a concentration of 1×10^4 cells per well and incubated overnight before the addition of lycopene. After that lycopene (L9879, Sigma-Aldrich) dissolved in tetrahydrofuran (THF) (final concentration of 2.5, 5, 10, and 20 µM) was applied to cells for 24 h. Untreated cells were used as a control. After incubation, 50 µL of XTT mixture was added to each well. After 4 h of incubation, the cells were shaken and the absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Altrincham, United Kingdom). Cell viability was assessed as a percentage of live cells versus control cells after each experiment was performed three times [10, 11].

The measurement of Bcl-2, cleaved caspase 3, BAX, cleaved PARP, and 8-oxo-dG levels

The human ELISA kits of Bcl-2 (BT Lab, catalog #E1832HU), cleaved caspase 3 (BT Lab, catalog # E6970HU), Bax (BT Lab, catalog #E1825HU), cleaved PARP (BT Lab, catalog #E6971HU), and 8-hydroxy-desoxyguanosine (8-oxo-dG) (BT Lab, catalog #E1436HU) were used to measure the levels of Bcl-2, cleaved caspase 3, BAX, cleaved PARP, and 8-oxo-dG in lycopene-treated and untreated control HT-29 cells. HT-29 cells were cultivated into a 6-well plate and treated with 7.89 µM lycopene for 24 h. HT-29 cells that had been treated with lycopene and those that had not were gathered and diluted in Phosphate-buffered saline (PBS). Then they were frozen and thawed three times. Following that, the quantities of Bcl-2, cleaved caspase 3, BAX, cleaved PARP, and 8-oxo-dG in cell lysates were assessed following the manufacturer's instructions. Bradford protein assay kit (Merck Millipore, Darmstadt, Germany) was used to calculate the total protein quantities in both experimental and control HT-29 cells [12].

Immunofluorescence staining

Cells were fixed using methanol for 5 min at −20°C and washed with PBS. They were then incubated with PBS containing 0.1% Triton X-100 for 15 min at room temperature. After washing, they were incubated with PBS containing 2% BSA for 60 min at room temperature. After rewashing, they were incubated overnight with monoclonal anti-gamma H2AX (Abcam, Catalog no. ab26350) and monoclonal anti-Cytochrome *c* (Abcam, Catalog no. ab110325) primary antibodies at a dilution ratio of 1/300 at +4°C. Cells were

washed with PBS and then incubated with goat anti-mouse FITC secondary antibody at a dilution ratio of 1/50 for 45 min at room temperature in the dark. Finally, 4′,6-diamidino-2-phenylindole (DAPI) was applied to the washed cells and examined under the fluorescence microscope. During the evaluation, positivity in the cells in the whole field was evaluated semi-quantitatively as follows; severe (+++), moderate (++), mild (+), absent (−).

Statistical analysis

The results were stated as a mean ± standard error of the mean (SEM). Statistical evaluation of the data was done with SPSS Version 23.0 for Windows using One Way ANOVA and a postdoc Tukey test. The results obtained from Bcl-2, cleaved caspase 3, BAX, cleaved PARP, and 8-oxo-dG levels tests were examined using Independent Samples *t* test. For anti-gamma H2AX and anti-Cytochrome *c* staining statistical evaluation of the data was performed by One-Way ANOVA. Differences were evaluated statistically significant at $*p < 0.05$, and $**p < 0.01$. GraphPad Prism 8.0 software (USA) was used for data analysis and graphical presentations.

Results

Cytotoxic effect of lycopene on HT-29 cells

The cytotoxic effect of lycopene was assessed in HT-29 cells. Lycopene at doses of 10 and 20 µM caused a significant decrease in the viability of HT-29 cells compared to the control ($p < 0.05$). The IC_{50} value of lycopene in HT-29 cells was found to be 7.89 µM for 24 h (Fig. 2).

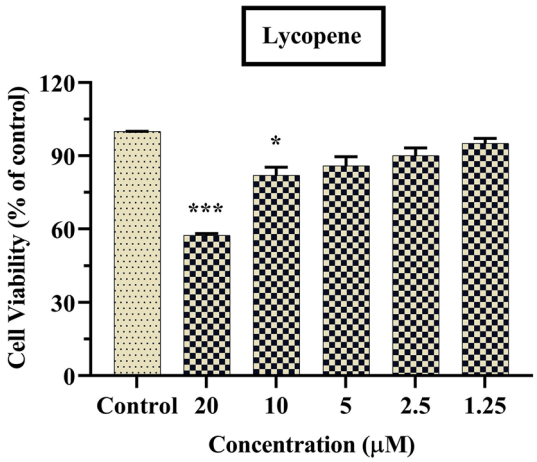

Fig. 2 The antiproliferative effects of lycopene on HT-29 cells. The findings are calculated as a percentage of viable cells versus control. The results are presented as the mean ± SEM of three samples. $*p < 0.05$ as compared to the control group

The effect of lycopene on Bcl-2, cleaved caspase 3, BAX, and cleaved PARP levels in HT-29 cells

ELISA was used to evaluate the expression of apoptosis-related proteins in HT-29 cells, such as Bcl-2, cleaved caspase 3, BAX, and cleaved PARP. The treatment with lycopene (7.89 µM) for 24 h significantly elevated cleaved caspase 3 level from 377.91 to 601.73 pg/mg protein ($p < 0.01$), BAX level from 5.32 to 12.09 ng/mg protein ($p < 0.05$), and cleaved PARP level from 390.5 to 503.64 pg/mg protein ($p < 0.05$). On the other hand, lycopene had no effect on Bcl-2 level ($p > 0.05$) (Fig. 3).

Effect of lycopene on 8-oxo-dG level in HT-29 cells

ELISA was used to assess 8-oxo-dG expression in HT-29 cells in order to determine the DNA-damaging effects of lycopene. Treatment with lycopene (7.89 µM) for 24 h significantly elevated the level of 8-oxo-dG from 31.61 to 49.81 ng/mg protein ($p < 0.05$) (Fig. 4).

Effect of lycopene on γ-H2AX levels in HT-29 cells

We stained 6 samples from both the experimental and control groups. The differences in gamma-H2AX foci and cytochrome *c* quantities were statistically significant ($p < 0.05$) (Table 1). It is obvious that the levels of γ-H2AX foci are higher while the levels of cytochrome *c* are lower in the microscope field of view 20× in the experimental samples compared with the control samples (Fig. 5).

Discussion

Colorectal cancer is the third most diagnosed cancer worldwide. About 60% of all colorectal cancer cases occur in developed countries due to their eating habits [5]. The most effective treatment for patients with advanced colon cancer the surgical resection followed by adjuvant treatment such as radiotherapy or chemotherapy to kill any cancer cells left. Moreover, Adjuvant chemotherapy may also be used for patients with cancer at early stages to promote the effects of primary chemotherapy. The drugs most commonly used to treat colon cancer are oxaliplatin combined with 5-fluorouracil or capecitabine. Drug resistance and serious side effects of these conventional chemotherapies such as cardiotoxicity, neurotoxicity, hair loss, bone marrow suppression, and gastrointestinal upsets make it important to look for new effective drugs with lower side effects for colon cancer treatment [2]. Natural products represent a rich source of bioactive compounds with therapeutic applications. In recent

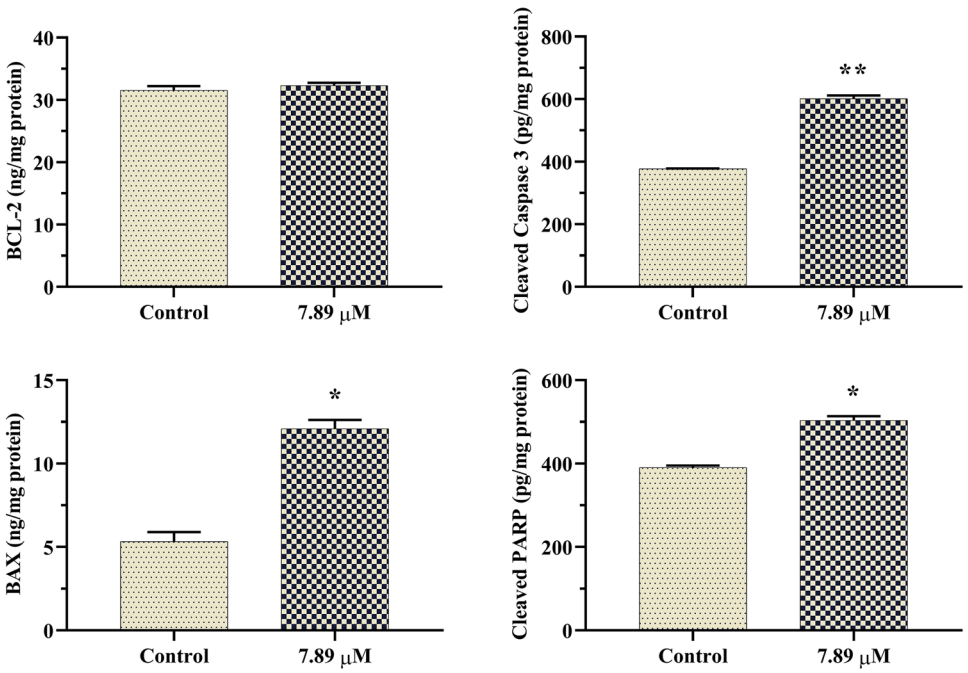

Fig. 3 Lycopene (4.382 μM) enhanced apoptosis of HT-29 cells. The cleaved caspase 3, Bcl-2, cleaved PARP, and Bax levels were calculated using the ELISA kits. Results are represented as mean ± SEM of three samples. *$p < 0.05$ and **$p < 0.01$ as compared to the control group

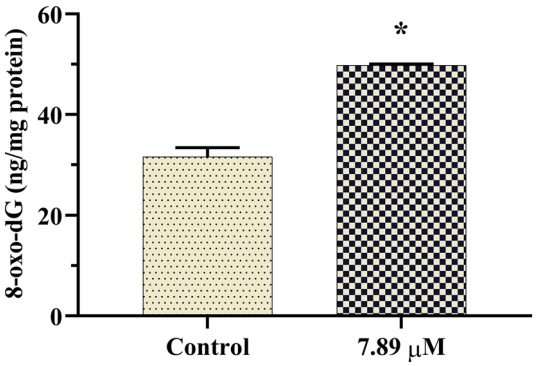

Fig. 4 Lycopene (4.382 μM) enhanced DNA damage of HT-29 cells. The 8-oxo-dG level was calculated using the ELISA kit. Results are represented as mean ± SEM of three samples. *$p < 0.05$ as compared to the control group

Table 1 The effect of lycopene on γ-H2AX levels in HT-29 cells

Samples	Gamma-H2AX	Cytochrome *c*
Control sample-1	+	+++
Control sample-2	+	++
Control sample-3	++	+++
Control sample-4	+	+++
Control sample-5	+	++
Control sample-6	+	+++
Experimental sample-1	+++	+
Experimental sample-2	+++	+
Experimental sample-3	+++	+
Experimental sample-4	++	+
Experimental sample-5	+++	–
Experimental sample-6	+++	+
Mean values ± standard deviation		
Control group	1.16 ± 0.40	2.66 ± 0.51
Experimental group	2.83 ± 0.40	0.83 ± 0.40

years, massive efforts have been made to isolate new natural products from plants, microbes, and other living beings, to evaluate their anticancer activity, and to investigate the mechanism of action. These efforts resulted in the discovery of many anti-cancer drugs. Between 1981 and 2019, about 25% of all newly approved anti-cancer drugs are derived from natural products [13]. Lycopene is a nonprovitamin A carotenoid, found abundantly in tomatoes and tomato products. Interest in lycopene has begun based upon observational epidemiologic studies demonstrating that people who consume more lycopene, or who have higher lycopene levels in plasma or in adipose tissue, are at lower risk of some chronic diseases, such as coronary heart disease and cancer [14, 15]. Lycopene exhibited promising anticancer activity in numerous studies performed on many types of cancer cell lines. The antiproliferative effect of lycopene was demonstrated in-vitro as monotherapy on HT-29 human colorectal adenocarcinoma cell line [16, 17] and also as an adjuvant therapy when used in combination with 5-fluorouracil as it enhanced both the cytotoxic and proapoptotic effect of 5-fluorouracil on Caco2 colon cancer cells [2]. Tomato extracts exhibited the same cytotoxic effect on HT-29 cell line [18]. Tang FY et al also indicated the potential of lycopene to suppress the growth and progression of colon tumors in vivo in a mouse xenograft model [19]. The

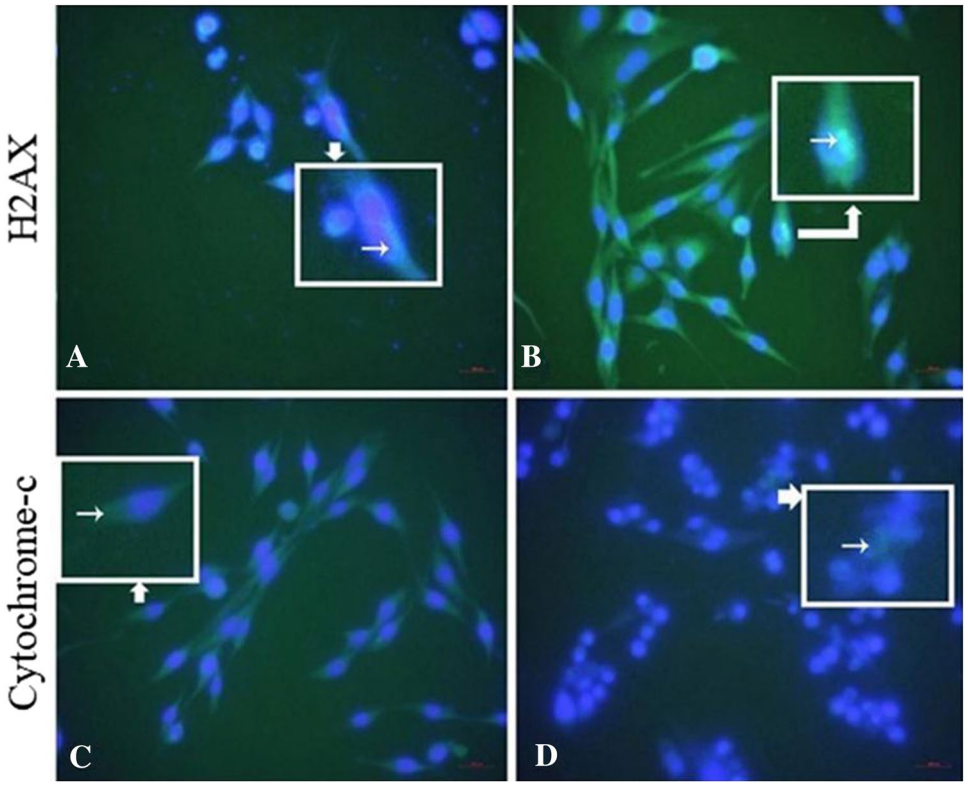

Fig. 5 Immunofluorescence staining of the effect of lycopene on γ-H2AX and Cytochrome *c* levels in HT29 cells. Immunopositive cells are pointed with arrows in × 20 magnification

antiproliferative effect of lycopene was also determined on prostate cancer cell line; Soares Nda C et al (2013) confirmed this cytotoxicity on both bone metastasis-derived (PC-3) and brain metastasis-derived (DU-145) human prostate cancer cell line, the treatment with lycopene significantly decreased cell viability, promoted apoptosis, and induced cell cycle arrest in G0/G1 phase [20]. Other studies reached the same results regarding the antiproliferative effect of lycopene on the prostate cancer cell line [21–23]. Several studies performed on breast cancer cell lines showed that lycopene treatment caused a significant decrease in the number of viable cancer cells, cell-cycle arrest and an increase in apoptosis [17, 24–26]. The antiproliferative and proapoptotic effects of lycopene were also investigated and demonstrated in other cancer types; in oral cancer cells (CAL-27 and SCC-9) [27, 28], gastric cancer cell line (AGS) [29, 30], human erythroleukemia (K562), B chronic lymphocytic leukemia (EHEB) and Raji, a prototype of Burkitt lymphoma cell line [31], ovarian cancer cell line (SKOV3) [32], pancreatic cancer (PANC-1) cells [33], and hepatic tissue from N-nitrosodiethylamine (NDEA) treated mice [34]. In a study conducted by Lingling Cui et al (2020), lycopene exhibited a protective effect against N-nitrosomethylbenzylamine (NMBzA) induced esophageal carcinogenesis in F344 rats via the potential mechanisms of anti-inflammatory and pro-apoptosis [35].

The aim of the present study was to evaluate the antiproliferative and pro-apoptotic effect of lycopene on colon cancer HT-29 cell line. Our findings showed that lycopene had a concentration-dependent cytotoxic effect on HT-29 cells. It significantly inhibited HT-29 cell proliferation in a concentration-dependent manner, with an IC_{50} value of 7.89 µM after 24 h.

Dysregulation in the apoptotic cell death mechanism is a feature of cancer. Alterations of apoptosis are responsible for both tumor development and tumor resistance to anticancer treatments. Most current anticancer therapies recruit the apoptosis signaling pathways to induce cancer cell death [36]. The levels of Bcl-2, cleaved caspase 3, BAX, and cleaved PARP were measured using ELISA technique to investigate the pro-apoptotic activity of lycopene on HT-29 cells. The intrinsic pathway is initiated by death stimuli such as DNA damage and ER stress leading to the activation of BH3-only proteins. Activator BH3s directly activate BAX and BAK to induce the homo-oligomerization of BAX and BAK, resulting in mitochondrial outer member permeabilization and release of mitochondrial intermembrane proteins like cytochrome *c* to cytosol, where it binds to Apaf-1 to form a procaspase-9-activating heptameric protein complex named apoptosome, which subsequently activates executioner caspase-3 and -7. Executioner caspases cleave target proteins resulting in cell apoptotic breakdown [37]. Caspases, in particular, caspase-3 and -7 are responsible for the proteolytic cleavage of poly (ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme involved in DNA repair, DNA stability, and transcriptional regulation. The cleavage

of PARP-1 thus prevents the recruitment of the enzyme to DNA damage sites [38]. In the present study, 7.89 µM lycopene treatment significantly elevated pro-apoptotic cleaved caspase 3 ($p < 0.01$), Bax, and cleaved PARP protein expressions ($p < 0.05$), while not altering anti-apoptotic Bcl-2 expression ($p > 0.05$). These results confirm the apoptotic activity of lycopene on HT-29 cells. Besides, the immunofluorescence staining of cytochrome *c* showed that the number of cells with intact mitochondria is significantly lower in the experimental group compared to the control group as they released more cytochrome *c* during apoptosis induced by lycopene. 8-oxo-dG is one of the major products of nuclear and mitochondrial DNA oxidation. Thus, the concentrations of 8-oxo-dG within a cell are a measurement of oxidative DNA damage, and as a biomarker to estimate the risk of different diseases including cancer [39]. The 8-oxo-dG ELISA kit was used in our study to investigate DNA fragmentation in HT-29 cells after 24 h of lycopene treatment. The results indicate the cytotoxic activity of lycopene is associated with oxidative DNA damage. Double-strand breaks (DSBs) are the most harmful DNA lesions. Cells react to DNA damage by activating the DNA damage response (DDR), a complex molecular mechanism developed to detect and repair DNA damage. The formation of DSBs leads to the activation of many factors, including phosphorylation of the histone H2AX, producing γ-H2AX. H2AX phosphorylation plays a critical role in DDR as it is required for the assembly of DNA repair proteins at the sites of damaged chromatin and also for activation of checkpoint proteins responsible for cell cycle arrest [40]. The results of our immunofluorescence staining of γ-H2AX, there was a significant increase in γ-H2AX expression which confirms the genotoxic effect of lycopene on HT-29 cells.

Conclusion

Lycopene significantly suppressed HT-29 cell growth in a concentration-dependent way. Lycopene treatment significantly elevated pro-apoptotic cleaved caspase 3, BAX, and cleaved PARP levels as well as mitochondrial cytochrome *c* release. Lycopene treatment also significantly elevated 8-oxo-dG quantities in HT-29 cells and thus the cytotoxic effect of lycopene may be linked to oxidative DNA damage. Therefore, the present study suggests that lycopene has the potential to be a promising therapeutic agent for colon cancer. However, these findings need to be supported by further in vivo and clinical studies.

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Data availability All results obtained in this experiment were presented in Figs. 2, 3, 4, and 5 and Table 1.

Declarations

Conflict of interest Authors declare no conflict of interest.

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