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(RESEARCH ARTICLE)

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In vitro anticancer and antiproliferative activity of crocin on HCT-116 cells

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Abstract

One of the world's main causes of cancer-related fatalities worldwide is colorectal cancer. The adherent epithelial cells known as HCT116 (human colorectal carcinoma) are frequently employed to examine inflammatory responses in colonic epithelial cells. They are derived from the human colorectal cancer cell line. Crocin, a potent antioxidant, anticarcinogenic was tested in this study to see how it affected the HCT-116 human colorectal cancer cell line. MTT analysis was used to assess cell viability. Crocin's antiproliferative activity to inhibit the proliferation of HCT 116 cancer cells was examined. Crocin displayed the outstanding activities for MTT test, IC50= 10.57 µL/mL for 24 hours was calculated as IC50= 3.29 µL/mL for 48 hours on HCT 116 cell line. Total antioxidant status (TAS) and total oxidant status (TOS) in the prepared cell lysate were determined by using commercial kits. Crocin is thought to be a high potential agent to be used in treatment.

Keywords: Colorectal cancer; Crocin; HCT 116; MTT

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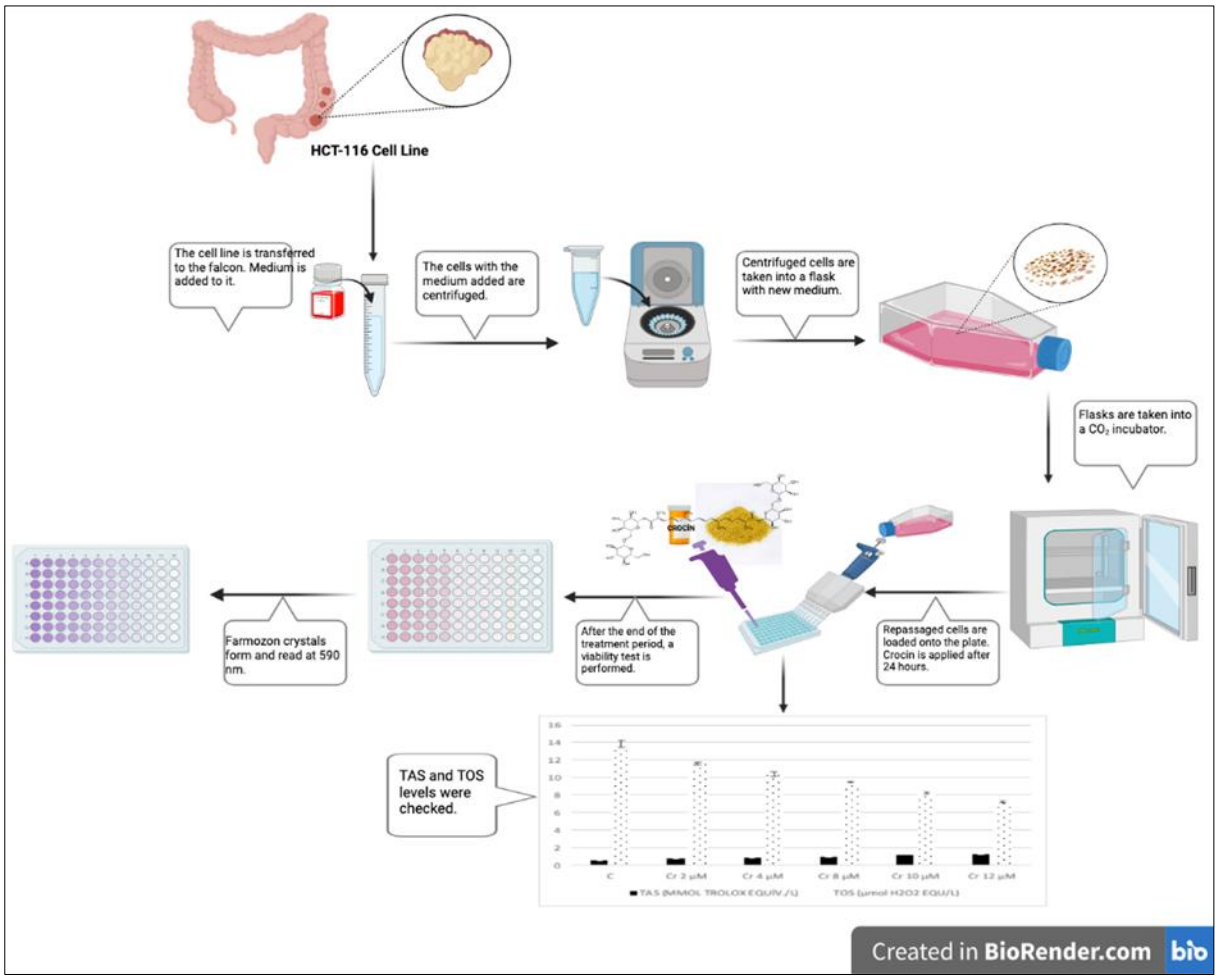
Graphical Abstract



1. Introduction

A tumor develops when cells in any part of the body begin to grow uncontrollably and spread throughout the body via the lymphatic system and blood circulation. This sickness is known as cancer (metastasis). Combinations of many external carcinogenic causes and hereditary variables can result in the development of cancer (UV light, viruses, pollutants, X-rays, chemical agents, stress, unknown factors) [1-5]. Colorectal cancer is a very common type of cancer affecting the colon and rectum, which usually develops with the spread of polyps in the colon. The serious side effects of drugs used in colorectal cancer cases direct people to develop new treatments. Recently the protection of the tumor cell from the immune system constitute the basic physiopathology and inability to perform apoptosis, the inactivation of tumor suppressor genes, the activation of oncogenes, the deterioration of DNA repair ability and thereprogramming of the energy metabolism of the studied cell [6].

The purest of *Crocus sativus* L. (saffron) can be purified to obtain crocin, a water-soluble carotenoid, and it can then be directly crystallized. All of these analogues, including crosin 1-4, are made up of transcrocetin glycosides, a derivative of the carotenoid crocin, which has chemical counterparts: crocin, alphacrocin, and crocetin digentiobiose ester. Numerous cellular proteins, including membrane transporters, enzymes involved in signal transduction and redox homeostasis, structural proteins, and enzymes responsible for ATP generation, are physically bound by crocin [7-9]. Crocin (Cr) is known to boost glutathione synthesis, superoxide dismutase, endogenous defense against oxidative stress, and overall antioxidant capability. According to reports, Cr promotes metal chelation, neutralizes free radicals, boosts antioxidant levels, modifies hemodynamic parameters, protects against ischemia-reperfusion injury, and protects against nephrotoxicity. In addition to having antidiabetic, antiatherosclerotic, antiarthritic, anti-tumoral, and anticarcinogenic properties, crocin also has antidepressant and antihyperlipidemic properties. It is well recognized to have antiinflammatory, genoprotective, memory impairing, neuroprotective, and metabolic syndrome management effects [10-14].

This study aimed to evaluate the effects of Crocin on the growth of HCT-116 human colorectal cancer cell line.

2. Material and methods

2.1. Chemicals

In this study, chemicals of analytical purity were used. Mccoy's Medium, trypsin, penicillin, streptomycin, fetal bovine serum (FBS) from American Type Culture Collection (ATCC, Manassas, VA, USA), phosphate buffered saline (PBS) from Sigma (St. Louis, USA) were used in the cell culture step. The product named Crocin as yellow powder used in the study was supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The human HCT116 colorectal cancer cell line was purchased from the American Type of Culture Collection (ATCC, Bethesda, MD, USA) CCL-247 LOT: 70009735 (Table 1). Cells were grown in McCoy's 5A medium containing 2mM L-glutamine, 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/ml streptomycin and incubated at 37 °C in a CO_2 oven containing 5% CO_2.

2.2.1. Cell passage

The cryo vial containing cells from ATCC was thawed by shaking in a 37 °C water bath, transferred to a 15 mL falcon and centrifuged at 1200 rpm for 4 minutes by adding 5 mL of medium. Cell precipitate was obtained at the bottom of the falcon. HCT 116 colorectal cancer cells at the bottom of the falcon were transferred to T-25 cm cell culture flasks, examined under an invert microscope, and incubated at 37°C in a CO_2 oven containing 5% CO_2. When the bottom of the flask was 80%, the medium in the flask was removed. PBS was used to purify cells from serum. After removing the PBS, 2 mL of 0.25% trypsin EDTA was added and left in the incubator for 5 minutes. At the end of this period, it was checked with an invert microscope whether the cells adhered to the flask were separated from the surface or not, and the cells were completely separated from the base by lightly tapping the flask. Adding 5 mL of medium, it was taken into a 15 mL falcon tube and centrifuged at 2500 rpm for 5 minutes, and the supernatant was poured away. 1 mL of medium was added and lightly pipetted. By adding 4 mL of medium to the new flask, the cell medium in the falcon was transferred to the flask. Cells were followed under an inverted microscope and incubated for 24 hours in a CO_2 oven.

Table 1 Details of ATCC HCT 116 cell line

ATCC HCT 116 Cell Line Description	
Organism	**Homo sapiens**
Tissue	Large intestine; Colon
Age	Adult
Gender	Male
Morphology	Epithelial
Growth properties	Adherent
Disease	Carcinoma; Colorectal

2.3. Determination of cell count with Trypan Blue

While dead cells are stained with trypan blue staining method, living cells are not stained with dye because the cell membranes of living cells are intact, so under invert microscope living cell appears transparent. After the cells covered the flask base by 80%, the medium of the cells was discarded by pulling with the help of a pipette. Afterwards, the flasks were washed with PBS without Ca2+ and Mg2+, and the remaining proteins in the flask were removed. The cells remaining in the flasks were incubated with 0.25% trypsin EDTA for 5 minutes in an incubator, 4 mL of medium was added, and the supernatant was removed by centrifugation at 1200 rpm at +4 °C for 5 minutes. 1 mL of medium was added and pipetted.

50 µL of cell suspension from eppendorf was taken into a separate 1.5 ml ependorph, the same volume (50 µL should be in equal volume) of trypan blue (0.4%) was added and pipetted so that it became homogeneous. 10 µL of the cell/trypan blue mixture was pipetted and dropped into the middle of the Thoma slide. The Thoma slide was covered

with a coverslip so that no air bubbles remained. The count lines of the Thoma slide placed on the microscope were counted with a 40x objective microscope. Only viable cells that were not stained blue in the 16 squares in the 1 mm^2 area where the lines intersect in the center were counted (Dead cells are all blue). This process was performed 5 times and the average was taken. The number of viable cells in 1 mL was calculated according to the formula below.

- Number of viable cells (cells/mL) = Average number of cells x 2 x 10^5
- Average cell number: It is obtained by dividing the total number of cells x 2 x 10^5
- Average cell number: It is obtained by dividing the total number of cells by 5 after counting 16 squares in 1 mm^2 area 5 times.

10^5: Multiply by 10^5 to find the number of cells per mL, since each 1 mm^2 area equals 0.1 mm^3 in volume, which equals 10^5 mL.

2: Since an equal amount of trypan blue is added to the sample volume taken, it will be diluted 2 times, so it is multiplied by 2 (if the sample taken for counting is diluted, the result found is multiplied by the final dilution factor).

2.4. Cell viability by MTT

The proliferative or cytotoxic effects of any therapeutic agent on the cell are determined with MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide), which is a method in which the amount of cell proliferation is determined based on the colorimetric measurement of enzymatic activity or formazone dyes depending on the decrease in MTT. The chemical basis of the experiment is based on the reduction of MTT The distinctive yellow color of MTT produces a blue formazan color as a result of chemical reduction.

The cytotoxic effect of crocin on the HCT116 cell line was performed according to the instructions for use specified in the commercially available MTT kit (Abcam ab211091). The method is based on the principle of colorimetric determination of color change in cells incubated with MTT agent. The resulting discoloration occurs as a result of the reduction of tetrazolium salt in the active cell mitochondria of formazone salts colored with yellow. The absorbance value of these compounds is proportional to the determination of their metabolic activities. Method;

One day before the application of the MTT method, 100 µL of medium was prepared with 1 x10^4 cells counted in a 96-well plate and inoculated into the wells. The cells were allowed to adhere to the surface by keeping the microplate in an incubator set at 37°C and 5% CO_2 for 24 hours. The reason for this is that the damage caused by the trypsin enzyme on cell membrane proteins and growth factor receptors can be eliminated, a 24-hour period is required for these proteins and growth factors to be resynthesized, and in this way, the cells gain metabolic activity before the therapeutic agents (Cr) in the medium are treated with the cell.

Stock solutions of the Cr was prepared in medium at different concentrations (2, 4, 8, 10, and 12 µL/mL) for MTT assay. After 24 hours of incubation, Cr was added at 2-12 µL/mL for varying time intervals (3, 24, and 48 h) MTT analysis was performed at the end of the determined periods by applying the determined concentrations.

2.4.1. MTT analysis

The medium in the 96-well plate is carefully emptied and 50 µL of serum free medium and 50 µL of MTT reagent are added to each well. It was incubated in an incubator at 37 °C for varying time intervals (3, 24, and 48 h). After three hours of incubation, 150 µL of MTT solvent was added to each well. It was mixed with foil and mixed in an orbital shaker for 15 minutes. At the end of the incubation, the absorbance value of 590 nm was measured with a microplate reader spectrophotometer (µ-Quant, Epoch BioTek Instruments, Winooski, Vermont, USA).

$$\% \text{ Cytotoxicity: } 100x(Abs_{Control}-Abs_{Sample}))/Control$$

The applied dose and % cell viability curve were determined with the help of Graphpad Prism 9.0 program, and the 50% inhibitory concentration (IC_{50}) value was calculated with a logarithmic slope graph.

2.5. Determining of TAS and TOS level

HCT-116 cells were cultured at 1 x 10^5 cells/mL in a 96-well plate and incubated at the determined Cr doses. After incubation, cells were harvested by washing them with cold PBS (10 mM, pH-7.5 in PBS). 1% TritonX-100 buffer was added to the cells and homogenized with Qiagen Tissue Lyser. The supernatant was obtained by centrifugation at 5000xg for 10 minutes at 4 °C and kept on ice for 10 minutes. In the obtained cell lysate, Total antioxidant status (TAS)

and total oxidant status (TOS) in the prepared cell lysate were determined using commercial kits (Rel Assay) were measured with Biotek ELISA Reader (Bio Tek µQuant MQX200 Elisa reader/USA) by applying the protocol specified in the commercially purchased ELISA kit.

2.6. Statistical analysis

Data were analyzed using SPSS software package, version 22.00. Statistical analysis was done by one-way analysis of variance (ANOVA). Post-hoc Tukey's test was used to compare the biochemical parameters between the groups. P values <0.05 were considered as significant. The results are expressed as mean±standard error (SEM) for each group.

3. Results and discussion

In order to crocin sensitivity HCT116 cells were exposed to 2-12 µL/mL for 3, 24, and 48 h using MTT analysis (Fig. 1). While the viability of HCT 116 cells were 100% at 3, 24 and 48 hours, it was 89.21%, 68.76%, 56.01%, 50.52% and 49.49% at 24 hours in the groups administered 2, 4, 8, 10 and 12 µL/mL crocin, while it was found to be 56.68%, 50.16%, 37.78%, 24.66% and 22.13% at 48 hours. After 24 h and 48 h treatment, cell viability decreased significantly in response to crocin.

The statistics of the data of MTT viability test obtained as a result of the study were made and presented in Figure 1.

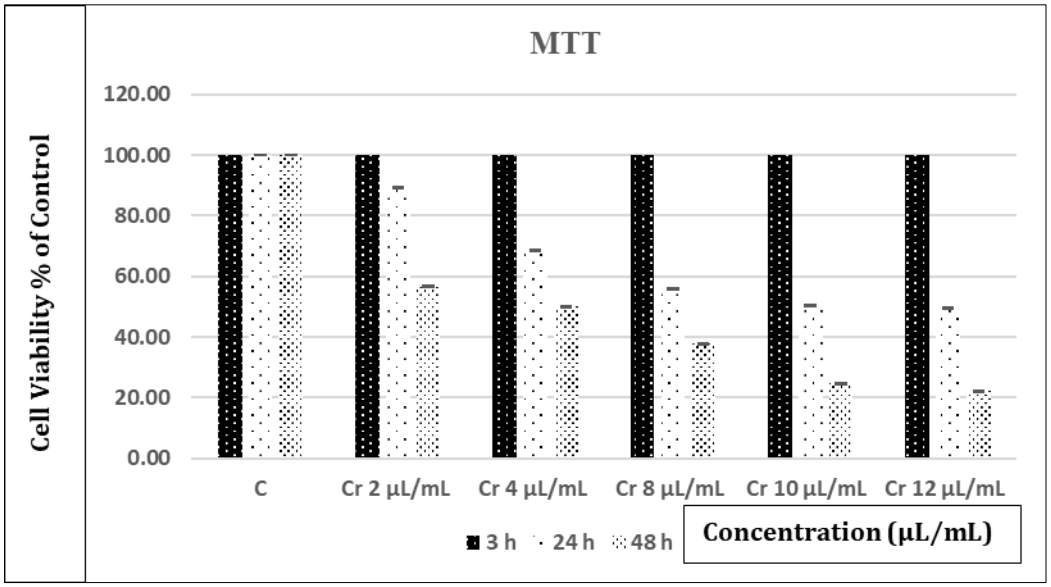


Figure 1 Percentage of cell viability on different concentration of Crocin against HCT 116 cells using MTT Assay. C: Control (HCT 116 cell), Cr: Groups treated with crocin to HCT 116 cancer cells. P <0.001 were considered as significant.

Table 2 The level of TAS and TOS l in HCT 116 cells after Crocin treatment

Group	TAS (mmol Trolox Equ./L)	TOS (µmol H2O2 Equ./L)
C	0.62±0.02^{f}	13.86±0.39^{a}
2 µL/mL	0.84±0.01^{e}	11.61±0.16^{b}
4 µL/mL	0.90±0.01^{d}	10.39±0.26^{c}
8 µL/mL	1.01±0.01^{c}	9.48±0.08^{d}
10 µL/mL	1.20±0.00^{b}	8.24±0.09^{e}
12 µL/mL	1.32±0.01^{a}	7.24±0.14^{f}
p	***	***

C: Control (HCT 116 cell), Cr: Groups treated with crocin to HCT 116 cancer cells. P <0.001 were considered as significant.

The TAS level of the cells treated with Crocin was significantly higher than the control group, while the TOS level was decreased compared to the control group (P<0.001) (Table 2, Fig. 2).

For MTT test, IC_{50}= 10.57 µL/mL at the 24th hour was calculated as IC_{50}= 3.29 µL/mL at the 48th hour.

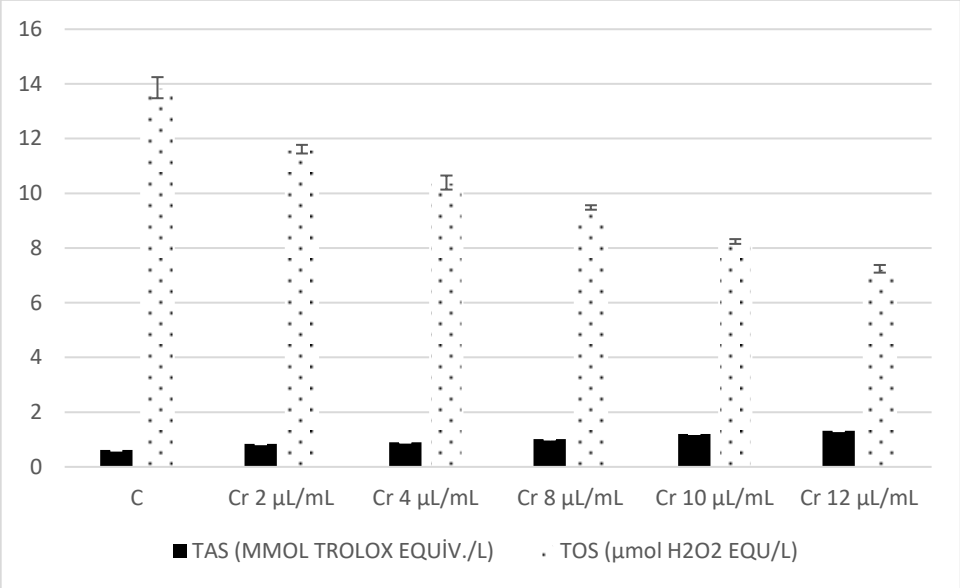


Figure 2 Effect of Crocin on HCT 116 cell TAS and TOS levels. C: Control (HCT 116 cell), Cr: Groups treated with crocin to HCT 116 cancer cells. P <0.001 were considered as significant

Crocin is a carotenoid compound obtained by drying the stigmas of the flower part of the saffron (*Crocus sativus* L.) plant. This substance is made by drying the stigmas found in the saffron plant's blossoms. According to studies, crocin has anti-inflammatory, antiischemic, neuroprotective, anti-diabetic, anti-obesity, anti-platelet, anti-hyperlipidemic, anti-apoptotic, and anticarcinogenic properties [15-21]. Crocin's antiproliferative activity in the HCT 116 cell line and its effects on TAS and TOS levels were investigated in the current investigation.

Increased redox imbalance in cancer cells causes ROS production from both endogenous and external sources to be activated, which alters biochemical processes by causing oxidative stress, inflammation, apoptosis, etc. The prognosis of many cancer types is influenced by ROS production, oxidative damage, or apoptosis, which is traditionally the inhibition of cellular proliferation or differentiation as a result of signaling pathways responding to changes in intracellular ROS levels, or by the absence of ROS production or antioxidant defense system [22, 23]. The TAS level of the cells treated with Crocin was significantly higher than the control group, while the TOS level was decreased compared to the control group.

Abbreviations

- HCT 116 (human colorectal carcinoma),
- MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide),
- IC 50 (The half-maximal inhibitory concentration).

4. Conclusion

As a result, crocin showed antiproliferative effect by decreasing TOS level while its application increased TAS level in cancer cells. It acts as an agent with a strong potential to be used in the treatment *in vitro*, and it should be supported by *in vivo* studies to determine the pathways affected by molecular studies and to determine their effects. For the clinical use of crocin in the prevention and treatment of colorectal cancer, its effect on other colorectal cancer cell lines should be determined.

Compliance with ethical standards

Acknowledgments

Apaydin Yildirim B, Dogan T and Ozturk S study design, literature review, statistical analysis of data and biochemical analysis. All authors approved the publication by checking the final version of the article.

Disclosure of conflict of interest

All author declares that there is no conflict of interest in the present study.

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