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ORIGINAL ARTICLE

Anticancer activity of safranal against colon carcinoma is due to induction of apoptosis and G2/M cell cycle arrest mediated by suppression of mTOR/PI3K/Akt pathway

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Summary

Purpose: *Colon cancer is among the deadliest malignancies and is responsible for a significant number of deaths across the globe. The treatment options for colon cancer are limited and with lot of side effects. In this study we evaluated the potential anticancer effects of safranal against colo-205 colon cancer cells along with evaluation of its effects on apoptosis, cell cycle phase distribution, reactive oxygene species (ROS) and PI3K/AKT/m-TOR signalling pathway.*

Methods: *Cell viability was examined by MTT assay. Apoptosis was checked by DAPI staining, comet assay and annexin V/propidium iodide (PI) staining involving the use of fluorescence microscopy and flow cytometry. ROS, mitochondrial membrane potential (MMP) and cell cycle phase distribution were checked by flow cytometry using different fluorescent probes. Effects of safranal on the protein expression of PI3K/AKT/m-TOR were studied by western blot assay.*

Results: *The results revealed that safranal suppressed the proliferation of colo-205 cancer cells with an IC_{50} of 20 μM. Furthermore, the anticancer effects of safranal were found to be due to accretion of ROS and decrease in the MMP, ultimately leading to apoptosis of colo-205 cancer cells. In addition, safranal caused increase in the expression of Bax in parallel with concomitant reduction in the expression of Bcl-2. Safranal also triggered G2/M cell cycle arrest and inhibition of PI3K/AKT/mTOR signalling pathway.*

Conclusion: *In conclusion, the above results indicate that safranal could prove a potential lead molecule in the treatment of colon cancer, provided further in vivo studies are carried out.*

Key words: *apoptosis, cell cycle, colon cancer, ROS, safranal*

Introduction

Plants synthesize a diversity of molecules that display impressive pharmacological activities [1]. Among the plant-derived secondary metabolites, carotenoids form a very large group. These compounds have a number of functions in plants [2]. In some plants carotenoids are broken down by incorporating molecular oxygen, leading to the formation of a group of metabolites called apocarotenoids. Among these apocarotenoids safranal is an important molecule that is mainly found in *Crocus sativus* [3,4]. Safranal exerts several neuroprotective and anti-inflammatory bioactivities. Moreover, there are several apocarotenoids, such as crocin and crocetin, that have been reported to exhibit anticancer activity. Constantly, we presumed that safranal may also exhibit anticancer activities. Over the years there has been drastic alterations in human lifestyles, accompanied with increased incidence of cancer around the globe. Studies have shown that colon cancer is among the

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top deadly malignancies in humans [5]. Currently, the treatment of colon cancer involves surgery followed by chemotherapy. Nevertheless the prognosis of this disease is rather poor and the mortality rate is high [6]. Hence, there is an imperative need to develop novel treatment strategies or explore novel targets for the treatment of this disease.

The main purpose of the present study was to evaluate the potential anticancer effects of safranal against colo-205 colon cancer cells along with evaluation of its effects on apoptosis, cell cycle phase distribution, ROS and PI3K/AKT/m-TOR signalling pathway.

Methods

Chemicals, reagents and culture conditions

All chemicals and reagents were procured from Sigma-Aldrich Co. (St. Louis, MO, USA) or otherwise mentioned. Antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Human colon cancer colo-205 line was purchased from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), penicillin and streptomycin (100 U/mL and 100 mg/mL, respectively) and maintained in air containing 5% CO_2 at 37°C.

MTT assay

For assessment of the cell viability, the colo-205 cells were cultured in a 96-well plate at a density of 5×10^3 cells/well. The cells were incubated overnight and then the RPMI-1640 medium was removed and replaced with fresh RPMI-1640 medium with safranal at different concentrations (0-200 µM) for 24 hrs. Thereafter, MTT solution of 0.5 mg/ml was added for the last 4 hrs of incubation and finally the sample absorbance was assessed at 570 nm using Eliza Plate Reader.

Apoptosis assay

For detection of apoptosis colon cancer HCT-116 cells were cultured (2×10^5 cells/well) in 6-well plates. The cells were then exposed to different doses of safranal and incubated for 24 hrs. DAPI staining was carried out by treating the cells in 6-well plates. The cells were then washed with phosphate buffered saline (PBS) and fixed with 10% formaldehyde. The DAPI-stained cells were then observed with fluorescence microscope for estimation of apoptotic cell populations. For the estimation of the percentage of the apoptotic cells a FITC-Annexin V/PI Apoptosis detection kit was employed as per the instructions of the manufacturer using a flow cytometer.

Comet assay

DNA damage triggered by safranal was assessed by the comet assay. Briefly, safranal-treated colo-205 colon cancer cells were harvested and suspended in cold PBS. The cells in the 0.5% low melting point agarose were kept on a slide precoated with a layer of 1% regular agarose. Afterwards, these two layers were allowed to solidify at 4°C, and then suspended in a cold lysis buffer for 50 min at 4°C. Then, the gel slides were allowed to dry and the dried slides were soaked in fresh electrophoresis solution for 25 min. Then, electrophoresis was carried out at 300 mA, 25 V for 25 min at 4°C. This was followed by staining with ethidium bromide (20 µg/ml) for 12 min, and neutralization of the slides with 0.4 M Tris-HCl (pH 7.5). Finally, the slides were washed and observed under a fluorescent microscope (BX51; Olympus, Tokyo, Japan).

Cell cycle analysis

For estimating the distribution of Colo-205 colon cancer cells in different phases of the cell cycle, the safranal-treated cells were harvested and washed twice with PBS. Then, the cells were fixed with 70% ethanol for about an hour and washed again with PBS. The cells were finally resuspended in solution of PI (50 µl/ml) and RNase1 (250 µg/ml). This was followed by incubation for 30 min at room temperature and fluorescence-activated cell sorting cater-plus cytometer was used with 10,000 cells/group.

Western blotting

The Colo-205 colon cancer cells treated with safranal were collected and treated with lysis buffer [Tris-HCl, sodium-dodecyl sulfate (SDS), mercaptoethanol and glycerol. The extracts were boiled for 10 min in the presence of loading buffer followed by separation of cell extracts using 15% SDS-PAGE gel. The samples were then put onto polyvinylidene fluoride membranes and blocked using 5% skimmed milk powder. Membrane incubation with primary antibodies was performed overnight at 4°C. The membranes were incubated with horseradish peroxidase-linked secondary biotinylated antibodies at 1:1,000 dilution for 2 hrs. Washing of the membranes with PBS was followed by visualization of the immunoreactive bands using the ECL-PLUS/Kit according to the manufacturer's instructions. The immune complexes development was carried out using an ECL detection kit according to the manual protocol (ECL GST western blotting detection kit, Pierce Biotechnology, Inc., Waltham, MA, USA). The bands were analyzed using GelGDoc2000 imaging system (Bio-Rad Laboratories GmbH, Munich, Germany).

Statistics

All the experiments were carried in triplicate and the values were expressed as mean ± SD. Differences between the control and treated cells were analyzed using Student's *t*-test, and statistical significance was considered at $p < 0.05$.

Results

Safranal exerts antiproliferative effects on Colo-205 cells

To examine the antiproliferative effects of the safranal (Figure 1), the Colo-205 cells were treated

with safranal at different doses and the cell viability was investigated by MTT assay. The results revealed that safranal (Figure 2) exhibited significant antiproliferative activity on the colo-205 cells which was concentration-dependent. The IC_{50} of safranal against colo-205 colon cancer cells was 20 µM.

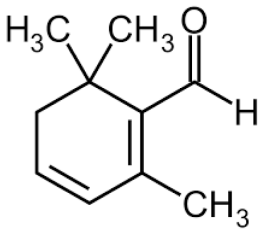

Figure 1. Chemical structure of safranal.

Safranal induces apoptosis in Colo-205 cells

Next we investigated by DAPI staining whether safranal also triggers apoptosis in colo-205 colon cancer cells. These cells were first exposed to safranal at IC_{50} concentrations and then subjected to DAPI and observed under fluorescence microscope. It was observed that safranal induced apoptosis in colo-205 colon cancer as evidenced from the increased number of cells with white color nuclei (Figure 3). Further, the percentage of apoptotic cell populations increased from 2.43% to 39.66 % at IC_{50} (Figure 4). To find out if safranal induced mitochondrial-related apoptosis we checked the Bax and Bcl-2 protein levels (Figure 5). The results clearly showed that safranal treatment increased the expression of Bax protein and decreased the Bcl-2 protein, indicative of mitochondrial apoptosis. The mitochondrial-mediated apoptosis was further confirmed by accretion of ROS by safranal (Figure 6) and reduction in MMP in a concentration-dependent manner (Figure 7).

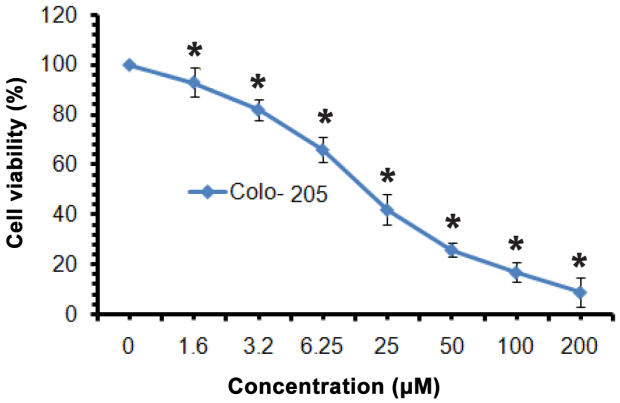

Figure 2. Effect of safranal on cell viability as determined by MTT assay. The experiments were carried out in triplicate and expressed as mean±SD (*p<0.05). The Figure shows that the cell viability decreased with increasing safranal concentration (dose-dependent response).

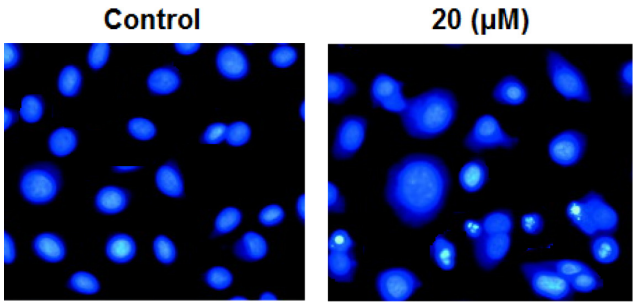

Figure 3. Induction of apoptosis at IC_{50} of safranal as indicated by DAPI staining. The experiments were carried out in triplicate and showed that safranal triggered apoptosis at IC_{50} (20 µM).

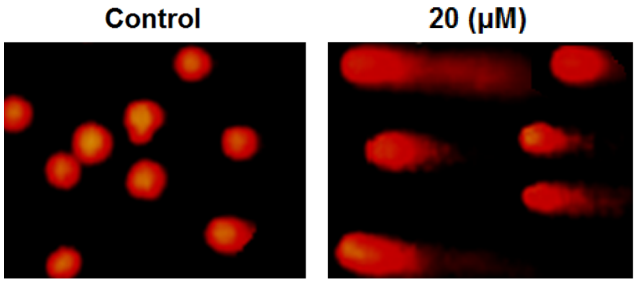

Figure 4. Comet assay showing DNA damage induced by safranal at IC_{50} (20µM).

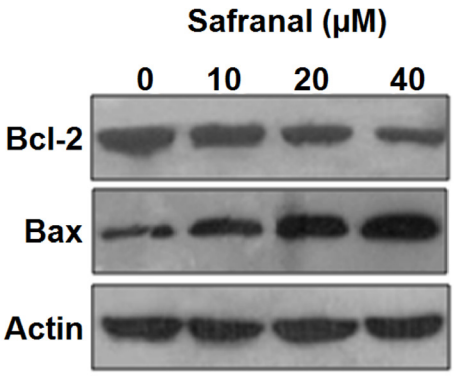

Figure 5. Estimation of apoptotic cell populations of colo-205 cells after treatment with safranal at IC_{50}. The experiments were carried out in triplicate.The Figure reveals that Bax expression increased and Bcl-2 decreased in a concentration-dependent manner.

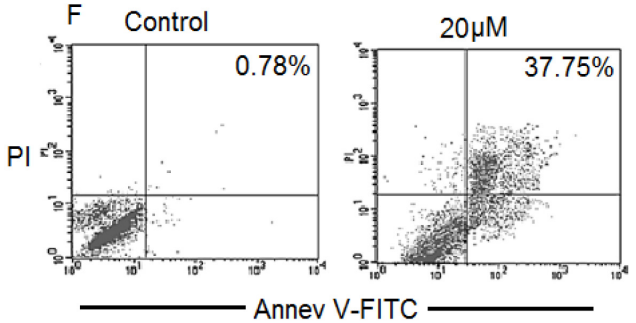

Figure 6. Annexin V/PI showing apoptotic cell populations. The experiment were carried out in triplicate. The Figure shows that safranal triggered apoptosis at IC_{50} (20µM).

Safranal induces DNA damage in Colo-205 cells

We also assessed whether safranal caused DNA damage in colo-205 colon cancer cells by comet assay. It was observed that safranal induced DNA damage in colon cancer colo-205 cells at IC_{50} concentrations. The DNA damage was evidenced from the formation of tail DNA (Figure 8).

Safranal causes G2/M cell cycle arrest in Colo-205 cells

The distribution of Colo-205 cells in the different cell cycle phases after treatment with safranal was studied at IC_{50} concentration. The results showed safranal led to accumulation of colo-205 colon cancer cells in G2/M phase of the cell cycle, ultimately leading to G2/M cell cycle arrest (Figure 9).

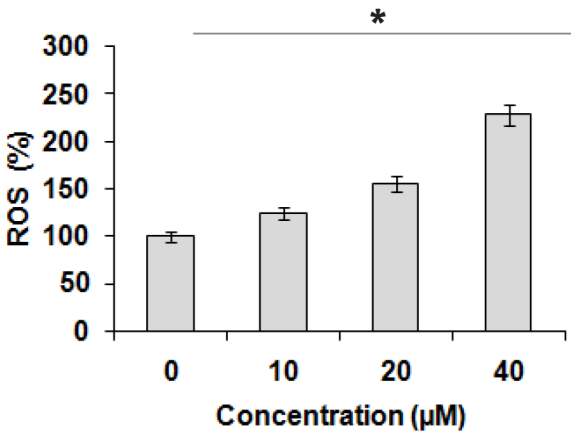

Figure 7. Effect of indicated concentrations of safranal on ROS generation. The experiments were carried out in triplicate and expressed as mean±SD (*p<0.05). The Figure shows that safranal led to the production of ROS in a concentration-dependent manner.

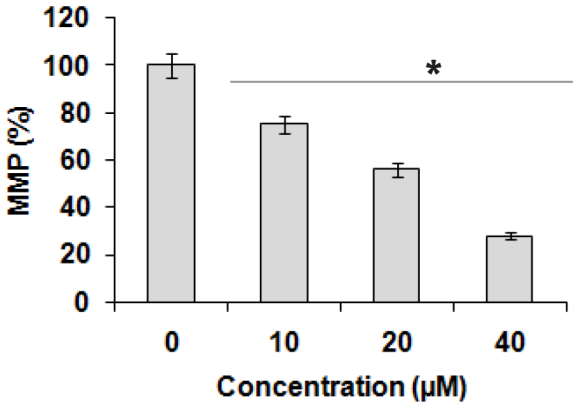

Figure 8. Effect of indicated concentrations of safranal on MMP. The experiments were carried out in triplicate and expressed as mean±SD (*p<0.05). The Figure shows that safranal led to reduction of MMP in a concentration-dependent manner.

Safranal inhibits PI3K/AKT/mTOR signalling pathway

Evaluation of the effect of safranal on the PI3K/AKT/mTOR signalling pathway revealed that safranal caused reduction in the protein expression levels of p-PI3K, p-AKT and p-mTOR. However, the expression of PI3K, AKT and mTOR remained almost unaltered (Figure 10).

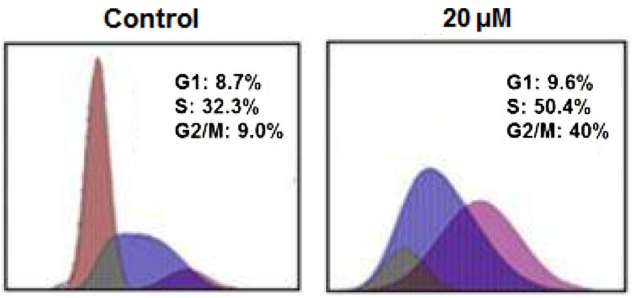

Figure 9. Effect of safranal on cell cycle phase distribution of colo-205 cells. The experiments were carried out in triplicate. The Figure depicts that safranal at IC_{50} caused increase in the G2 phase cells, leading to G2/M cell cycle arrest.

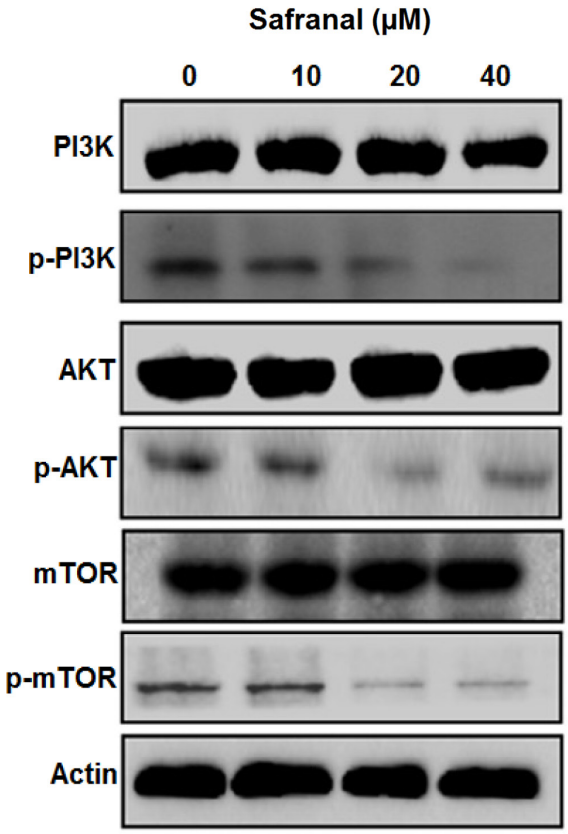

Figure 10. Western blot showing the effect of safranal on the protein expression of PI3K/AKT/mTOR signalling pathway. The experiments were carried out in triplicate The Figure shows that the expression of p-PI3K, p-AKT and p-mTOR decreased with increasing concentrations of safranal.

Discussion

Colon cancer is the second most important cause of cancer-related deaths among malignant tumors [7]. The treatment options for colon cancer are limited and also have side effects [8]. Thus, novel plant-derived molecules are being screened for their anticancer activities every now and then [9]. In this study we investigated the anticancer potential of safranal against the colo-205 colon cancer cells. Safranal reduced the viability of these cells in a dose-dependent manner and exhibited an IC_{50} of 20 μM. It has been reported that safranal together with other saffron apocarotenoids inhibited the growth of human cancer cells [10]. To further decipher the mechanism by which safranal reduced the viability of colo-205 cancer cells, we first carried out DAPI staining and observed that safranal could prompt apoptosis in cancer cells which was further confirmed by the comet assay. Then, annexin V/PI staining revealed that the apoptotic cell populations were 39.66% at IC_{50} concentration in comparison to 2.43% in control. Apoptosis is considered one of the important mechanisms to prevent the development of diseases, such as cancer, and the organism eliminates the harmful cells via this process [11]. Furthermore, the anticancer agents that are able to induce apoptosis of cancer cells are considered proficient as they also curb the development of drug resistance by cancer cells [12]. Next, to unveil if the safranal-triggered apoptosis followed the mitochondrial pathway, we evaluated the expression of Bax and Bcl-2 proteins and observed that safranal upregulated the expression of Bax and downregulated the Bcl-2 expression in a concentration-dependent manner. This was further associated with accretion of ROS and reduction MMP. All these changes clearly indicate that safranal induced-apoptosis follows the mitochondrial pathway. Apart from apoptosis, cell cycle is another target by which anticancer agents induce their anticancer effects [13]. In this study it was observed that safranal triggered G2/M cell cycle arrest in the colo-205 cancer cells. Finally, we examined the effect of safranal on the PI3K/AKT/mTOR signalling pathway and the results revealed that safranal could inhibit this pathway. PI3K/AKT/mTOR signalling pathway is one important pathway that is reported to be activated in several cancer types [14]. Therefore, drugs targeting this pathway may prove very useful in the treatment of malignancies.

Conclusion

To conclude, the results of the present study indicate that safranal inhibits the growth of cancer cells via induction of mitochondrial apoptosis and induction of G2/M cell cycle arrest. These results clearly indicate that safranal could prove a lead molecule for the treatment and management of colon cancer.

Conflict of interests

The authors declare no conflict of interests.

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