The Function of Saffron and its Constituent in Gastroenterological Tissues

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Abstract: Japan has been moving towards a super aging society, resulting in a rapidly increasing prevalence of lifestyle diseases, including colon cancer. Japanese patients survey by the Ministry of Health, Labour and Welfare reported 235,000 colon cancer patients in 2015, and this number is quickly increasing due to the change of dietary life style from the typical Japanese food to the westernized style food. Although the cancer chemotherapy has been widely developing recently, some natural product support, having wide spectra of bioactivity, however mild, are required. Saffron finds use as folk medicines as well as a flavoring and a coloring agent. Saffron consists of three main chemical components; red color, crocetin glycosides; a bitter taste, picrocrocin; and spicy aroma, safranal. In this chapter, we evaluate the activities of saffron extracts and a major crocetin glycoside, crocin, against colorectal cancer in vitro and in vivo trials. Saffron crude extracts, which contain approximately 40% of crocin, significantly inhibited the growth of colorectal cancer cell lines HCT-116, HT-29 and SW-480, although crocin did not affect for non-cancer cells. Crocin significantly inhibited the development of colonic adenocarcinomas induced by azoxymethane and dextran sodium sulfate in mice during 18 weeks feeding. The crocin feeding experiment for 4 weeks evidently inhibits the dextran sodium sulfate induced colitis and, then, the clear suppression for the mRNA expression of tumor necrosis factor α, interleukin-1β, interleukin-6, interferon γ, NF-κB, cyclooxygenase-2, and inducible nitric oxide synthase, and the increase of Nrf2 mRNA expression in the colorectal mucosa occurred. From these results we suggest that crocin can suppress chemically induced colitis and colitis-related colon carcinogenesis in mice mainly through the inhibition of inflammation related cytokines, indicating that saffron and crocin are suitable candidates for the prevention of colitis and inflammation-associated colon carcinogenesis. We further review the supporting phenomena like strong anti-oxidant and anti-inflammation activities of crocin using our previous publications.

Keywords: Crocus sativus, saffron, crocin, colorectal cancer cell line, colon carcinogenesis, anti-inflammation activity.

1. INTRODUCTION

Japan has been moving towards a super aging society, resulting in a rapidly increasing prevalence in lifestyle diseases, including colon cancer. Japanese patients survey by the Ministry of Health, Labour and Welfare reported 235,000 colon cancer patients in 2015, and this number is quickly increasing due to the change of dietary life style from the typical Japanese food to the westernized style food. Although the cancer chemotherapy has been widely developing recently, some natural product support, having wide spectra of bioactivity, however mild, are required. This is the reason why natural products having preventive activities for cancers are particularly desirable in Japan. Considering such recent health circumstances in Japan, we select saffron for preventing and increasing quality of life against cancers, and its function will be reviewed in this chapter because we have been clarified the multifunctional activity of saffron and its constituent, crocin [1], although saffron was not listed in the NCI report indicating the 40 foods having anti-cancer activity [2].

Crocus sativus L. (Iridaceae) is a perennial herb that is widely cultivated mainly in Iran, which produces 90% of saffron, and in other countries like Greece, Spain and Morocco for its red stigmatic lobes that constitute saffron from 3500 years ago. This plant blooms only once a year and the manual harvest of stigmas should be performed within a very short time [3].
The manual cultivation methods practiced with saffron crocus contribute greatly to its high price. About 100,000 flowers give about 1,000 g of the dried saffron. The stigmas can be collected from full blooming *C. sativus* (Figure 1 Left). We confirmed that the concentration of crocin increases until full blooming and then decreases. Therefore, stigmas can be collected in full blooming season in order to keep the higher concentration of crocin [4].

Saffron finds use as folk medicines and traditional Chinese medicine (TCM) as well as a flavoring and a coloring agent. Saffron has three main chemical components: the bright yellow coloring carotenoids, a bitter tasting picrocrocin, and spicy aroma, safranal. The carotenoid pigments consist of crocetin-diglucoside, crocin-2, crocin-3, crocin-4 and crocetin-di-(β-D-digentiobiosyl)-ester (crocin) (Figure 2). More recently we succeeded to isolate a novel crocetin glycoside, trans-crocetin-1-al 1-O-β-gentiobiosyl ester (Figure 2) [5]. We confirmed that drying is important because an endogenous β-glucosidase is still active when moisture remains [4]. Therefore, drying is completed in about 30-45 min, after which the drug is cooled and stored under dry condition [4].

Saffron can be used as an antispasmodic, anticatarrhal, and nerve sedative ingredient, and is reported to be useful in treating various human disorders such as heart and blood disorders [6]. Crocin has a wide range of activities including antioxidant [7,8], hypolipidemic [9,10,11] like lowering of cholesterol and triglyceride levels in serum by crocin and crocetin [12], an inhibitory effect on the increase of bilirubin in blood [13] and anti-inflammatory effects [14-16]. The neuroprotective activities of crocin have also been demonstrated in various experimental animal models of brain disorders, such as cerebral ischemia [17], Alzheimer disease [18], depression [19], memory impairment [20-22] and neuroprotective activity [7,17,23,24].

Since it becomes clear that saffron and its constituent, crocin have the wide pharmacological activities as described above, we focus to confirm the incorporation of crocin into cells first and the anti-colorectal cancer activities of saffron and crocin *in vitro* and *in vivo* in this chapter.
2. PREPARATION OF ANTI-CROCIN MONOCLONAL ANTIBODY (MAB) AND CONFIRMATION FOR INCORPORATION OF CROCIN INTO CELLS BY IMMUNOSTAINING

In the first stage of anti-colorectal cancer investigations, we prepared monoclonal antibody (MAb) against crocin [25]. In the first step for preparation of MAb against crocin, the conjugate of crocin with carrier protein for immunization is necessary. Therefore, crocin was treated with NaIO₄ to cut sugar moiety releasing aldehyde in a molecule following addition of carrier protein. As the other way, the crocin-hemisuccinate was prepared first, and then conjugated with BSA to give crocin-hemisuccinate BSA conjugate as indicated in Figure 3. The molecular weight of prepared schiff base was analyzed by MALDI-tof-mass spectrometry to determine the hapten number in the conjugate for suitability of immunization. Since the hapten number in crocin-hemisuccinate BSA conjugate was determined to be 8.6, which was suitably enough for immunization rather than that of crocin-BSA conjugate prepared by NaIO₄ treatment, the former was used as an antigen. Hybridoma producing MAb reactive to crocin was obtained by general procedure, and classified into IgG2a, which had λ light chains. The reactivity of IgG type MAb 12a was tested by varying antibody concentration and by performing a dilution curve, and then the antibody concentration was selected for competitive ELISA. The measuring range of this ELISA system extends from 10 to 200 ng/ml of crocin [25].

In order to confirm the incorporation of crocin and the localization of crocin into PC-12 cells, we immunostained cells using the anti-crocin MAb prepared. Clear incorporation of crocin into PC-12 cells was confirmed after 30 min comparing with the control cells as indicated in Figure 4 [24]. The incorporation after the addition of crocin in the medium was not enough after 15 min (Figure 4B). After 30 min, the clear staining occurred (Figure 4C and D). From this evidence we confirmed that crocin can be incorporated into the cell and be functioned.

3. ANTI-PROLIFERATION ACTIVITIES OF SAFFRON EXTRACT AND CROCIN AGAINST HUMAN COLORECTAL CELL LINES

Anti-tumour activity of saffron on mice transplanted with sarcoma-180, Ehrlich as cites carcinoma and Dalton’s lymphoma as cites tumours [26], inhibitory effects of saffron on chemical carcinogenesis in mice using two-stage assay system [27,28,29] and the effect of crocetin on skin papillomas and rous sarcoma [30]. Escribano et al. (1996) reported crocin inhibits the growth of Hela cells and suggested apoptosis induction [31]. Effects of saffron extracts and crocin on the proliferation of colorectal cancer cell lines, we investigated HCT-116, SW-480 and HT-29 [32].

Figure 5A shows the relationship between saffron extract concentration and the inhibition of proliferation for the three cell lines. At 0.25 and 0.5 mg/ml levels of saffron extract no inhibition occurred in three cell lines. When 1.0 mg/ml of saffron extract was added, the decrease of proliferation ratio was observed resulting in 45.5, 91.0 and 79.2 %, in HCT-116, SW-480 and HT-29 cells, respectively. In the case of 3.5 mg/ml of saffron extract, strong inhibition appeared in all cells as 6.8, 17.6 and 12.9 %, respectively (Figure 5A). From these results HCT-116 cells were the most sensitive to saffron extract, suggesting the major constituent of saffron, crocin, might be strongly affective for three cell lines.

The same tendency was observed with the addition of crocin (Figure 5B). At a 1.0 mM concentration of crocin, HCT-116, SW-480 and HT-29 cells proliferation was significantly reduced to 2.8, 52 and 16.8%.
proliferation, respectively ($P < 0.01$), although at 0.03, 0.1 and 0.3 mM the affect was not strong. Consistent with the saffron data, crocin has the most significant anti-proliferative effect on HCT-116 cells. Since the concentration of crocin in the saffron crude extract is approximately 40% [4], 3 mg of saffron extract and 1 mM of crocin are nearly equivalent. Therefore, the above two results (Figure 5A and B) are satisfactory although the other minor crocetin-glycosides are contained in saffron extract as previously documented [4].

![Figure 4: Immunostaining of crocin in PC-12 cells using anti-crocin monoclonal antibody.](image)

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<td>A Control cells.</td>
<td>B Culturing for 15 min</td>
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<td>C Culturing for 30 min</td>
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![Figure 5: Effects of saffron extract (A) and crocin (B) on proliferation of human colorectal cancer cells, HCT-116, SW-480 and HT-29.](image)

![Bar charts](image)
In the above evaluation of saffron extract and crocin, we confirmed the specificity of saffron and crocin against cancer cell lines. We also evaluated the effects of saffron extract and crocin on non-small cell lung cancer (NSCLC) cells in addition to colorectal cancer cells. Our data showed that at 1.0 mg/ml and 3.0 mg/ml, saffron extract reduced the NSCLC proliferation to 83.9% ($P < 0.05$) and 34.1% ($P < 0.01$), respectively. At 1.0 mM crocin concentration, the cell proliferation was reduced to 43.3% ($P < 0.01$). Saffron extract did not affect the proliferation of non-cancer (young adult mouse colon) YAMC cells. In this part of the study, the effect of saffron extract on YAMC cells was compared to that of the HCT-116 cells. At the tested concentration range, saffron extract did not show any significant inhibition of the YAMC cells, while cell growth was significantly inhibited in HCT-116 cells at 1.0 mg/ml ($P < 0.01$).

4. ANTI-COLORECTAL CANCER ACTIVITY OF CROCIN IN VIVO

Previous in vitro investigation clearly showed that the real constituent having anti-cancer activity in saffron was crocin. First of all, we confirmed that no observable clinical toxicity was found in the mice by histopathological survey of liver and kidneys, by weights of the whole body and liver, and by the colon length after feeding of crocin for 8 weeks. This is in good agreement with the previous data that the oral LD$_{50}$ of saffron was approximately 20 g/kg [33], meaning it is a very safe food.

Following the in vitro data of crocin using three colorectal cell lines we started the in vivo experiments using mice with the feeding of azoxymethane (AOM) and dextran sodium sulfate (DSS), which were applied as the promotional agent for the induction of colorectal lesions [34]. The feeding of AOM and/or DSS in mice was investigated. The AOM and/or DSS treatment indicated the occurrence of several colorectal lesions, such as colitis with mucosal ulcers, dysplastic crypts, tubular adenoma and tubular adenocarcinoma resulting in the clear incidences and multiplicity of colorectal inflammation with mucosal ulcers and the presence of dysplasia after 18 weeks.

The incidence of inflammation with mucosal ulcers significantly decreased after feeding of all three concentrations of crocin (50, 100 and 200 ppm) compared to AOM + DSS group. Similarly, the inflammation score decreased after crocin treatment at the higher concentrations (100 and 200 ppm). The incidence of high-grade dysplastic crypts significantly decreased by feeding the mice with all three concentrations of crocin compared to the AOM + DSS group. The multiplicity of high-grade dysplastic crypts also decreased by the crocin treatment at the higher concentrations (100 and 200 ppm).

The incidence and multiplicity of colonic tumors after 18 weeks of feeding were observed. The AOM + DSS group clearly indicated colonic adenocarcinoma with an incidence of 90% and a multiplicity of 3.15 ± 1.87. On the other hand, the treatment with three concentrations of crocin significantly reduced the incidence and multiplicity of adenocarcinoma. Crocin also significantly decreased the incidence of adenomas and the multiplicities of colonic adenoma.

The expression of minichromosome maintenance protein 2 (MCM2) related to DNA replication in colorectal adenocarcinoma areas was surveyed by the immunohistochemical analysis using anti-MCM2 rabbit MAb in order to determine the effects of crocin on the proliferation of cancer cells. From this analysis, a clear decrease of staining in adenocarcinoma was observed in the treatment with crocin compared to that of AOM + DSS group, indicating that crocin evidently decreased the cancer cell proliferation (Figure 7).

To make sure the expression of NF-κB and Nrf2 by the treatment with or without crocin, the immunohistochemical analysis in the adenocarcinomas that will be developed into the colon was investigated. When compared with the AOM + DSS group, the treatment of crocin at 100 ppm and 200 ppm significantly suppressed the immunohistochemical score for NF-κB, while significantly enhancing the...
expression of Nrf2 at 200 ppm crocin. In the crocin alone group (200 ppm) and the untreated group, the immunohistochemical expressions of NF-κB and Nrf2 in the colonic mucosa were very weak.

The colonic mucosa of mice treated with 200 ppm of crocin alone showed almost normal histology. On the other hand the DSS treatment induced severe colitis with mucosal ulcers. The induction of colitis in the mice treated with DSS and crocin at 100 ppm or 200 ppm decreased, and regenerative crypt cells covered and healed the mucosal ulcers as shown in Figure 8.

![Figure 8: Histopathological survey of colorectal mucosa. Treated with DSS and 200 ppm of crocin.](image)

The inflammation scores of the DSS + 50 ppm crocin (P <0.05), DSS + 100 ppm crocin (P <0.01), and DSS + 200 ppm crocin (P <0.001) groups were significantly decreased dose-dependently than those of the DSS alone group as indicated in Figure 9.

![Figure 9: Inflammation score in colorectum with DSS and/or crocin.](image)

5. CONCLUSION

In cancer chemotherapy, the induction of cancer cell apoptosis has been emphasized, and the cell apoptosis is mediated by many factors. Among them, p53 gene is a transcription factor placed at the nexus of a number of pathways that mediate apoptosis in response to a wide range of cellular stresses [35]. HCT-116 cells are p53 gene wild-type, while SW-480 and HT-29 cells are mutant in the p53 tumor suppressor gene. Since the effects of saffron extract and crocin on HCT-116 are stronger than that of HT-29 and SW480, it suggests that some activity of p53 gene may be linked to the saffron extract and crocin to express the anti-cancer effects [36]. Furthermore, crocin has anti-tumor effects...
on cellular DNA and RNA synthesis [26,37]. Another mechanism for the anti-tumor action of saffron extract and crocin is the inhibitory effect on free radical chain reactions [38], because most carotenoids are lipid-soluble and might act as membrane-associated high-efficiency free radical scavengers, connects with their anti-oxidant properties [7,39,40].

The feeding of crocin significantly suppressed several inflammatory events and NF-κB expression in the colorectal mucosa of the mice fed with DSS. Inflammatory genes, such as COX2, iNOS, TNF-α, and IL-1β, are the most common target genes participating in the activation of NF-κB and are associated with a number of chronic inflammatory diseases, including inflammatory bowel disease (IBD) and IBD-related colorectal carcinogenesis [41-45]. We observed decreases in the mRNA expression levels of NF-κB, COX-2, TNF-α, IL-1β, and IL-6 in the mice treated with DSS and crocin compared to the mice with DSS alone. These evidences suggest that crocin suppressed the mouse colonic inflammation induced by DSS by modulating the NF-κB signaling pathway. The NF-κB signaling pathway also has a major role in inflammation-associated carcinogenesis [46]. Therefore, NF-κB is a target for cancer chemoprevention [47,48], and natural compounds that suppress NF-κB expression may be useful for cancer chemoprevention [49]. As previously documented crocin has the anti-inflammatory effects [14-16], these evidences might be related to a range of inflammation gene expression. Since rocin did not produce any chromosome damage in mammalian cells in culture [50], the clinical trials of crocin may be possible.

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