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SHORT COMMUNICATION

Suppression of Polyps Formation by Saffron Extract in *Adenomatous polyposis coli*$^{Min/+}$ Mice

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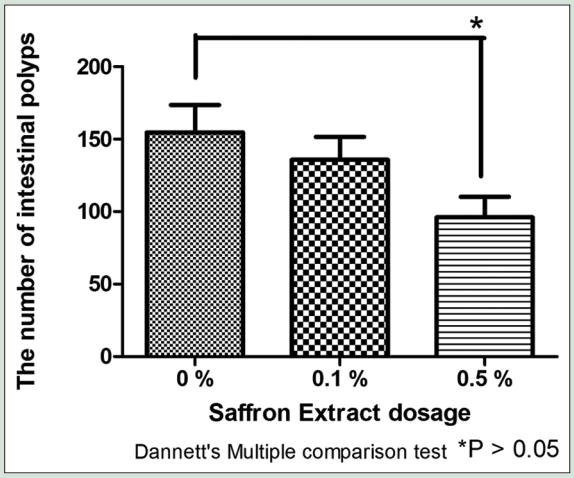
ABSTRACT

Saffron (*Crocus sativus* L.) has been used both as a food additive for flavoring and coloring and in traditional medicine. Saffron extract and its main component crocin decrease the growth of several types of human cancer, including colorectal cancer *in vitro*. Numerous polyps develop in the small intestine in the *Adenomatous polyposis coli* (*Apc)* deficiency mice. *Apc*$^{Min/+}$ mice are models for human familial adenomatous polyposis and human colon cancer patients. In this study, we examined the efficacy of saffron extract added to diet on reducing the polyp density in *Apc*$^{Min/+}$ mice. *Apc*$^{Min/+}$ mice were either given a placebo or saffron extract (0.1% and 0.5%) diet for 4 weeks. At 12 weeks of age, intestines were analyzed for polyp number in the small intestine. Our analysis confirmed that crocin (1), crocin-2 (2), and crocin-4 (4) are the major compounds in the saffron extract and the content of 1 in the tested saffron extract was 29.2%. Saffron extract decreased the number of intestinal polyps in a concentration-dependent manner in *Apc*$^{Min/+}$ mice. Notably, the number of polyps in the distal small intestine of the mice fed with 0.5% saffron extract was significantly decreased compared with the placebo. These results indicate that saffron extract can reduce the polyp number in the *Apc*$^{Min/+}$ mice.

Key words: *Adenomatous polyposis coli*$^{Min/+}$ mice, crocin, *Crocus sativus*, intestinal polyps, saffron

SUMMARY

- Saffron has been used both as a food additive and in traditional medicine
- *Adenomatous polyposis coli* (*Apc)*$^{Min/+}$ mouse is a model of familial adenomatous polyposis (FAP)
- We investigated the effect of the saffron extract on polyp formation using *Apc*$^{Min/+}$ mice
- Saffron extract suppressed the number of intestinal polyps in *Apc*$^{Min/+}$ mice
- Saffron extract might be effective in preventing intestinal polyp formation for FAP patients.



Abbreviations Used: FAP: Familial adenomatous polyposis; *Apc*: *Adenomatous polyposis coli.*

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INTRODUCTION

Crocus sativus L. is a perennial herb belonging to the iris family (Iridaceae). When dried, it is commonly called "Saffron" and is used both as a food additive for flavoring and coloring and as a drug in medicine.[1-3] Saffron is cultivated worldwide, especially in Iran, India, Greece, Morocco, Spain, and China. Phytochemical research reported that the main component of saffron is crocin [Figure 1], an ester glycoside of crocetin. Other typical components such as picrocrocin and safranal, related to the flavor of the herb, have been isolated from saffron.[4,5]

Pharmacological studies have revealed that saffron extracts and/or the active constituents have properties to improve learning and memory and have anticonvulsant, antidepressant and anti-inflammatory properties.[6-10] Free radical scavenging, antioxidant activity, and promotion of the diffusion of oxygen in different tissues were also reported to be the properties of saffron extracts or their bioactive constituents.[11,12] Other biological effects of saffron and its constituents include the induction of apoptosis, antihyperlipidemic effects, immunomodulation, and antineurodegenerative effects.[13-17] Our previous studies have demonstrated several bioactivities of saffron and/or crocin. Crocin promotes nonrapid eye movement sleep in mice.[18] Saffron and crocin exhibit neuroprotective activities *in vivo* and *in vitro*.[19-23] In addition, we prepared monoclonal antibodies against crocin.[24]

Several studies have revealed the effects of saffron and its ingredients on carcinogenesis.[25,26] Saffron extract and crocin were reported to inhibit the growth of several types of human cancer cells.[27-29] We also indicated the prevention of skin tumor formation in mice and a decrease in the proliferation of human colorectal cancer cells.[30,31]

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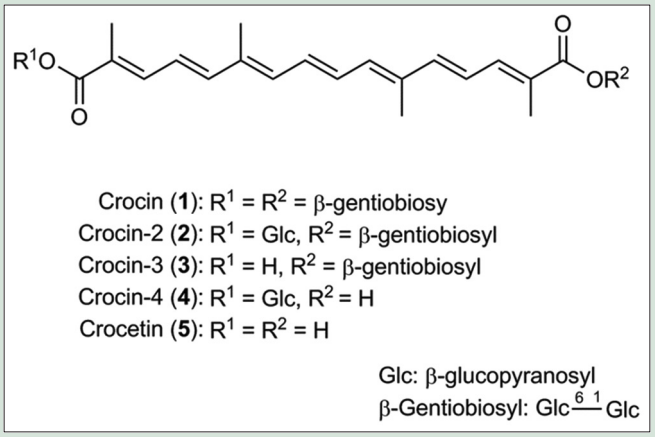

Figure 1: The chemical structures of crocetin glycosides (1–4) and crocetin (5)

However, the effects of saffron on intestinal carcinogenesis *in vivo* have not been clarified yet.

Penetrant dominant mutation of *Adenomatous polyposis coli* (*Apc*) is known to lead the numerous intestinal polyp development.[32,33] Familial adenomatous polyposis (FAP) is the hereditary disease that develops hundreds of intestinal polyps. In this disease, intestinal polyps develop from young generation and increase the risk of transformation that leads to colorectal cancer development.[34] The $Apc^{Min/+}$ mouse is a model of FAP that is lacking a functional *Apc* gene product. By using this well-recognized mouse model, we examined the effect of the saffron extract on intestinal polyps *in vivo* for the first time whether saffron extract can inhibit the development of intestinal polyps.

MATERIALS AND METHODS

Plant material

The stigmas of *C. sativus* (saffron) were collected in Oita Prefecture, Japan, in 2010 and were authenticated by one of the authors (Y. S.). A voucher specimen was deposited in the Department of Pharmacognosy, Nagasaki International University, Japan.

Preparation of saffron aqueous extract for *in vivo* study

The air-dried and shade-dried saffron (500 g) was pulverized and then extracted with 50% aqueous EtOH (2.0 l × 3 times) at 40°C under sonication. The combined extracts were concentrated into dark brown syrup (280 g).

High-performance liquid chromatography analysis

The pure main pigment crocetin glycosides, crocin (1), crocin-2 (2), crocin-3 (3), and crocin-4 (4), were dissolved in MeOH at a concentration of 1.0 mg/ml and the saffron extract (10.0 mg) was prepared in MeOH (10 mg/ml). All samples were filtered with 0.45-μm syringe filters and stored at −20°C until use. Peaks in the saffron extract were unambiguously assigned by comparison of their retention time with those of authentic specimens. Figure 1 shows the chemical structures of crocetin (5) and crocetin glycosides (1–4).

For the quantitative analysis of 1, various concentrations were prepared to determine the calibration curve. The calibration curve was constructed using the content versus peak area ($y = 28.082x + 28.563$, $R^2 = 0.9998$, linear range: 0.015–0.25 mg/ml). The content of 1 in the saffron extract was calculated using the standard curve.

High-performance liquid chromatography instruments and conditions

The high-performance liquid chromatography tests were performed on a TOSOH 8020 series (Tosoh, Tokyo, Japan) equipped with an intelligent ultraviolet (UV)/visible detector (UV-8020), two dual pump (DV-8020), and a degasser (SD-8020). The chromatographic separation was performed on a TSK Gel ODS-100V (4.6 mm × 250 mm with 5-μm particle size; Tosoh Co., Tokyo, Japan) in the following conditions: mobile phase A (MeOH) and B (H_2O containing 1.0% acetic acid), gradient program, 0–5 min (30:70, v/v), 5–20 min (30:70 → 50:50, v/v), 20–30 min (50:50 → 70:30, v/v), and 30–45 min (70:30 → 100:0, v/v). The flow rate was 1.0 ml/min, the injection volume was 10.0 μl, the detection was performed at 442 nm, and the program was held at ambient temperature throughout the analysis.

Mice and diet

The $Apc^{Min/+}$ (C57BL/6J) mice were supplied by Jackson Laboratories (Bar Harbor, Maine, USA). The mice were maintained under specific pathogen-free conditions and 12:12 light/dark cycles at the Animal Center of Nagasaki International University (Nagasaki, Japan). In this research, we analyzed 30 mice in total (16 females, 14 males). Four weeks after birth, all baby mice were genotyped from a tail sample. The examined $Apc^{Min/+}$ genotype mice were separated in three groups, including two test groups and one control group. The mouse diet was compounded with two different doses of saffron extract, 0.1% and 0.5% (i.e., 0.25 and 1.25 g/kg body weight/day, respectively). The control mice were not given any saffron extract. The two test groups (from 8 weeks old) were given food mixed with the saffron extract for 4 weeks. According to standard dietary intake guidelines provided by the Ministry of Health, Labour and Welfare, we calculated the appropriate dose for the mice. Mice were sacrificed at 12 weeks of age for the analysis of intestinal polyps. All animal experiments were conducted according to the Guidelines for Animal Experiments from the Faculty of Pharmaceutical Sciences, Nagasaki International University (approval number 124).

Genotyping

Mouse tails were genotyped using a KAPA Express Extract (NIPPON Genetics Co., Ltd., Japan) according to the manufacturer's protocol. Allele-specific polymerase chain reaction primers for the *Apc* gene were produced according to genotyping protocols of the Jackson Laboratory (https://www2.jax.org/protocolsdb/f?p=116:5:0:NO:5:P5_MASTER_PROTOCOL_ID,P5_JRS_CODE:21922,002020).

Counts of intestinal polyps and statistical analysis

After the 12-week-old mice were sacrificed, their small intestines were removed and divided into three equal segments. These segments were incised longitudinally and then washed with chilled phosphate-buffered saline. The washed intestines were laid flat on filter paper and fixed in 10% neutral-buffered formalin. The number of intestinal polyps was counted under light microscopy. All statistical analyses were carried out using the GraphPad Prism 5 program (GraphPad Software Inc., San Diego, CA). Statistical analysis of the number of polyps was performed by Dunnett's multiple comparison test.

RESULTS AND DISCUSSION

There are several arguments regarding absorption of glycosides and one of them is the direct absorption and the other hydrolysis then absorbs. It has not been evident in the case of crocin.[35,36] However, it is clear that crocin and/or crocetin can be incorporated into cell since previously we prepared anti-crocin monoclonal antibody which has

Table 1: The average number of polyps in the small intestine and large intestine of mice fed with saffron extract

Group of saffron extract	Small intestine				Large intestine	Total
	Proximal	Middle	Distal	Total		
0% (placebo group) (*n*=9)	10.2±2.6	55.8±7.1	77.6±10.5	143.6±19.3	1.3±0.2	144.9±19.4
0.1% (*n*=10)	10.3±2.1	59.7±7.0	63.5±7.4	133.4±15.3	2.4±0.7	135.8±15.8
0.5% (*n*=11)	7.8±1.7	42.5±7.1	53.9±8.0* (*P*=0.023)	104.3±16.0* (*P*=0.048)	1.7±0.4	106.0±16.2* (*P*=0.05)

*$P<0.05$ versus placebo. *n*: Number of mice

affinity against both components and confirmed by immunostaining of cell.[21,24]

The polyp number was significantly decreased in *Adenomatous polyposis coli*$^{Min/+}$ mice

The average number of polyps in the small intestine of mice fed with saffron extract at doses of 0% (placebo), 0.1% and 0.5% was 143.6 ± 19.3, 133.4 ± 15.3, and 104.3 ± 16.0, respectively. The difference in polyp number was statistically significant compared the mice fed with 0.5% saffron extract to that of placebo ($P < 0.05$, Dunnett's multiple comparison test) [Table 1]. The number of the polyp was most decreased in a distal part that located next to the large intestine (0.5% of saffron extract versus placebo, $P = 0.023$). The number of intestinal polyps in the proximal and middle part was decreasing but failed to obtain the significant difference.

Interestingly, we found that the distal part of the small intestine seems to display the highest sensitivity to the saffron extract. We assumed that the underlying reason might be due to the longer residence time of intestinal contents in the distal part of the small intestine than the proximal part.[37] As is exposed longer to the saffron extract, as the effects of saffron may be remarkable. A time-dependent effect of saffron extract could be evident in the *in vitro* experiment.

The content of crocin in saffron extract

A chromatogram of crocin (1) is shown in Figure 2a. The retention time and purity of crocin was 29.6 min and 95.7% (relative pic area), respectively.

Figure 2b shows the chromatogram of the saffron extract. As illustrated, crocin (1), crocin-2 (2), and crocin-4 (4) are the major compounds in the saffron extract, and they were clearly separated from each other. Because 1 has been used as the compound for the quality control of saffron products, the content of 1 was examined quantitatively. The amount of 1 in the tested saffron extract was 29.2%.

In this study, the intake amount of saffron extract was that could be able to intake in daily life. Intake of 0.1% and 0.5% of the saffron extract was calculated based on the dietary fiber reference intakes for Japanese determined by the Ministry of Health, Labour and Welfare (approximately 300 mg/kg/day). As 29.2% of the saffron extract was crocin, we calculated that the content of the crocin in 0.1% and 0.5% of saffron extract could be 0.073 g/kg/day and 0.365 g/kg/day.

CONCLUSION

This is the first report using *Apc*$^{Min/+}$ mice to examine the effect of saffron extract on polyp formation. We assume that saffron extract might be effective in preventing intestinal polyp formation for FAP patients. As is already used in medicine, further experiments, especially for its safety evaluation is needed. Furthermore, we found that saffron and/or crocin can prevent the colon cancer by inhibition of colitis-associated colorectal carcinogenesis because the anti-inflammatory effects of crocin are suggested to be based on its strong antioxidant activity rather than that of alpha-tocopherol.[21,38] Therefore, we have reached to further the ability of cancer preventive activity of crocin because in this investigation we have found the inhibitory activity against polyp formation which will be transformed and lead to colon cancer.

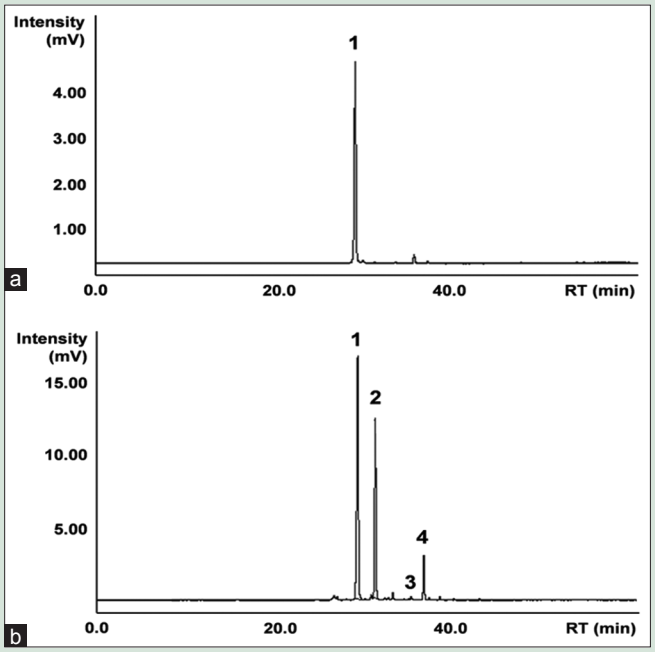

Figure 2: (a) A high-performance liquid chromatography chromatogram for standard crocin (1) (concentration 1.5 mg/ml). (b) A high-performance liquid chromatography chromatogram for saffron extract (concentration 2.5 mg/ml). Number to peak identity; crocin (1), crocin-2 (2), crocin-3 (3), and crocin-4 (4)

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Nil.

Conflicts of interest

There are no conflicts of interest.

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