Multifunction of Saffron and Its Components in Brain

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Abbreviation: BSO; Buthionine sulfoximine, DMEM; Dulbecco's modified Eagle's medium, ELISA; Enzyme-linked immunosorbent assay, FB1; Fumonisin B1, c-GCS; c-Glutamylcysteinyl synthase, GPx; Glutathione peroxidase, GR; Glutathione reductase, GSH; Glutathione, IL-6; Interleukin-6, JNK; c-Jun kinase, LTP; Long-term potentiation, MAb; Monoclonal antibody, NGF; Nerve growth factor, NMDA; N-methyl-D-aspartate, N-Smase; Neutral sphingomyelinase, PS; Phosphatidylserine, SAPK; Stress-activated protein kinase, SD; Step down, SM; Sphingomyelin, SOD; Superoxide dismutase ST; Step through, TNF-a; Tumor necrosis factor

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Inroduction

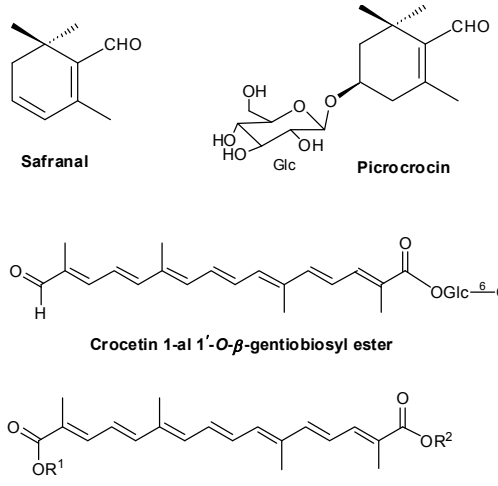
Crocus sativus L.(Iridaceae) is a peren nialherb that is widely cultivated mainly in Iran and the other countries like Greece, Spain and Morocco for its red stigmatic lobes that constitute saffron (Fig.1, Fig. 2) from 3500 years ago. This plant blooms only once a year and the manual harvest of stigmas should be performed within a very short time [1].

Figure 1 Flowering of Crocus sativus L. and saffron (right)

The manual cultivation methods practiced with saffron crocus contribute greatly to its high price. About 90000-100000 flowers give 5000 g of fresh stigmas or about 1000 g of the dried drug. Weather conditions affect the quality of saffron. Therefore, an indoor cultivation system was established in Japan from 100 or more years ago in Oita prefecture in Japan (Fig. 2).

Figure 2 Indoor cultivation of *Crocus sativus*in Japan

The stigmas can be collected from full blooming *C. sativus* in the room. This is the reason why the indoor cultivation method is advantageous for the achievement of a homogenous quality of saffron and for saving time. [2]. Saffron finds use as folk medicines and traditional Chinese medicine as well as a flavoring and a coloring agent. Saffron has three main chemical components. The bright yellow coloring carotenoids, a bitter taste, 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) (Fig. 3). Previously we confirmed that drying is important because an inner β-glucosidase is still active when moisture contains [2]. Therefore, drying is completed in about 30-45min, after which the drug is cooled and stored under dry condition [2]. More recently we succeeded to isolate a novel crocetin glycoside, trans-crocetin-1-al 1-O-β-gentiobiosyl ester. (Fig. 3) [3].



Crocetin: $R^1 = R^2 = H$
Crocin-3: $R^1 = H$; $R^2 = Glc \xrightarrow{6} Glc$ (β-gentiobiosyl)
Crocetin diglucoside: $R^1 = R^2 = Glc$
Crocin-2: $R^1 = Glc$; $R^2 = \beta$-gentiobiosyl
Crocin-4: $R^1 = CH_3$; $R^2 = \beta$-gentiobiosyl
Crocin : $R^1 = R^2 = \beta$-gentiobiosyl

Figure. 3 Components of saffron

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 [4-7]. Crocin has a wide range of activities including antioxidant [5-7], anti-cancer [4,8], hypolipidemic [5,9], anti-atherosclerotic [10-12] and anti-inflammatory effects [13,14]. The neuroprotective activities of crocin have also been demonstrated in various experimental models of brain disorders, such as cerebral ischemia [14],

Alzheimer disease [15], depression [16], and memory impairment [17-19].

It is well known that saffron has anti-cancer activities against several cancer cell lines and *in vivo* investigations. We also investigated the anti-cancer activity of saffron and its constituent, crocin. In the first investigation we tested the inhibitory activity for skin tumour promoted by chemicals by using saffron and crocetin- glucosides. When crocin was applied before each 12-O-tetradecanoylphorbol-13-acetate treatment, it delayed the formation of papillomas, only 10 % of mice borepapillomas at 9 weeks of promotion. The effect of crocin was not mimicked by gentiobiose or glucose alone [20].

Furthermore, we investigated *in vitro* anti-cancer active evidences of saffron extract and its constituent, crocin using several cancer cell lines like HTC-116, SW-480 and HT-29. Saffron extract and crocin significantly inhibited the growth of colorectal cancer cells while not affecting normal cell [21]. From these data we started *in vitro* experiments using mice. The development of colonic adenocarcinomas in mice was induced by azoxymethan and dexran sodium sullate. Crocin significantly inhibited the colonic adenocarcinomas depending on the inhibition of inflammation phenomenan resulting in the prevention of colitis and inflammation-associated colon carcinogenesis [22].

The development of natural products with properties for alleviating the symptoms of learning and memory impairments has been expected by clinicians and researchers in the field. In the brain, the hippocampusis a very important region in the learningand memory processes, and the LTP induced from the brain tissue is closely related to learning and memory [23]. In earlier publications, we reported the effects of an ethanol extract of *C. sativus* and its purified components on the central nervous system interms of learning behaviors in mice and LTP in the dentate gyrus of hippocampus in anesthetized rats and in the CA1 region of rat hippocampus slices[24-26]. Neuronal cell death is required for the development of the nervous system. However, recent studies suggest that neurons die from programmed cell death(apoptosis) in brains deprived of oxygen by stroke[27] and trauma [28] and in the brains of Alzheimer's patients [29]. Therefore, prevention of neuronal apoptosis has been considered to be a desirable therapeutic strategy for treating such neurodegenerative diseases, although the value of this approach is not yet evident.This review discusses the value of folk medicines interms of learning and memory and also in modulating apoptotic cell death, together with our recent data of crocin's effect on neuronal cell death.

Neuroprotection by crocin

1. Inhibitory activity of crocin for PC-12 cell death induced by serum/glucose deprivation

In the first stage of neuronal investigations, we prepared monoclonal antibody (MAb) against crocin [30].In order to confirm the incorporation of crocin and the localization of crocin into PC-12 cells, we immunostained cells using the anti-crocinMAb prepared. Clear incorporation of crocin into PC-12 cells was confirmed comparing with the control cells [31].

Cells cultured in serum/glucose-containing Dulbecco's modified Eagle's medium [DMEM (+)] had a normal morphology at 24 h, while those cultured in the serum- and glucosefree medium [DMEM (–)] for 24 h were round inshape and showed the characteristic properties of necroticand/or apoptotic cells. We confirmed that approximately 60% cell death had occurred in thel atter culture using the Trypan blue dye exclusion method. The addition of crocin (10 μM) significantly suppressed both the morphological changes and thePC-12 cell death induced by the DMEM (–) conditionsas crocin inhibited TNF-a-induced PC-12 cell death[32], resulting in 85% survival. It is wellknown that serum [33-35] or nerve growth factor(NGF) [36,37] deprivation induces apoptosis in PC-12cells. Colombaioni et al. [38] demonstrated that serum deprivation increased the intracellular ceramide levelsin undifferentiated HN9.10e cells, resulting in apoptosis. These findings easily suggest a possibility that ceramide levels increase in PC-12 cells under DMEM(–) conditions. PC-12 cells cultured for 3 h in DMEM (–) showed asignificant increase (3.5-fold increase) in the level of ceramide compared to the basal level in cells cultured in DMEM (+) conditions. The suppressive effect of crocin was dose-dependent. We also tested the effect of fumonisin B1 (FB1), which inhibits de novo ceramide synthesis in cells at a concentration of 10–30μM [39,40]. However, FB1 had no significant effect on ceramide levels, suggesting that the accumulation of ceramide through an enhancementof de novo synthesis following a 3-h culture in DMEM(–) was in itself not sufficient to explain the increase. It has been suggested that the sphingomyelin (SM) pathway and SAPK/JNK signaling systems may function together [41] in stress-induced apoptosis of U937cells and BAE cells. Since the environmental stress under DMEM (–) conditions may activate the stress activated protein kinase (SAPK)/JNK cascade in PC-12 cells, we compared the amounts of phosphorylatedJNK in the cells cultured in DMEM (+) and DMEM(–) for 6 h. The DMEM (–) conditions stimulated the phosphorylation of JNK in the cells byapproximately 3.7-fold relative to the control cells.

2. Inhibitory effect of crocin on the activation of N-SMase induced in serum/glucose-deprivedPC-12 cells

In order to confirm the resource of the accumulated ceramide, we measured the activity of magnesium dependent N-SMase in the PC-12 cell homogenate. N-SMase activity in cells cultured in DMEM (–) reacheda maximum at 1 h and decreased to around the level of the control cells at 3 h. However, there was no time dependent change in the N-SMase activity during a 3-hculture in DMEM (–). This assay method can detect N-SMaseactivity in these supernatants by substitution of the reaction medium for 50 mM sodium acetate buffer(pH 5.6). The results demonstrated that the activity ofN-SMase in PC-12 cells was unaffected by serum/glucosedeprivation for at least for 3 h. The addition of crocin in the culture medium suppressed the enzyme activities at 1 and 2 h in a dose-dependent manner. To determine whether or not the inhibition of N-SMase is a direct action of crocin on the enzyme, we added crocin

to the reaction medium, whose cells had been cultured in DMEM (–) for 2 h.The addition of 1 or 10 μM crocin had no inhibitory effect on N-SMase activity in the reaction medium. However, the addition of GSH at concentrations of 1 and 10 mM inhibited the enzyme activity in a dose-dependent manner. Earlierreports indicate that GSH is a physiological inhibitor of magnesium-dependent N-SMase in plasma membranes[42-44]. N-SMase is inactive in the presence of physiological concentrations (1–20 mM) ofGSH. Therefore, these results suggest that theN-SMase activity in the reaction medium is derived from magnesium-dependent N-SMase contained in plasmamembranes and that the observed N-SMase inhibitionby crocin does not occurred through its direct action on the enzyme.

3. Increase of intracellular GSH levels in serum/glucose deprivedPC-12 cells through an increase in the activities of GR and *c*-GCS by crocin

In an investigation aimed at testing the above-mentioned hypothesis, we examined the effect of crocin on intracellular GSH levels in serum/glucose-deprived PC-12 cells. The GSH levels in PC-12 cells exposed for 3 h to serum/glucose-free DMEM decreased to half that found in the control cells, and the reafter remained constant. However, the addition of crocin to the medium increased the intracellular GSH level dose-dependently, maintaining it at the 3-h time-point at a higher level. The most significant effect of crocin occurred at a concentration of 10 μM. The concentration of GSH was high enough to inactivate N-SMase.We then investigated the mechanism by which crocinin creased the GSH levels. The GR activities in serum/glucose-deprived PC-12 cells decreased in a timedependent fashion, whereas the co-presence of 10μl Mcrocin enhanced GR activity each hour (approximately four-fold elevation at 6 h). This result indicates that crocin has no significant effect on the GPx activity in the cells.GSH synthesis is regulated by the rate-limiting enzyme c-GCS. This enzyme is thought to be regulated by several mechanisms. In mouse endothelial cells, theTNF-a- or IL-1b-induced increase in c-GCS activity is associated with an increase in mRNA expression [45].IL-6 also stimulates the expression of c-GCS mRNA and increases the activity of this enzyme, which leads to increased GSH levels in PC-12 cells [46]. In contrast,Nakajima et al. [47] reported that NGF had anability to increase the activity of c-GCS at the transcription level by extending the half-life of c-GCSmRNA.The addition of crocin (10 IM) doubled c-GCS mRNA expression in PC-12 cells in serum/glucose-freeDMEM, while it had no effect on the mRNA levels ofthe control PC-12 cells.The crocin-induced increase in c-GCS mRNAexpression is reflected in an increase in the activity of this enzyme in the cells. These results suggest that crocin can increase GSH levels by increasing the 1 h DMEM.

4. Antioxidant effect of crocin in preventing neuronal celldeath

The effects of crocin on PC-12 cells deprived of serum/glucose in comparison with those of a-tocopherol havebeen reported [31].

Depriving the PC-12 cells of serum/glucose caused changes in the morphology and peroxi dation of their membrane lipids and decreased intracellular superoxide dismutase (SOD) activity. Although hosphatidylserine (PS) residues are normally present in the inner membrane, the oxidativestress transferred these into the outer membrane leaflet. PS externalization is known as an early sign of apoptotic induction. Annexin binds to the negatively charged PS, and the conjugated FITC shows a ring-likestain along the cellular boundary. The cells deprived of serum/glucose show strong ring-like stains compared to the control cells. Crocin keptthe cell's morphology more intact than a-tocopherol. InPC-12 cells deprived of serum/glucose for 6 h, the levelof peroxidized membrane lipids increased 1.8-fold incomparison to the control cells, and SOD activity decreasedto 14% of that in the control cells. However,crocin significantly decreased the formation of peroxidizedmembrane lipids and restored SOD activitycompared to a-tocopherol activity. The restoration ofSOD activity suggests that crocin has an important rolein modulating antioxidative effects. Crocin also suppressedthe activation of caspase-8 caused by serum/glucose deprivation, this activation was suppressed in a concentration-dependent manner (0.1–10 lM). Crocin did not inhibit caspase-8 activity in the cell lysates and its inhibitory effect may be caused indirectly by the antioxidant activity.

Learning and memory by saffron and crocin

1. Effects of saffron extract on memory registration

There were no differences between control and saffron extract treated groups in either ST or SD test, suggesting that saffron extract had n effect on memory registration in normal mice (data not shown).

Saffron extract improved the short-ening of latency induced by ethanol significantly, but had no effect in increasing the number of successful mice decreased by ethanol in ST test. It also ameliorated the increase of errors induced by ethanol significantly, but gad bi effect on the decrease of the learned mice induced by ethanol in SD test. Saffron extract ameliorated the etha-nol-induced memory registration impairment in both ST and SD test. The ameliorating effects were dose-dependent [49].

Saffron extract significantly amelio-rated the ethanol-induced impairment of memory retrieval on SD test, but not in ST test. The in-crease of errors and the decrease in number of learned mice were significantly ameliorated by saffron extract in SD test. The improving effects were dose-dependent.

The crude extract of saffron prevents the ethanol-induced impairment of memory acqui-sition in stepthrough (ST) and step down (SD) tests [49]. On thebasis of these results it is rela-tively easy to suggest that some components of saffron are capable of antagonizingthe blocking effect of ethanol for memory acquisition. Frac-tionation of the crude extract based on activity

revealed that crocin is actual active componentin saffron.

Single oral administration of crocin had no effect on mice in passive avoidance tasks. Oral administration of30% ethanol induced an impairment in memory acquisition in ST and SD tests. However, the subsequentoral administration of crocin (50 mg/kg) improvedthe impairment of memory acquisition in both tests in a dose-dependent manner [48].

2. Effect of saffron and crocin on LTP

We previously confirmed that the saffron crude extract can improve the blocking effect of ethanol on the LTP in a dose-dependent manner. However, since the active component had not yet been isolated at that stage, we discuss our search for the active compound in this review. The saffron extract were injected intracerebroventricularly, and the blocking effect of ethanol on the LTP decreased dose-dependently. These results led us to hypothesize that crocin might antagonize the blocking effect of ethanol on the induction of LTP, as already discussed in the context of the improvement of impairments in memory acquisition [18, 19]. Following the activity-guided separation from the crude extract, we confirmed that crocin is the actual active component in saffron. When a 50 mg/kg dose of crocin was injected 5 min before the administration of ethanol, LTP was induced at 84% of that of control, suggesting that the LTP blocking effect of ethanol was improved dose dependently with the administration of crocin. The activities of the crocetin gentiobiose glucose ester and crocetin di-glucose ester, which are analogs of crocin, on the LTP blocking effect of ethanol was investigated at the same dose scale. These activities were found to be distinctly lower than that of crocin. The active improvement effect against blocking was clearly proportional to the number of glucoses because crocin, which possesses four glucoses in a molecule, showed the highest improvement effect while the activity of crocetin di-glucose ester was almost the same as the control. From this result we concluded that crocin is the actual active component in saffron related to learning and memory phenomenon.

Non rem sleeping effect of crocin

It is known that saffron with traditional Chinese medicines promotes the sleep activity in the field of therapy of mental disorders and is used for sleep promotion as a folk medicine in Japan. From these evidences we started to search the activity of saffron and its component, saffron.We examined the sleep-promoting effect of crocin and crocetin on mice after an intraperitoneal administration at 20:00 during the wake period. Fig 2A shows time-courses of the hourly amounts of non-REM and REM sleep and wakefulness after the administration of vehicle or crocin (100 mg/kg). During the period of 20:00 to 01:00, mice with vehicle-treatment spent more time in wake than in sleep. When 100 mg/kg of crocin was injected on the experimental day, the amount of non-REM sleep was increased immediately after the injection, and the effect was statistically significant from 2 to 4 hr after the administration. Crocin did not change the REM sleep after the administration.

This augmentation effect on non-REM sleep time was accompanied by reduction in wakefulness. The increase in non-REM sleep and decrease in wake lasted more than 4 hr after the injection. There was no further disruption of the sleep architecture during the subsequent period (8:00 to 20:00). These data indicated that crocin induces non-REM sleep without occurrence of adverse effects, such as rebound insomnia after the sleep induction. Similar time-course profiles were observed with a low dose of 30 mg/kg, but the effect on sleep was small and lasted only about 1–2 hr after the injection (data not shown).

We calculated the total time spent in non-REM and REM sleep and wakefulness for 4 hr after the crocin or crocetin injection (Fig. 2B). Crocin at 10 mg/kg did not affect the cumulative amounts of non-REM and REM sleep and wakefulness for 4 hr after injection. Crocin given at 30 and 100 mg/kg statistically significantly increased the total amount of non-REM sleep by 160 % and 270 %, respectively, and decreased the total amount of wakefulness by 20 % and 50%, respectively, without changing the amount of REM sleep during a 4 hr period as compared with the vehicle control. The increase in non-REM sleep and the decrease in wakefulness were statistically significantly between two doses of 10 and 100 mg/kg. Crocetin at a dose of 100 mg/kg significantly increased the total amounts of non-REM sleep by 160% and decreased the total amount of wakefulness by 25% without changing the REM sleep amount during a 4-hr period as compared with the vehicle control [50].

Conclusion

It is well known that saffron is very safety food because oral administration of saffron extract at concentrations of up to 5 g/kg is still nontoxic in mice [51]. Crocin increases the intracellular glutathione leverl and prevents cell death in serum-deprived and hypoxic PC-12 cells, a cell culture model for brain ischemia, by its antioxidant property. The antioxidant capacities of crocin have been reported in association with a variety of neuroprotective potentials. In PC-12 cells, the generation of ROS activates neutral SMase to generate ceramide, which induces cell death. Glutathione directly inhibits the activation of the SMase. Therefore, we hypothesized that crocin might prevent the activation of N-SMase in serum/glucose-deprived PC-12 cells by a GSH-depen dent inhibition mechanism.

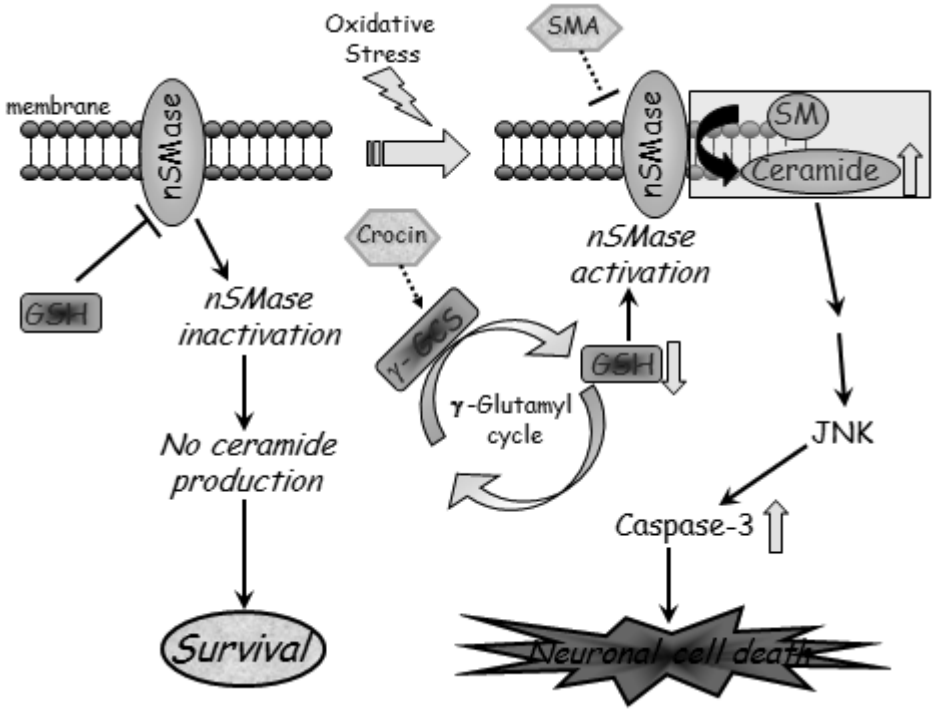


Figure 4 Summary of occurrence of apoptosis in terms of GSH biosynthesis

Ceramide release and activate the caspase family as discussed already. Although we have not discussed in this review, we found the effect of crocin on an infarcted ared caused by MCA occlusion of the middle cerebral artery in mice. This evidence also helps the brain healthcare by saffron and/or crocin.

In the early stage of our investigation on the pharmacological activity of crocin, we reported that crocin is the actual active component involved both in the improvement of learing and memory and with the preventive effect of LTP blocked by ethanol *in vivo* although oral administrations of saffron and/or crocin had no effect on memory acquisition in normal mice. Recently Naghibi et al. investigated the effect of saffron extract on morphine-induced memory impairment and concluded that the saffron extract attenuated morphine-induced memory impairment [51]. We further demonstrated for the first time that crocin selectively antagonizes the inhibitory effect of ethanol on N-methyl-D-aspartate (NMDA)-receptor-mediated responses in hippocampal neurons [17]. The sugar numbers in crocetin glycosides reflected the LTP blocking activity by ethanol like that crocin having 4 glucoses in a molecule attenuated strongest compared to the other smaller molecule. This tendency is a good agreement with the previous reports that the sugar residues are important for the activities of some drugs like cardiac steroids [52], streptozotocin [53], ginsenosides [54] and so on.

Patient related to sleeping problem are increasing in Japan, and now 25% of population

have some sleeping problems. Safron can be prescribed with TCM for mental disordering patient having sleeping problem in Japan. Therefore, crocin was tested for sleep promotion and we found crocin increased the total time of non-REM sleeping. This finding promotes the clinical use of saffron since saffron has been used as safety food from thousands ago. Moreover, crocin was approved by State Food and Drug Administration for the clinical trial in 2006 and now a drug for angina in China. From various kind of pheno mena described in this review saffron and/or crocin have a multifunctional natural product in brain.

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