Review Article

The effect of crocin on neuroprotective activity *in vitro*: a narrative review

Shinji Soeda[1], Tomoe Ohta[2], Takuhiro Uto[2], Yukihiro Shoyama[2]

[1]Faculty of Pharmacy, Fukuoka University, Fukuoka, Japan; [2]Faculty of Pharmacy, Nagasaki International University, Sasebo, Nagasaki, Japan

Contributions: (I) Conception and design: Y Shoyama; (II) Administrative support: T Uto; (III) Provision of study materials or patients: Y Shoyama; (IV) Collection and assembly of data: T Ohta; (V) Data analysis and interpretation: S Soeda; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Yukihiro Shoyama. Faculty of Pharmacy, Nagasaki International University, Sasebo, Nagasaki 859-3298, Japan.
Email: shoyama@niu.ac.jp.

Background and Objective: With the notable pharmacological activity crocin in saffron has been investigating widely resulting in anti-apoptosis, anti-oxidation, anti-inflammation, learning and memory and anti-dementia activities *in vitro*. However, since the mechanisms of neuroprotective, learning/memory promoting and anti-dementia activities for crocin are not exactly clear yet. This review is focused on the neuroprotective activity of crocin *in vitro*.

Methods: A narrative review of data published on the pharmacological value of crocin was surveyed and collected references gathering from the Google search engine from January 1, 1980 to May 31, 2022 and publications in English. The suitable data of investigations have been compared, analyzed and reorganized resulting in newly assembled clarification.

Key Content and Findings: Regarding the neuroprotective activity of crocin, several processes were associated. Among these pathways, programmed cell death, apoptosis in neurons occurs in the brain deprived of oxygen after stroke, as well as in patients with Alzheimer's disease. The oxidative stress decreases the cellular levels of glutathione, the potent inhibitor of neutral sphingomyelinase. PC-12 cells showed a rapid increase in cellular ceramide levels, followed by an increase in the phosphorylation of c-Jun kinase, leading to apoptosis. Exploration of the crocin's preventive mechanism in oxidative stress-induced cell death revealed that the activities of glutathione reductase and γ-glutamylcysteinyl synthase in the γ-glutamyl cycle affected the stable glutathione supply resulted blocking the activation neuronal sphingomyelinase.

Conclusions: Glutathione-dependent inhibitory mechanism in oxidative stress-mediated cell death suggested the importance of crocin as a neuroprotective agent candidate. Regarding the neuroprotective activity related to memory and dementia, several phenomenon and proteins including enzyme were closely associated *in vitro*. Crocin improved memory inhibited by hyoscine resulting in no change of CaMKII, NMDA and AMPA except ERK although CaMKII is closely correlated to LTP expression. This result indicated that the combination of *in vitro* investigation, *in vivo* experiments and/or the clinical trial is necessary for understanding the real function of crocin.

Keywords: Saffron; crocin; anti-apoptosis; neuroprotection; dementia

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Introduction

Crocus sativus L. belonging Iridaceae cultivation was recorded in Crete 3,500 years ago, and it has been expanding in Iran, Greece, Spain, Morocco, China and Japan, for its red stigma (*Figure 1*).

C. sativa flowers once in a year and its stigma should be harvested during very short period. Therefore, saffron is too expensive comparing with the other herb medicines.

Crocetin glycosides, picrocrocin, and safranal are the major components of saffron (*Figure 2*). Among them, crocin concentration is around 15 % or more (1,2). Crocin 2, 3 and 4 are contained as the minor constituents.

Since indoor cultivation systems were started in Japan from 1910 (1) because the quality of saffron depends on weather conditions (*Figure 3*). Indoor cultivation systems have several benefits such as adjusting full blooming period resulting in labor reduction. Moreover the quality control of saffron is improved since saffron contains strong β-glucosidase which cleavages glycoside linkage to give crocin 2, 3 and 4 as shown in *Figure 2* under moisture condition (1). This is the reason why the short time drying of saffron is needed. The relation between biological activity and sugar number conjugated was reported that the saponin's haemolytic activity of di- and triglycoside saponins were higher than that of monoglycoside (3). From this evidence, it is easily suggested that the higher glycoside of crocetin, crocin might possess higher activity. This is an important issue for the quality control of saffron (4). Therefore, dried saffron is then frozen and stored free of moisture to preserve its high quality (1).

We present the following article in accordance with the Narrative Review reporting checklist (available at https://lcm.amegroups.com/article/view/10.21037/lcm-22-4/rc).

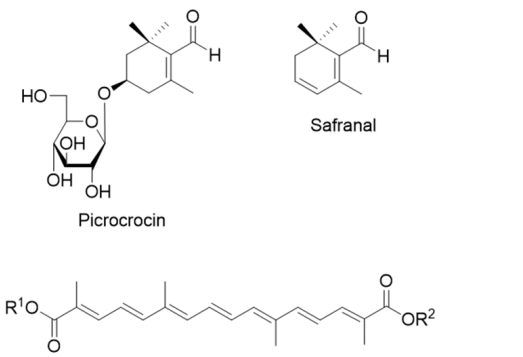
Methods

Studies published over the last 20 years were identified via a PubMed search using different combination such as "Crocus sativus", "Saffron", "Crocin", "neuroprotective", "PC-12 cell", "apoptosis". Most of search formulas were shown in *Table 1*. The search strategy was summarized in *Table 2*.

Additional papers are identified by reviewing reference lists of publications. Publications with relative low credibility and publications without English are removed. Data are extracted depending on justness for the topic and its detail are shown in *Table 2*.

Saffron was listed in the Bencao Gangmu published in China in 1578 for its cognitive functions, and has been known for its blood disorder (5), anti-cancer (6-8), anti-oxidant activity (9,10), memory impairment (11-15), learning and memory (12,15,16) which are closely related to dementia. Brain dysfunction models mimic the protection of neuronal function such as in anti-amyloid-βaggregation (17), NMDA receptor (13,18). Naghibi *et al.* investigated the effect of saffron extract on morphine-induced memory impairment (19). The number of sugars in crocetin glycosides reflected the ethanol-mediated LTP inhibition. Crocin showed the highest activity comparing with crocin 2, 3 and 4. This evidence is consistent with previous reports which sugar numbers are important for the activities of saponins such as cardiac glycosides (20), ginsenosides (21), saikosaponins (22) and hemolytic saponins (3,23). Recently natural products preventing dementia is strongly desired under the rapid increase of dementia patients. With respect to the safety of natural products, the most important factor for investigation is the confirmation of no toxicity of the major constituents. Saffron is chosen in this review since it has been used from approximately 3,000 years ago as a spice, coloring and medicine recorded in *materia medica* 2,000 years ago. In the case of health volunteers saffron (200 and 400 mg) are safe drug on coagulation system under a double-blind, placebo-controlled study for 1 week (24). When old AD patients were administered saffron extractives (30 mg) or donepezil (10 mg) as a positive control under a double-blinded/phase II study, the effect of saffron extract was evaluated to be the same with donepezil for mild to moderate AD patients. The major adverse effect of saffron, vomiting was lower than that of donepezil (25). Volunteers received crocin tablet (20 mg) or placebo resulted that the crocin tablet showed no major side effect except decreases of amylase, white blood cells and partial thromboplastin time (26). Therefore, the neuroprotective activities of saffron and crocin will be incorporated in the current study. Regarding the neuroprotective activity of saffron related to dementia, several processes were associated including enhancing nerve growth factor (NGF) secretion (27), choline acetyltrasferase (ChAT) activity and its mRNA levels (28), inhibition of β-amyloid secretion (29), promotion of energy metabolism in neurons via protection of mitochondrial damage (30) and sirtuin 1 (SIRT1) protein activation (31,32). Among these pathways, Crowe *et al.* reported that programmed cell death (apoptosis) in neurons occurs in the brain deprived of oxygen after stroke (33,34), as well as in patients with Alzheimer's disease (35). The prevention of neuronal apoptosis might become a promising

Figure 1 Flowering of *Crocus sativus* L. (A) and collected saffron (B).



	R^1	R^2
Crocetin digentiobioside (Crocin):	Geb	Geb
Crocin 2:	Geb	Glc
Crocin 3:	Geb	H
Crocetin diglucopyranoside (Crocin 4):	Glc	Glc
Crocetin:	H	H

Geb: gentiobiose (*β*-D-glucopyranosyl-(1-6)-*β*-D-glucopyranoside)
Glc: *β*-D-glucopyranoside

Figure 2 Major phytochemical constituents in saffron.

Figure 3 Indoor cultivation of system *Crocus sativus* L in Oita prefecture in Japan.

Table 1 The search terms used

("Crocus sativus"[Mesh]) AND "neuroprotective"[Mesh]
("Saffron"[Mesh]) AND "neuroprotective"[Mesh]
("Crocin"[Mesh]) AND "neuroprotective"[Mesh]
("Crocus sativus"[Mesh]) AND "PC-12 cell"[Mesh]
("Saffron"[Mesh]) AND "PC-12 cell"[Mesh]
("Crocin"[Mesh]) AND "PC-12 cell"[Mesh]
("Crocus sativus"[Mesh]) AND "apoptosis"[Mesh]
("Saffron"[Mesh]) AND "apoptosis"[Mesh]
("Crocin"[Mesh]) AND "apoptosis"[Mesh]

therapeutic strategy for a neurodegenerative disease. The neuronal cell death, apoptosis was occurred by many pathways and their mechanisms will be confirmed in this review. Among them anti-oxidative stress, γ-glutamyl cycle promotion and inhibition of ceramide release which were found firstly by authors, and the proteins related to memory were included in this review. The wide field data related to the neuronal investigations has been accumulated by the rat *pheochromocytoma* (*PC-12*) cells easily differentiated into neuron-like cells. Therefore, the neuroprotective activity of crocin using PC-12 cells is reviewed.

Preparation of anti-crocin monoclonal antibody and immunostaining of PC-12 cells

Crocin possessing 4 glucoses in a molecule in saffron is

Table 2 The search strategy summary

Item	Specification
Date of search	2021/12/15–2022/02/20
Database and other sources searched	PubMed
Search terms used	Details in *Table 1*
Time frame	1980–2021
Inclusion and exclusion criteria	Inclusion criteria: research articles and reviews in English about themes such as Crocus sativa/saffron/crocin and neuroprotection/PC-12 cell/apoptosis
	Exclusion criteria: some papers which we considered with low reliability
Selection process	Yukihiro Shoyama conducted the selection, authors attended a discussing the literature selection and obtained the consensus
Any additional consideration	Some papers were identified by reviewing reference lists of relevant publications if applicable

unstable because saffron contains inner β-glycoside resulting in hydrolysis occurrence under moisture condition (1). Moreover, the polyene structure in crocin is unstable under the existence of oxygen. From these reasons the quality control of saffron and/or crocin is needed before research. In our ongoing research of monoclonal antibody (MAb) for natural products the preparation of anti-crocin MAb has been started.

In order to prepare MAb against crocin the conjugate of crocin and carrier protein is necessary. Synthesis of crocin-carrier protein conjugate has two pathways because crocin possess 4 glucoses in a molecule, one is the cleavage method by $NaIO_4$ to prepare aldehyde body which can be conjugated with carrier protein via shiff's base bond as prepared for many MAbs against saponins like ginsenosides (36,37), paeoniflorin (38), saikosaponin (39), glycyrrhizin (40) and so on. In the first stage of MAb preparation the exact structure of crocin-carrier protein is unknow because crocin has many cleavage points by NaIO4. As the second method a hydroxyl group on C-6 of terminal glucose was specifically reacted with succinate anhydrate to give a hemisuccinate which combined with carrier protein producing crocin carrier protein conjugate. The hapten number in conjugate was determined by MALDI-tof-MS. When compared the hapten numbers prepared by $NaIO_4$ and hemisuccinate pathway, respectively the later method was clearly higher than that of $NaIO_4$ method (41). Crocin-hemisuccinate-HAS conjugate was used because the higher hapten number in the conjugate promoted the preparation of MAb (42) and prepared MAb against crocin by usual way via hypoxanthine- aminopterin-thymidine (HAT)-sensitive mouse myeloma cell (43,44). Single cell cloning by limited dilution way (45) was succeeded vir HAT-selection and screening with direct enzyme linked immuno solvent assay (ELISA) (42). The cross-reactivity was not specific for crocin, for example 66.9%, 61.6% and 1.5% for crocin 2, crocin 3 and crocin 4, respectively. However, it did not react with monoterpenoids, carotenoids and saponins. This wide reactivity is the main advantage of the antibody. It is better than a specific antibody for the metabolic study on crocin and the study of pharmacologically active mechanism of crocin in the central nervous system because it is suggested this MAb should be helpful to the further study on the presumable receptor in the brain.

To verify the incorporation of crocin and its localization in PC-12 cells, cells were immunostained using the anti-crocin MAb. *Figure 3* presents the time course of 10 μM crocin in PC-12 cells over a 30 min time span. Clear incorporation of crocin after incubation of 30 and 60 min into PC-12 cells was confirmed in comparison with control cells as presented in *Figure 3*. Incorporation of crocin in cells was recently confirmed by bone marrow mesenchymal stem cells (46) and plant cells (47) too. From these findings the incorporation of crocin into cells was exactly confirmed by anti-crocin MAb (9). It is easily suggested that transformation and/or metabolism of crocin will be occurred in cultured PC-12 cells because it became clear that alpha-glycosyl rutin, higher soluble rutin is hydrolyzed *in vitro* (48). Fortunately, the prepared anti-crocin MAb can cover the metabolites if it will be occurred in neuronal cells. Immunostaining using the anti-crocin MAb confirmed its incorporation and localization of

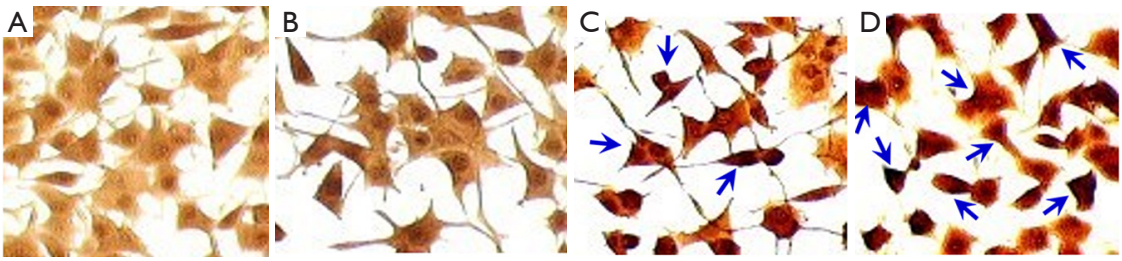


Figure 4 Incorporation of crocin in PC-12 cells. Control (A), incubated for 0 min (B), 30 min (C), 60 min (D), arrows show crocin stained by anti-crocin monoclonal antibody. Olympus CK-40 microscope at 100× magnification analysis.

crocin in PC-12 cells (9) as shown in *Figure 4* resulted that PC-12 cells can be used for further neuronal investigation together with anti-crocin MAb such as the immunostaining analysis for several proteins related to neuroprotective activity in PC-12 cells similar to that Li *et al.* reported the immunostaining of Cx36 and CaMKII in the investigation of neuroprotective natural product, leonurine in PC-12 cells (49).

Inhibitory activity of crocin against PC-12 cell death induced by serum/glucose deprivation (SGD) medium

PC-12 cells have been used widely for the neuronal investigation because it is easily differentiated into neuron-like cells. Furthermore, the clear cell death is induced by serum and/or glucose deprivation in culture medium.

Ochiai *et al.* found that 60% cell death was occurred after 1 day of serum/glucose deprivation (50). This phenomenon was due to apoptotic cell death via the oxidative stress induced by the increase of malonyldialdehyde (MDA) and the decrease of glutathione (GSH) levels resulting in the activation of caspase-3, -8, and -9, and increased Box levels with the decreases of Bcl-2 levels and NF-κB binding activity (48). Moreover, serum deprivation was linked with the deactivation of mitochondrial function, like confirmed by cytosolic release of cytochrome c and JC-1 (51). Glucose deprivation caused apoptosis in HeLa cell, which was prevented by knockdown of caspase-8 resulting in caspase-8-driven and receptor-independent apoptosis with no occurrence of mitochondrial outer membrane permeabilization (MOMP) (52). From this evidence we confirmed that serum/glucose deprivation (SGD) medium can be used for the evaluation of activity for crocin using PC-12 cells.

Cultured cells in Dulbecco's modified Eagle's medium contained serum and glucose showed a normal morphology at 24 h. When cells were cultured in the SGD medium for 24 h, the apoptotic cells such as rolling shape were observed. It became clear that approximately 60% of cell death were determined by the Trypan blue dye method (50).

The addition of crocin (10 μM) significantly inhibited the morphological changes indicated that crocin blocked TNF-α-induced PC-12 cell death (53) showing 85% surviva1 compared to control cells. It is well known that serum deprivation gives apoptosis in PC-12 cells (54-56). Colombaioni *et al.* found that serum deprivation increased intracellular ceramide levels in undifferentiated HN9.10e cells inducing apoptosis. This evidence suggests that ceramide concentration increase in PC-12 cel1s incubated in SGD medium. When PC-12 cells are cultured in SGD medium for 3 h, ceramide increase significantly (3.5-fold) compared to the cells cultured in SGE medium (57). The addition of crocin inhibited PC-12 cell death dose-dependently. Verheij *et al.* postulated that the SAPK/JNK signaling systems and the sphingomyelin (SM) pathway formed a mixed function in apoptotic phenomenon induced by stress in U937 cells and BAE cells (58). It becomes evident that environmental stress under SGD conditions activates the stress-activated protein kinase (SAPK)/JNK cascade in PC-12 cells when compare the level of phosphorylated JNK in cells cultured in SGD medium and positive medium for 6 h. In fact, the SGD medium stimulates JNK phosphorylation in PC-12 cells approximately 4-fold comparing with control cells (58).

Antioxidant effect of crocin against neuronal cell death

A large number of investigations has been focused on antioxidant plants and their constituents. Among them, the antioxidant activities of saffron and crocin were investigated by many groups (59-68). Ochiai *et al.* also investigated

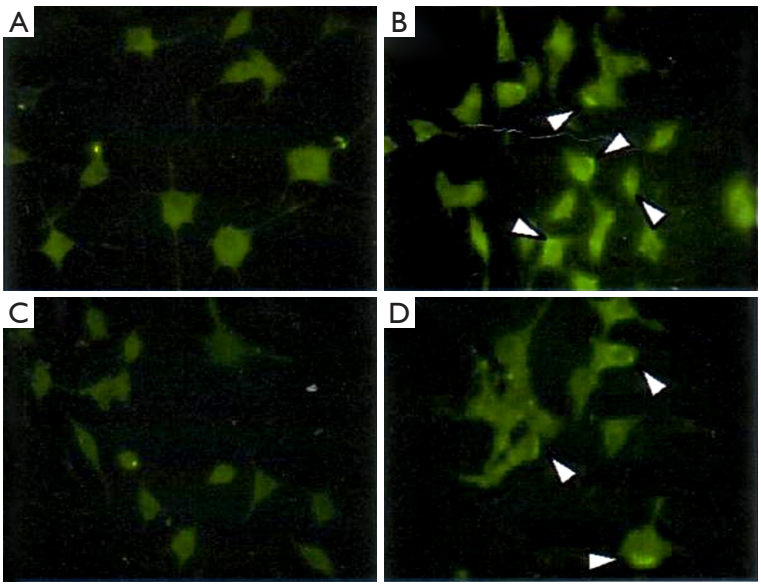


Figure 5 Annexin V staining of PC-12 cells exposed for 3 hr in serum/glucose-deprived DMEM medium. (A) Control cells in serum/glucose added medium; (B) cells in serum/glucose deprived medium; (C) cells in 10 μM crocin added serum/glucose deprived medium; (D) cells in 10 μM α-tocopherol added serum/glucose-deprived medium, arrows indicating ring-like stains. Olympus CK-40 microscope at 100× magnification analysis. DMEM, dulbecco's modified eagle medium.

the antioxidant activity of crocin using different assay system and the effects of crocin on PC-12 cells in DSG medium comparing with a-tocopherol (9). PC-12 cells were cultured in SGD medium resulted that morphological changes including membrane lipids were occurred whereas intracellular superoxide dismutase (SOD) activity was confirmed to be decrease by survey of Annexin V staining (*Figure 5*). Phosphatidyl serine (PS) residues are unusually sticked to the inner membrane, however under oxidative stress PS externa1ization appears as an ear1y stage of apoptosis. Since the negative1y charged PS attaches to Annexin, a ring-1ike staining occurs by conjugation with fluorescein isothiocyanate (FITC). When PC-12 cells are cultured in secoisolariciresinol diglucoside (SDG) medium, clear ring-1ike staining appears comparing with control cells. Crocin showed the more intact cell morphology than that of α-tocopherol. PC-12 cells cultured in SDG medium for 6 h indicated a 1.8-fold increase of peroxidized membrane lipid contents and a 14-fold increase of SOD activity comparing with control cells. On the other hand, crocin significantly inhibited the formation of peroxidized membrane lipids and the SOD activity compared with the α-tocophero1 activity as shown in *Figure 5*. The decrease of SOD activity indicated that crocin has an important duty for modu1ating antioxidative activities and for inhibiting the activation of caspase-8 released by DSG medium concentration-dependently in the concentration of 0.1–10 μM. Crocin did not affect caspase-8 activity in cell lysates and its inhibitory effects might be indirectly mediated by its antioxidant activities (9).

Increase of intracellular glutathione levels in serum glucose deprivation PC-12 cells through an increase in the activities of glutathione reductase and c-glutamyl cysteinyl synthase by crocin

It was known that the decrease of glutathione (GSH) levels gave apoptosis by the oxidative stress (69). GSH levels in PC-12 cells cultured for 3 h in SGD medium reduced to approximately half comparing with control cells and kept constant then after. However, the addition of crocin in the medium raised levels of intracellular GSH dose-dependently and its level continued for 3 h. A 10 μM of crocin indicated the highest levels of intracellular GSH. The GSH levels were satisfactory for inactivation of N-SMase. GR activities of PC-12 cells in the SGD medium reduced time dependently, however a 10 μM crocin addition promoted the activity of GR around a 4-fold increase at 6 h. This finding shows that crocin has no major outcome

on the GPx activity in cells. GSH synthesis is regulated by the rate-limiting enzyme c-GCS which may be controlled by different pathways. TNF-α and IL-lβ promote c-GCS activity associated with an increase of mRNA expression in mouse endothelial cells. IL-6 also induces the expression of c-GCS mRNA and increases its activity, reaching to increased GSH levels in PC-12 cells. NGF accelerated the c-GCS activity at the transcription level by prolonging the half-1ife of c-GCS mRNA. Addition of 10 μM crocin promoted a two time increase of c-GCS mRNA expression in PC-12 cells incubated in SGD medium although it did not affect on the mRNA levels of control PC-12 cells. The crocin-mediated increase in expression of c-GCS mRNA relates an increase of this enzyme activity in cells. From these findings it is suggested that crocin can increase the levels of GSH by increasing the incubation of DMEM (50).

Proteins in central nervous system

The neuroprotection was associated with several proteins and/or enzymes such as acetylcholine esterase inhibition (28), inhibition of β-amyloid secretion (29) and improving β-amyloid-induced behavioral damage (70-73), ERK (74), AMPA receptor (74), NMDA receptor (13), CaMKII (74,75), promotion of energy metabolism in neurons via protection of mitochondrial damage (30) and SIRT 1 protein activation (31,32). We found that LTP induced by strong tetanic stimulation in the presence of 20 μM crocin and 75 μM ethanol was significantly bigger than that of the presence of 75 μM ethanol alone (12). This phenomenon indicated that crocin attenuated the action of ethanol. The strength of LTP was proportional to the sugar numbers conjugated to crocetin indicating that crocin was significantly strong, and reduced to crocin-2, -3 and -4 (15). This tendency is the same with saponins such as ginsenosides (21), saikosaponins (22) and cardiac saponin (20). Gene disruption of CaMKII in animal clearly inhibited memory and LTP occurrence resulted that CaMKII and LTP are closely related (76). Moreover, serious intellectual disability in human being was occurred by the genetic mutation of CaMKII (77). Therefore, the relation between LTP and CaMKII was suggested that the induction of Ca^{++} into cells activated CaMKII of which Ca^{++} moved to synapse and bound to the NMDA receptors and prepared LTP by mainly phosphorylating and accomplice subunits of AMPA receptors. Adabizadeh *et al.* investigated that crocin improved spatial memory inhibited by hyoscine, and NMDA, AMPA, ERK and CaMKII were analyzed by Western blot resulting in no change except ERK. The authors suggested that crocin only affected the elevation of ERK level in rat hippocampus (74). However, Chang *et al.* found that CaMKII was activated but the stimulation term was very short just about 1 min (78). From these evidence, the change of CaMKII stimulated by crocin can not be detected contrary LTP continued for 1 hr or more (13). Learning/memory and LTP behaviors are detected easily, but the transmitter and/or enzyme reaction are sometimes no detection although they are closely associated together. So that it is needed to confirm the pharmacological activity and the enzymatic activity and/or the transmitter activity together.

Conclusions

Saffron possesses a wide spectrum of pharmacological activities as crocin increases the intracellular glutathione levels and prevents cell death in serum-deprived and hypoxic PC-12 cells by its antioxidant properties. The antioxidant potential of crocin has been previously described in association with a variety of neuroprotective properties. In PC-12 cells, generation of ROS activates neutral SMase to generate ceramide resulting in cell death while glutathione directly inhibits the activation of the SMase. It is hypothesized that crocin could prevent the activation of N-SMase in PC-12 cells growing in SGD medium by a GSH-dependent inhibition mechanism. Ceramide release activates the caspase family as previously discussed as shown in *Figure 6*.

As discussed previously the neuronal enzyme reaction related to for example LTP is sometimes no detection although they are closely related. This evidence indicated that both *in vitro* and *in vivo* investigations are necessary for understanding real function of crocin. Therefor it is needed to confirm the pharmacological activity together.

We have also discovered that crocin can have an effect on cerebral infarction area caused by middle cerebral artery (MCA) occlusion in mice as indicated in *Figure* 7 (10). It is suggested that apoptosis causes neuronal cell death after acute brain ischemia although the mechanism is not completely evident (79). This evidence also supports the correlation of saffron and/or crocin with brain health supported by that crocin prevented neuronal cell death *in vitro* as described previously and MCA in mice via neuronal cell death which may be started from apoptosis *in vivo*. As previously discussed crocin prevented neuronal cell death *in vitro* and we found the prevention of MCA

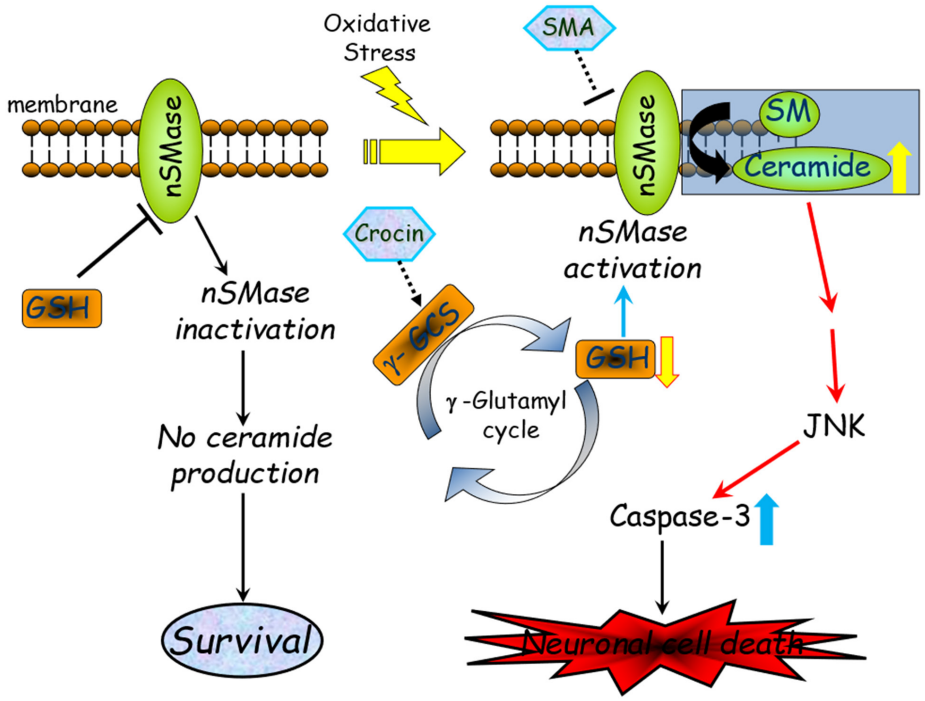


Figure 6 Neuroprotective function of crocin in PC-12 cells.

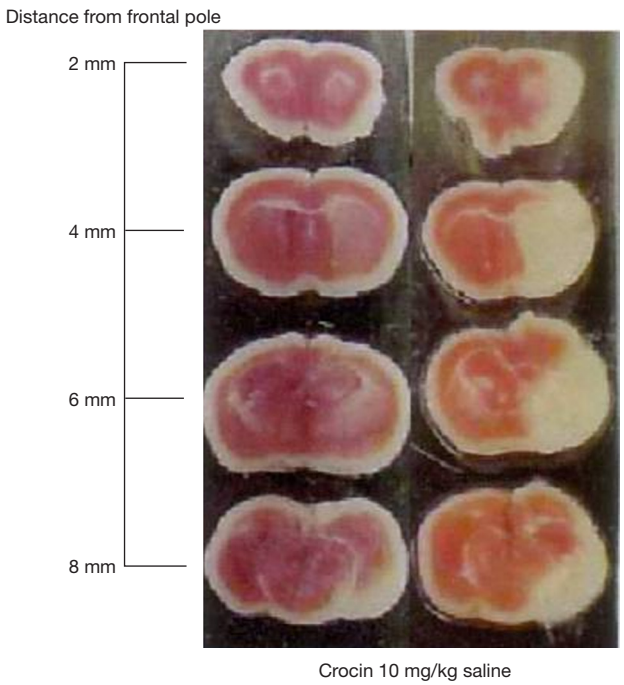


Figure 7 Cerebral infarction prevention by crocin. Left indicates crocin 10 mg/kg administration, right is addition of saline as a control.

by administration of crocin in mice. This result suggested that the combination of *in vitro* investigation which may cover a wide range of mechanism and *in vivo* experiments which shows the exact phenomena is necessary. From this suggestion we reviewed *in vitro*, *in vivo* and clinical trial related to neurological disorders using saffron and/or crocin.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://lcm.amegroups.com/article/view/10.21037/lcm-22-4/coif). The series "Multifunctional Saffron" was commissioned by the editorial office without any funding or sponsorship. YS served as the unpaid Guest Editor of the series. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This article is not concerned with any animal investigation and clinical trial, only limited to publication analysis.

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