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REVIEW ARTICLE

Review on Pharmacological Active Saffron and Crocin

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ABSTRACT

Saffron has been used for medicinal, coloring, aroma and flavoring purposes for thousands of years. In recent years, research on the pharmacological activity of saffron has become increasingly active, with particular attention being paid to saffron's anti-cognitive activity. Saffron is known to contain a number of components, among which crocetin glycosides, i.e. crocin has been found to be the active substance. Anti-crocin monoclonal antibody having wide cross-reactivity was prepared and carried out immunostaining using PC-12 cells to confirm the uptake of crocin into the cells. Saffron extract and/or crocin ameliorated learning and memory performance inhibited by ethanol in mice. Long term potentiation blocked by ethanol shows recovery by the addition of saffron extract and/or crocin to mouse hippocampus tissues. Mice injected with crocin expressed non-rem sleeping even during an activating period. The clinical trial of saffron extract ameliorated symptoms in Alzheimer's patients. A Chinese formula prescribed with saffron reversed brain vascular dementia. PC-12 cells were used to study the mechanism of cell death by apoptosis. Crocin showed strong anti-oxidant and anti-inflammatory activities. Crocin decreased the level of ceramide by inhibition of sphingomyelinase activity and increased glutathione concentration resulting in inhibition of apoptosis. From these findings saffron and crocin can potentially be applied for treatment and prevention of dementia in patients.

Keyword: Crocus sativus, crocin, quality control, anti-cognitive activity, non-LEM sleeping.

1. Introduction

Dioscorides, Materia Medica, states that saffron is the fresher the better, and is used for hangovers, for poor circulation and uterine medicine, for bowel movements and as a tonic. It was also used as an aphrodisiac in Greek times. Saffron was introduced to China from India around the 13th century and has been used medicinally until today. Saffron was described by Li Shi-zhen in his book 'Honcho-net-me' as "to make use of the blood when the mind is depressed and anxious and cannot be dissipated, and to make the spirit happy if taken for a long time". It has been used since ancient times as an herbal medicine for depression, breathing problems, haematemesis, chills, hysteria, fear, ecstasy, female menopause, postpartum haemorrhage and abdominal pain. In Japan, it is commonly used as a raw material for home remedies for sedation, analgesia and menstruation. It is also cultivated for use as a dye. While field cultivation has been practiced throughout the world since recorded history, a unique indoor cultivation method was developed in Oita Prefecture, Japan, about 110 years ago (Fig. 1-1), and has been applied until today.

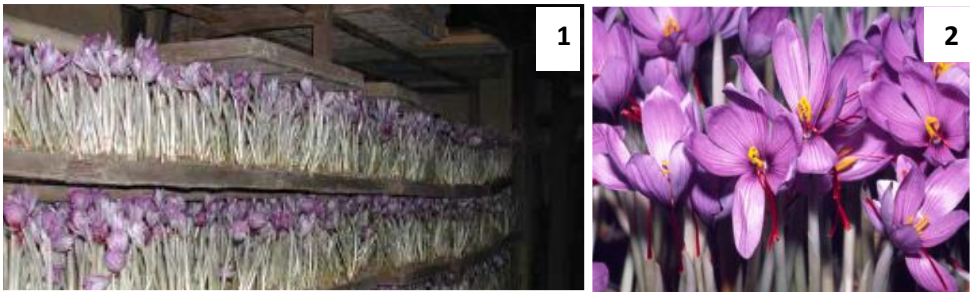


Fig.1 Indoor cultivation system and flowering of saffron

The three pistils of the flower (Fig.1-2) are collected and dried to produce saffron. 5 kg of pistils are harvested from 90,000 to 100,000 saffron flowers, which are about 1 kg when dried. Because of this, saffron is a very expensive natural medicine.

Approximately 150 constituents have been determined in saffron, such as terpenoids, amino acids, anthraquinones, alkaloids and others. Among them crocin, safranal, picrocrocin and crocetin are major constituents. [1] With regard to illnesses dementia has recently become a major social issue worldwide. The type of dementia is clearly indicated in "The Diagnostic and Statistical Manual of Mental Disorders" specified dementia. [2] The rapid increase in the number of people with dementia in recent years has created a major crisis worldwide. To give an example of the estimates in Japan, the number of sensitized persons is expected to exceed 10 million in 2050, compared to 7.3 million in 2025 (Fig. 2). [3] This situation is seen all over the world. The ratio of the number of people with dementia to the global population aged 60 and over is higher in Europe, the USA and Japan, at more than 6%, and lower in Asia and African countries, in the 3% range. [4] We consider it an important matter to investigate the factors behind this. In fact, epidemiological studies on lifestyle and eating habits have shown that wine [5] or fish [6] reduce Alzheimer's disease (AD) in older people. For these reasons, there is a strong need for methods and natural products for the prevention of AD all over the world. AD is the most common form of dementia in older people, accounting for 30% of cases, and is estimated to affect 33 million people worldwide. It is generally accepted that AD is influenced by inflammation and oxidative stress in the brain, which leads to the accumulation of amyloid-β (Aβ) plaques and phosphorylation of tau protein in the brain, resulting in necrosis of cranial nerves and the development of dementia. AD generally increases with age, but as described above, it is also clear that eating habits and lifestyle are associated with the dementia development. Research into natural products with anti-cognitive activity, particularly those with acetylcholinesterase inhibitory activity, has been widely developed and many active components have been identified. [7,8] Nevertheless, the only natural product with a medicinal use as an acetylcholinesterase inhibitor is galantamine, which was first isolated from *Galanthus caucasicus* and *G. woronowii*. [9]

In this review, the components of saffron, the stability of its main active ingredient crocin, the preparation of monoclonal antibodies (MAbs) against crocin and its applied research, the memory learning and long-term potentiation effects of saffron and crocin, the non-REM sleep effects of crocin, clinical cases of saffron in AD patients and the elucidation of the mechanisms of the anti-cognitive activity of crocin will be discussed.

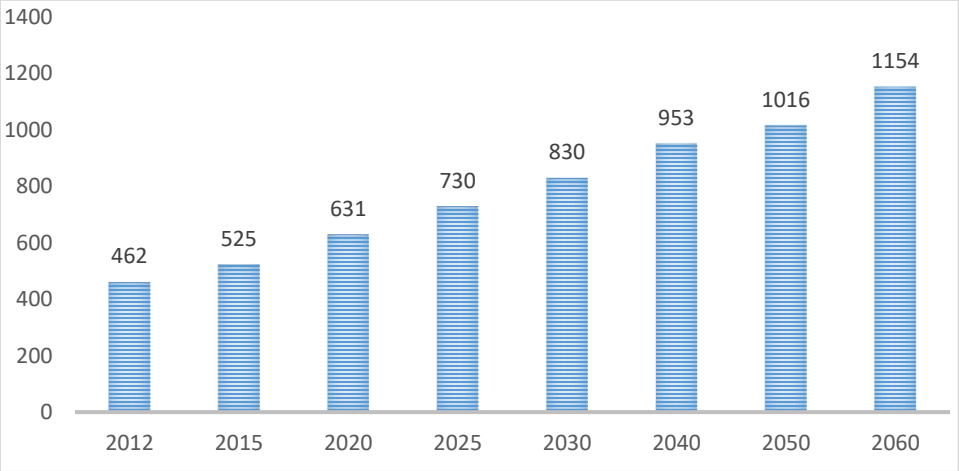


Fig. 2. Increases of dementia patients in Japan

2. Constituents of saffron and stability

Nearly 150 components have been confirmed in saffron [10], such as terpenoids, amino acids, anthraquinones, alkaloids and others. Also many papers indicated that saffron contained crocetin glycosides [11, 12, 13, 14, 15], picrocrocin [14,16] and safranal [13, 14, 16] as major constituents exhibiting red color, characteristic bitterness, and distinctive saffron odor, respectively (Fig.3). Among them crocins 1–4 [11, 13, 14] are marker compounds for quality control and standardization of saffron [11, 17] because the authors previously showed in vivo experiments that crocin promotes memory learning [18] and *non-rapid-eye-movement* (non-REM) sleep effects. [19]

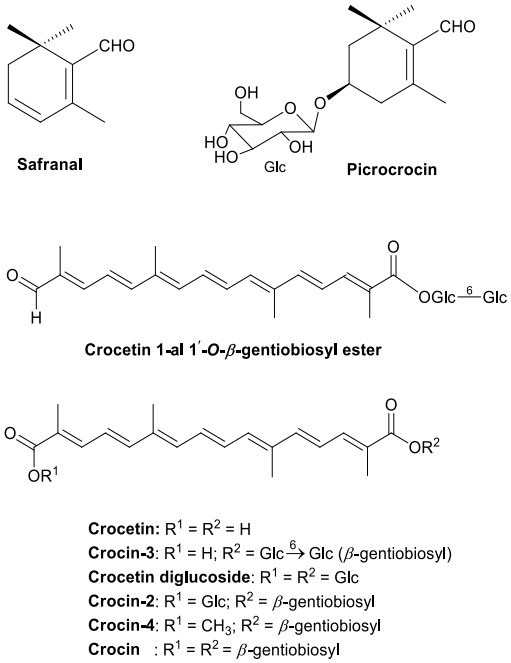


Fig.3 Major constituents of saffron

Saffron was stored under the combining conditions in the long-term storage period resulting that the existence of air, light irradiation and higher temperature promoted the degradation of crocin. [17] Also several researches indicated that saffron was sensitive to light, heat, oxidation and pH condition. [20, 21, 22] More recently Alehossseini et al. investigated the stability of saffron extract through encapsulation in zein protein. [23] Moreover, Morimoto et al. confirmed that inner β-glucosidase is stable and reactive under moisture condition, and cleavages the glycoside linkage in crocetin glycosides resulting in crocin 4 via crocins 2 and 3 indicated the degradation steps of crocin by inner β-glucosidase. [17]

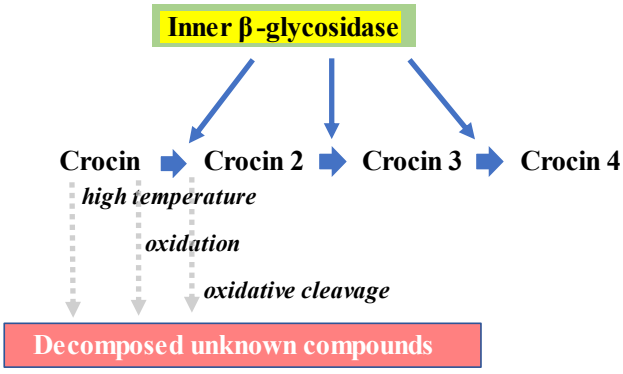


Fig.4 Degradation step of crocin

The activity of crocin with the highest number of glucose on crocetin is the most potent, and decreases as the number of glucose decreases, i.e., crocin 2, crocin 3 and crocin 4 (Fig. 4) as will be discussed later on long-term potentiation (LTP) [24]. This tendency was found in the biologically active natural saponins such as cardiac steroid saponin [25], ginseng saponin [26] saikosaponin [27] and haemolytic saponin [28]. From the results saffron and saffron extractives should be stored under cool and dry condition until use and assessed the exact crocin concentration before use because crocin in saffron

is changeable as already discussed. [17] Therefore, the quality control of saffron and its extractives is necessary for in vitro, in vivo and clinical investigations.

3. Preparation of monoclonal antibody against crocin and its incorporation into cells

Many MAbs against pharmacologically active natural products having small molecule, nearly 40 compounds such as phenolics including marihuana component, phenolic glycoside including sennoside A, B, naphthoquinone, terpenoid like forskolin and artemisinin, terpenoid glycosides including paeoniflorin, steroidal glycosides including ginsenosides, glycyrrhizin, solasodine glycoside have been prepared for their standardizations and quality controls as previously reviewed. [29] New assay system using MAb, eastern blotting system was newly developed [30] and applied for pharmacologically active compounds. [29] MAb preparation against crocin was performed as a part of the MAb research against the above pharmacologically active natural products.

Three crocin-carrier protein conjugates were synthesized and their hapten numbers were determined by matrix-assisted laser desorption/ ionization time of flight mass spectrometry. [31] Three MAbs against crocin were produced by hybridomas fused with the splenocytes immunized with crocin hemisuccinate-bovine serum albumin conjugate and hypoxanthine-aminopterin-thymidine-sensitive mouse myeloma cell line, P3-X63-Ag8-653. They were identified as IgG2a and IgG2b possessing λ light chain, respectively. [32]

Although anti-crocin MAb is much more sensitive than thin-layer chromatography (TLC) and High Performance *Liquid Chromatography* (HPLC) analysis [17], the enzyme-linked immuno-sorbent assay (ELISA) was not specific for crocin. However, it did not react with monoterpenoids, carotenoids and sugars. This wide reactivity is the main advantage of the MAb used in this ELISA. It is better than a special antibody for the metabolic study on crocin and the study of pharmacologically active mechanism of crocin on LTP in the central nervous system (CNS). It is suggested this MAb should be helpful to the further study on the presumable receptor in the brain.

In order to confirm the incorporation of crocin and its localization in PC-12 cells, cells were immunostained using the anti-crocin MAb. Fig.5 shows the cell staining with anti-crocin MAb. No clear cell staining was observed 15 minutes after the addition of crocin, but incorporation of crocin after incubation of 30 and 60 min into cells was clearly observed comparing with control cells. [33]

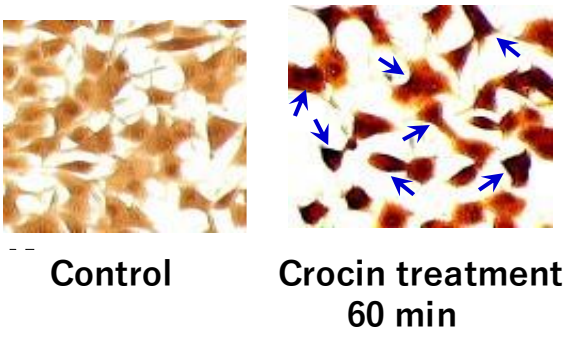


Fig.5 Immunostaining of crocin in PC-12 cells

4. Ameliorative effects of saffron and crocin on alcohol-impaired memory learning and long-term potentiation in vitro and in vivo

Although ethanol, acetaldehyde, scopolamine, hyoscine or amyloid β (Aβ) and so on is used as an inhibitor in memory learning and LTP experiments, ethanol is generally used because of its high sensitivity and convenient weighing. The saffron extract (SE) activity on memory in aged mice was associated with the antioxidant activity. [34] Roustazade et al. found that the SE dose reflects the activity of brain function in stressed rats. [35] The SE were studied on learning and memory in step through (ST) and step down (SD) tests in normal mice as well as in learning- and memory-impaired mice. A single administration of SE had no effects on memory registration, consolidation or retrieval in normal mice. SE (125-500 mg/kg) dose-dependently reduced the ethanol-induced impairment of memory registration both in ST and SD tests and the 30% of ethanol-induced impairment of memory retrieval in SD test. The SE decreased the motor activity and prolonged the sleeping time induced by hexobarbital resulting that SE ameliorate the impairment effects of ethanol on learning and memory processes, and possesses a sedative effect. [36] Since SE showed ameliorative effects on alcohol-blocked memory learning deficits, Morimoto et al. purified by silica-gel column chromatography using activity-guided separation to reach crocin as active principle. This result is easily supposed, given that crocin is a major constituent, around 40% of the SE. [17, 24]

It became evident that no effect on memory possession by a single-oral investigation of crocin in normal mice was found in ST and SD tests, respectively. If crocin was administered 10 minutes

before the ethanol injection, the latency in the ST test dose-dependently increased compared with that of ethanol-injected mice. The investigation of 200 mg/kg crocin increased the number of successful mice decreasing the error numbers compared to the ethanol-treated group in the test trial. [18]

Since LTP is an important phenomenon related to short-term memory, Sugiura et al. evaluated the activity of SEs in the rat hippocampal using ethanol as a LTP inhibitor. LTP does not occur when SE alone is administered orally. On the other hand, the administration of SE prior to ethanol administration clearly reduced the inhibition of LTP expression by ethanol. SE at 125-250 mg/kg was found to exhibit dose-dependent manner. [37]

Next Sugiura et al. evaluated the activity of crocin in the dentate gyrus of rat hippocampal slice using ethanol as a LTP inhibitor. LTP is significantly suppressed by 50-75 nM alcohol administration. This suppression of LTP by alcohol was found to be dose-dependently enhanced by the pre-administration of crocin 10-30μM 5 min before alcohol administration although crocin alone did not attenuate the LTP. [38]

5. non-REM sleep by crocin

Mice have a habit of being active at night. The authors took advantage of this habit to test the non-REM sleep-inducing effects of crocin. The electroencephalogram (EEG) of mice pre-administered with crocin was measured using an EEG measuring device, and the results showed that non-REM sleep was apparently induced by the administration of at least 10 mg/kg crocin even at 8 pm (Fig. 6).

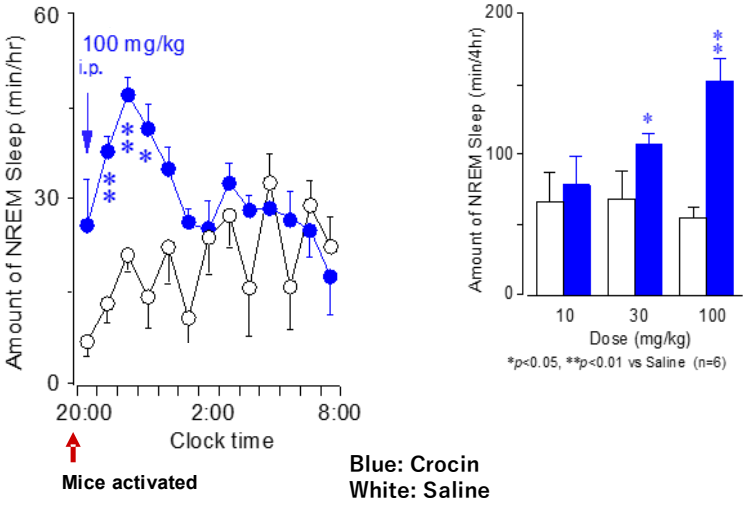


Fig.6 Non-REM sleeping by crocin

The intensity of non-REM sleep increased dose-dependently by the administration of crocin (10-100 mg/kg). It became also evident that the sleep effects of crocin were not due to inhibition of exercise. [19] As previously indicated the SE prolonged the sleeping time induced by hexobarbital by a sedative effect. [39] These results indicate that saffron and crocin induces good sleep. [40, 41]

The combination of Kampo medicine and saffron can be used for several clinical departments in Japan. For instance, 100 mg of saffron was received daily for sleep disturbance patients, combining with Kampo prescriptions such as Sansoninto formula (prescription: Poria sclerotium, Tibet sweetflag rhizome, Anemarrhena rhizome, Licorice root and Jujube seed), Saikokaryukotuboreito formula (prescription: Bupleurum root, Pinellia tuber, Cinnamon bark, Poria sclerotium, Scutellaria root, Jujube, ginseng, Oyster shell, Longgu and Ginger), and Daijyokito formula (prescription: Magnolia bark, Immature orange and Rhubarb). [42] These results might be closely related to the non-REM sleep effects of crocin. [19]

6. Clinical studies of saffron and its prescribed formula

Saffron has been used as herb medicine, spice and pigment for thousands of years [43] and is admitted in Generally Recognized as Safe (GRAS) by the American Food and Drug Administration (FDA). [44] This message indicates that saffron and crocin are safe herb medicine without adverse effects and can be used in clinical trials.

Forty-six AD patients were treated with SE capsules 15 mg twice daily and placebo as well. After 16 weeks, the saffron group showed clear improvement in cognitive function. No side effects were observed, as was the case with placebo. The results concluded that short-term administration of SE is safe and effective for patients with mild to moderate AD. [45]

A 22-week clinical trial was conducted on 54 patients with AD using the Cognitive subscale and clinical dementia rating scale. Fifteen mg of SE was administered twice daily, and 5 mg of donepezil was administered twice daily as a positive control. The results concluded that the SE 22-week treatment was as effective as donepezil in treating mild to moderate AD. Nausea was also observed in saffron extract as a side effect, but more frequently than in donepezil. Based on the above, SE is useful as a safe therapeutic agent for AD.

Since sleep deprivation and depression have been shown to be closely related to dementia, clinical cases for healthy volunteer will be presented, respectively. The results of sleep effects of SE on healthy subjects were conducted and clearly showed sleep effects.[41] Furthermore, the antidepressant effect of SE on healthy subjects was also studied and found to be effective. [47]

One of the most popular traditional Chinese medicines (TCM) formulas is Sai Luo Tong which is an extract of ginseng, saffron and ginkgo biloba. In clinical trials on dementia, saffron is mentioned above, and ginseng shows anti-dementia activity. [48, 49,50] Alternatively *G. biloba* containing flavone glycosides and diterpene lactones is confirmed to be anti-dementia activity [51, 52] resulting in an OTC medicine for the prevention of vascular dementia in Europe. [53] Therefore, the effect of Sai Luo Tong can be easily supposed. The standardized ingredients of the ginseng ginsenoside 20.46 mg (45.5%), ginkgo biloba flavonoid glycosides 20.46 mg (45.5%), and saffron crocin 4.09 mg (9.1%) are contained in the Sai Luo Tong capsule and the clinical studies have been performed as following. Three hundred and twenty-five patients with cerebrovascular dementia were randomly assigned to the high-dose group (360 mg/day), the low-dose group (240 mg/day), or the placebo group for 52 weeks and evaluated using the VaD Assessment Scale-cognitive subscale score. The results showed that the placebo group had higher cognitive function than the low-dose group. The results showed that the high-dose group was more effective than the placebo group, indicating that Sai Luo Tong was effective in treating cerebrovascular dementia. [54]

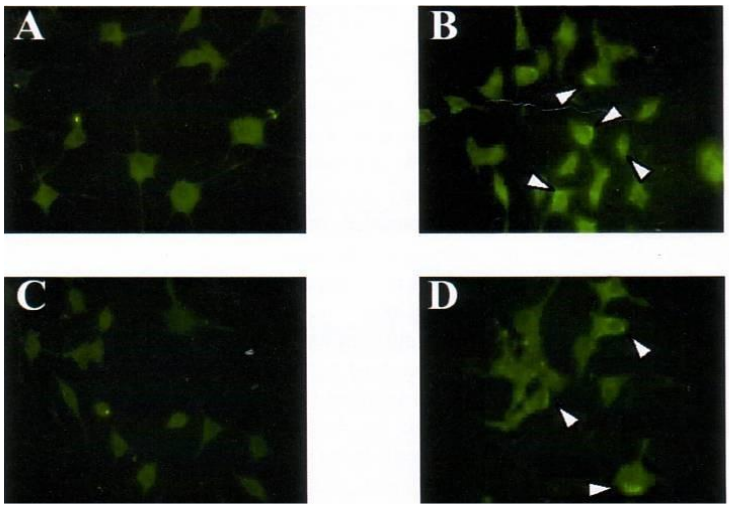
As the other prescription of saffron Singaporean pharmaceutical company fully incorporated the aforementioned saffron research data by the authors to prepare Hul Tong Jiao Tong capsule (慧通胶囊), which is a combination of Ding Zhi Wan, a Chinese medicine prescribed with Ginseng, Tibet sweetflag rhizome, Polygalae radix, Poria sclerotium and saffron for the prevention of dementia, and approved as an OTC drug in 2018 as shown in Fig.7.

Fig.7 Anti-cognitive Chinese medicine prescribing saffron

7. Mechanism of anti-cognitive activity for crocin

1) Inhibitory effect of crocin on morphological changes and membrane oxidation of PC-12 cells in serum and glucose-free medium

The effect of crocin on PC-12 cells under serum and glucose-free conditions was compared with that of α-tocopherol. Fig.8 shows the effect of crocin on the morphology and membrane lipid oxidation of PC-12 cells under serum and glucose-free conditions, as well as the reduction of intracellular supeoxide dismutase (SOD) activity. Phosphatidylserine (PS), normally present in the endothelium of cells, is translocated to the outer membrane of cells by oxidative stress, and this translocation is considered an early signal for apoptosis induction. FITC-bound AnnexinV binds to negatively charged PS and exhibits a ring-shaped fluorescence. PC-12 cells under serum and glucose-free conditions (B) clearly show ring-shaped fluorescence compared to A. No ring fluorescence is observed in cells (C) in medium with 10 μM of crocin. 10 μM of α-tocopherol (D) detects rings, although the number is small. It is clear that the cells in the crocin-added medium maintained a more normal state when compared to those in the α-tocopherol added medium. [55, 56]



A: control cells in serum/glucose positive medium B: cells in serum/glucose negative medium C: cells in serum/glucose negative plus 10 μM crocin D: cells in serum/glucose negative plus 10 μM α-tocopherol Allows show oxidized membrane

Fig.8 Effect of crocin on morphology and membrane lipid oxidation of PC-12 cells

Oxidative stress is known to cause many brain disorders, and is deeply involved in AD, Parkinson's disease, and Hutchinson's disease as diseases of the CNS. Since the antioxidant effect of crocin is stronger and safer than that of α-tocopherol, it is considered to be applicable to neurological disorders, and further animal and clinical experiments are desirable. Recent studies have shown that apoptosis is induced by oxygen deficiency in the brain and brain damage, and that apoptosis also occurs in the brain of AD patients. Therefore, the inhibition of apoptosis of brain neurons by natural products is strongly required.

2) Induction of apoptosis in serum- and glucose-free medium and inhibitory effect of crocin

With regard to apoptosis of PC12 cells, it is known that removal of serum and nerve growth factor (NGF) causes an increase in ceramide levels inducing apoptosis. PC12 cells remain normal in medium containing serum and glucose, but when serum and glucose are removed from the same medium, 60% of cell death were confirmed by the Trypan blue dye method. In contrast, the addition of 10 μM crocin significantly inhibited the morphological phenomenon showed that crocin blocked TNF-α-induced PC-12 cell death resulting in 85% survival compared to control cells. [57]

3) Inhibitory effect of crocin on ceramide production in serum- and glucose-free medium

Serum deprivation increased intracellular ceramide levels in HN9.10e cells resulting apoptosis. This finding suggests that a similar phenomenon in PC-12 cells will be occurred. In fact, the ceramide concentration significantly increased like as 3.5-fold by culturing in serum and glucose deprivation medium compared to the control medium. [58] Sphingomyelinase acts on sphingomyelin to produce ceramide, and sphingomyelinase activity in PC-12 cell homogenates was measured, reaching a maximum at 1 hr and decreasing to control levels

at 3 hr. Next, sphingomyelinase activity increased markedly under serum- and glucose-free conditions, but the addition of crocin decreased the activity in a dose-dependent manner. However, no direct effect of crocin on sphingomyelinase activity was observed. [55] The SAPK/JNK signaling system and the sphingomyelin pathway might be conjugated to give a mixed function by stress in U937 and BAE cells [59] resulting that environmental stress conditions activated the stress-activated protein kinase SAPK/JNK cascade in PC-12. Rukenstein et al. found that serum and glucose deprivation medium activated JNK phosphorylation in PC-12 cells nearly 4-fold when compared with the control cells. [60]

4) Inhibitory effect of crocin on the decrease of glutathione (GSH) concentration in serum and glucose-free medium

GSH levels in PC12 cells under serum and glucose-free conditions were measured. GSH levels were also measured under crocin- and crocin-free conditions. 3 hr of treatment of PC12 cells under serum- and glucose-free conditions resulted in half the concentration of GSH compared to the control. On the other hand, the addition of crocin increased GSH levels in a dose-dependent manner, and GSH levels remained higher than those of controls after 3 hr. Furthermore, the effect of crocin on glutathione synthetase was investigated resulted that crocin increased GSH levels by activating the enzymes. [61]

8. Conclusion

Saffron is an extremely safe natural medicine that does not cause toxicity even when administered to mice at 5 g/kg. [62] It became evident that crocin ameliorates alcohol-induced inhibition of memory learning and LTP suppression as previously mentioned and the inhibitory effects of ethanol via N-methyl-D-aspartate (NMDA) receptors in hippocampal neurons resulting that crocin may improve memory learning based on the above action. [63] Crocin protects brain neurons by inhibiting antioxidant stress, inhibiting ceramide production, promoting GSH production resulting in inhibiting apoptosis activity. Furthermore, crocin inhibits apoptosis by activating GSH biosynthesis and inhibiting ceramide production. Oxidative stress is well known to cause many brain diseases. It has already been mentioned that the anti-oxidant effect of crocin is stronger than that of α-tocopherol. In addition, the anti-cancer effect in the colon was examined by administering a drug that induces colon cancer to mice pre-treated with crocin, and it was found to be due to the strong anti-inflammatory effect. [64] The anti-inflammatory effect is closely related to cognitive function, and the combination with the strong antioxidant effect of crocin, as indicated previously, may protect nerve cells resulting in the prevention of dementia.

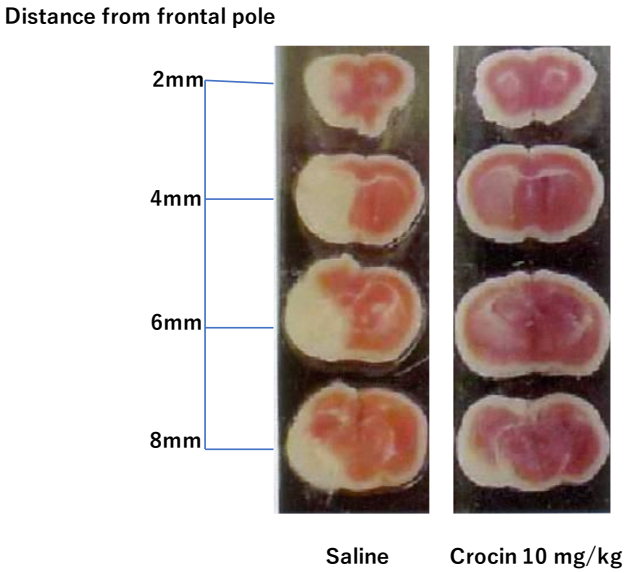


Fig.9 Prevention of cerebral infarction by crocin

Fig. 9 shows the prevention of cerebral infarction by crocin 10 mg/kg, [65] and it supposes that saffron may be effective in preventing dementia of the cerebral circulatory system too. Based on the above, saffron and/or crocin is a useful natural medicine for improving brain diseases. Therefore, saffron including crocin, is expected to be of great clinical interest.

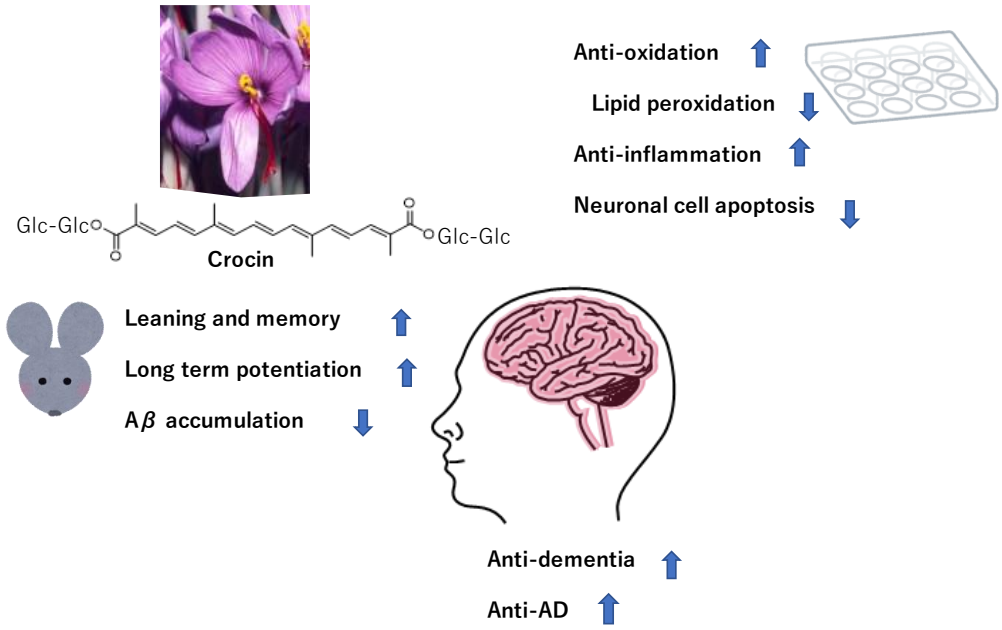


Fig. 10 Mechanism of anti-dementia activity of saffron and crocin

Fig.10 indicated the mechanism of anti-dementia activity on saffron and crocin. As Fig. 10 shows, saffron and/or crocin have many points of action for their anti-cognitive activity, and new mechanisms are likely to be discovered in the future.

This review mainly focuses on studies relating to the anti-cognitive activity of saffron and crocin, but other activities have also been widely reported, and it is no exaggeration to say that saffron is a multifunctional natural medicine, as it contains safranal and picrocrocin, which have been reported to have various pharmacological activities. [66]

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