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Antioxidant effect of crocin on scopolamine-induced memory deficits and oxidative stress in experimental animal model

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Abstract:

Alzheimer's disease (AD) is a term of neurodegenerative diseases that has characteristic clinical and pathological symptoms. Natural substances are thought to offer distinct biological properties. Crocin, a bioactive component of *Crocus sativus*, has a variety of therapeutic effects on the brain. This study aims to investigate whether pre- and/or post-treatment with crocin is sufficient to slow down or halt the progression of Alzheimer's disease (AD). Fifty-six adult male rats were divided into seven groups: GI: control, GII: Crocin (50mg/kg b. wt.), GIII: crocin (Cr) (50mg/kg b. wt.) + scopolamine (Sc) (3mg/kg b. wt.), GIV: scopolamine (Sc) (3mg/kg b. wt.), GV: scopolamine (Sc) (3mg/kg b. wt.) + memantine (M) (10mg/kg b. wt.), GVI: scopolamine (Sc) (3mg/kg b. wt.) + crocin (Cr) (50mg/kg b. wt.), and GVII: scopolamine (Sc)+ memantine (M) + crocin (Cr) (3 mg/kg b. wt., 10 mg/kg b. wt. and 50mg/kg b. wt., respectively). AD rats exhibited an obvious rise in lipid peroxidation (LPO) levels and a significant decline in superoxide dismutase (SOD) and catalase (CAT) activity in the homogenate of the brain hippocampus. Pre- and/or post-treatment of crocin exhibited a significant decline in LPO and a noticeable rise in SOD and CAT. Additionally, Bielshowsky silver stain showed an increase in tau protein deposition in section from scopolamine group, whereas treatment with crocin, memantine and their combination revealed a reduction in tau deposition. The perception that Alzheimer's disease (AD) is pharmacologically unresponsive is gradually shifting. Emerging evidence suggests that treatment with crocin –either alone or in combination with memantine – can lead to improvements in the symptoms of this debilitating condition.

Keywords: Alzheimer's disease, Crocin, Memory deficits, Oxidative stress

1- Introduction

Alzheimer's disease is a destructive neurodegenerative disease **(Alzheimer's Association Report, 2016; Association, 2019; Kochanek et al., 2019)**. AD is categorized by reduced spatial orientation, memory failure, agnosia, mood disturbance, language disturbance, and other neuropsychiatric and mental tasks that decrease the capability to perform the activities of daily life. AD progression results in changes in the brain, as a reduction in the transfer of information leading to the death of neurons **(Alzheimer's Association Report, 2016).**

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A wide range of strategies is used to induce AD in animal models nowadays, including the use of natural aging processes, transgenic AD mice, various surgeries to obstruct cerebral arteries and the administration of several types of toxic substances. Scopolamine is an extensive model used to study dementing-related disorders as it can induce cognitive and memory deficits. Scopolamine is used in numerous studies as an excellent behavioral model to study disorders of dementia such as AD **(Zhang et al., 2017; Demirci et al., 2017)**.

To date, no conventional therapy is presently accessible to treat AD. Currently, most drugs on the market are single-target drugs. These medications have modest effectiveness and severe side effects **(Forchetti, 2005; Rodda and Walker, 2009; Pitsikas, 2015).** Memantine, an N-methyl D-aspartate (NMDA) receptor antagonist, approved is the only anti-AD drug for the treatment of AD on the market that targets just the glutamatergic system. It is an NMDA receptor non-competitive antagonist that prevents glutamate overstimulation. It regulates the Ca^{++} influx and departure from the cell membrane via NMDA receptors **(Wang and Reddy, 2017).**

Natural substances are thought to offer distinct biological properties, such as antioxidant, antiapoptotic, and anti-inflammatory properties **(Elufioye et al., 2017; Angeloni and Vauzour, 2019).** Saffron, known as red gold, used in traditional medicine **(Shokrpour, 2019).** Crocin, a metabolite of saffron, has a variety of therapeutic properties including antioxidant, anticancer, anti-atherosclerotic, and genoprotective properties. Neuroprotective characteristics of crocin have been recognized in cases of cognitive damage **(Mohamadpour et al., 2013; Sebastin Santhosh et al., 2013).**

Crocin also improves memory by eliminating free radicals efficiently, decreasing the production of per-oxidized lipids, and returning SOD activity **(Hosseinzadeh et al., 2012).** Because of its antioxidant effect, crocin successfully protects hippocampal neurons thereby enhances spatial cognitive skills **(Hosseinzadeh et al., 2012; Finley and Gao, 2017; Ahmed et al., 2020).** Also, crocin prevents stress-induced deficits in spatial memory via improving SOD and GPx activities **(Mecocci et al., 2014; Justice, 2018; Bisht et al., 2018).**

Therefore, evidence from animal models challenges the earlier expectations about prooxidants and antioxidants in the processes of AD. The present study aims to evaluate the action of crocin against oxidative stress induced by scopolamine.

2-Materials and Methods

2.1-Chemicals:

Scopolamine (product no. 37022) was bought from Fluka BioChemika Co. USA, and crocin (product no. 17304) was bought from Sigma Aldrich Co, USA. Memantine hydrochloride 10 mg was purchased as an Ebixa drug from Rottendorf Pharma GmbH, Germany. Lipid peroxidation (product no. MD 2529), catalase (product no. CA 257), superoxide dismutase (product no. SD 2521), and total protein (product no. TP 2020) kits were gotten from BioDiagnostic Co, Egypt. The other chemicals are of the best purity available.

2.2-Animals: Fifty-six adult male albino rats (120 ± 20 g) attained from the Serum and Vaccine Lab - Farm of Helwan . Rats were held in a fit-air clean cage kept under a 12 h:12 h schedule of light-dark set at 25 ± 2ºC with a humidity of 50 ± 5%. All experimental practices were authorized by the Animal Ethical Committee. **Ethical approval code:** ASWU/ 05/SC/ZO/24- 01/08

2.3-Experimental design: The rats were erratically split up into seven groups (n = 8). They received daily treatments for 28 consecutive days and were assigned to the following groups based on their respective administrations:

Group I: **Control Group** – Rats were administered saline only via intraperitoneal (IP) injection.
Group II: **crocin (Cr) Group** – Rats were orally administered crocin at a dose of 50 mg/kg **(Ghofrani et al., 2022)** for 28 consecutive days.
Group III: **Cr+Sc** (**Protective) Group** – Rats were orally administered crocin (50 mg/kg) for 28 days and scopolamine (3 mg/kg) for 7 days, starting from the 21st to the 28th day.
Group IV: **Induced (Sc) Group** – Rats were orally administered scopolamine (3 mg/kg) for 7 days, from day 1 to day 7 **(Biradar, 2020).**
Group V: Sc + M Group – Rats were orally administered scopolamine (3 mg/kg) for 7 days, followed by oral treatment with memantine (10 mg/kg) for 21 days **(Rani et al., 2023).**
Group VI: Sc + Cr (Curative) Group – Rats were orally administered scopolamine (3 mg/kg) for 7 days, followed by crocin treatment (50 mg/kg) for 21 consecutive days.
Group VII: Sc + M + Cr (Combination) Group – Rats were orally administered scopolamine (3 mg/kg) for 7 days, followed by a combination treatment of memantine (10 mg/kg) and crocin (50 mg/kg) for 21 consecutive days.

2.4-Biochemical estimation of LPO levels and CAT and SOD activity:

Levels of LPO, CAT and SOD were estimated in the brain hippocampus homogenate. Lipid peroxidation was evaluated by quantifying malondialdehyde (MDA) levels through the thiobarbituric acid reactive substances (TBARS) assay, pink chromogen detectable at 534 nm **(Ohkawa et al., 1979)**. Catalase (CAT) activity was determined by monitoring the decomposition of hydrogen peroxide at 240 nm. **(Aebi, 1984)**, and Superoxide dismutase (SOD) activity was measured using the nitroblue tetrazolium (NBT) method, where one unit of SOD is defined as the amount of enzyme causing 50% of NBT reduction. The resulting absorbance read at 560 nm **(Nishikimi et al., 1972).**

2.5-Estimation of total protein:

Total protein was assessed with biuret test kits **(Gornall et al., 1949).** This assay relies on the interaction between copper ions and peptide bonds in an alkaline environment, forming a violet-colored complex, which was quantified spectrophotometrically at 540 nm.

2.6-Histochemical examination:

Bielschowsky's silver stain: Nerve fibers were prepared with a silver solution then treated with an ammoniacal silver solution and reduced to a detectable metallic silver atoms. Sections were deparaffinized and washed three times. The slides were immersed in pre-warmed (40 ºC) 10% silver nitrate solution for 15 minutes until the sections converted to light brown color. Slides washed 3 times with distilled water (d.H_2O). Concentrated ammonium hydroxide was added drop by drop to the silver nitrate solution until the formed precipitation is completely clear. The slides were placed in this ammonium silver solution and incubated at 40ºC for 30 minutes or until sections turned dark brown color. The slides were directly immersed without washing in developing working solution for about 1 minute or less. To stop the silver reaction, slides were dipped in ammonium hydroxide solution (1%) for 1 minute. Afterward, the slides were washed 3 times with d.H_2O then placed for 5 minutes in 5% sodium thiosulfate and washed

3 times with d.H_2O. Finally, the slides were dehydrated and cleared out using 95% ethyl alcohol, absolute alcohol and xylene **(Crookham and Dapson, 1991; Mirra et al., 1993)**.

2.7-Statistical analysis:

Data for biochemical analysis were presented as means ± S.E. Variances in means were evaluated using one-way ANOVA, after that the Student-Newman-Keuls T-test using Minitab 19 software. Significance was determined at $p < 0.05$.

3-Results and Discussion

3.1-Levels of Lipid Peroxidation (LPO):

LPO is a renowned example of oxidative injury in cells. Our data revealed a highly significant rise ($p<0.01$) in LPO levels in the Sc-treated group (212.48±9.38) compared with the control group. In contrast, protective and curative groups of crocin reversed the influence of scopolamine, causing a significant decrease ($p<0.001$) in the LPO level, showing values of 121.63±4.5 and 104.74±8.91, respectively. Also, memantine treatment showed a highly significant decline ($p<0.01$) with a value of 151.68±1.78 compared with scopolamine treated rats. In addition, the combination of memantine and crocin induced an extremely significant reduction ($p<0.001$) with a value of 87.72±3.01 compared to Sc-group **(Table 1 and Fig. 1).**

Table (1): The effect of crocin, memantine and their combination on lipid peroxidation (LPO) levels and SOD and CAT activity in homogenates of brain hippocampus of control and differently treated rats.

Groups	Control	Cr	Cr+Sc	Sc	Sc+M	Sc+Cr	Sc+M+Cr
LPO (µmole/mg protein)	106.23±1.3	96.57±5.74*	121.63±4.5$^{\#\#\#}$	212.48±9.38$^{\#}$	151.68±1.78$^{\#\#}$	104.74±8.91$^{\#\#\#}$	87.72±3.01$^{\#\#\#}$
SOD (U/mg protein)	143.3±0.28	149.52±0.23*	149.6±0.28$^{\#\#\#}$	71.391±0.08$^{\#}$	114.62±0.32$^{\#\#}$	141.87±0.21$^{\#\#\#}$	195.16±0.42$^{\#\#\#}$
CAT (U/mg protein)	1.317±0.12	1.236±0.15*	1.124±0.09$^{\#\#}$	0.4789±0.04$^{\#}$	0.895±0.07**	1.065±0.07$^{\#\#}$	1.376±0.06$^{\#\#\#}$

values are presented as means ± S.E. of 8 animals in each group.
*Non-significant compared to control group (P≥0.05).
Very high significant compared with control group (p<0.001).
Highly significant compared with AD group (p<0.01).
Very high significant compared with AD group (p<0.001).
**Significant compared with AD group (p≤0.05).

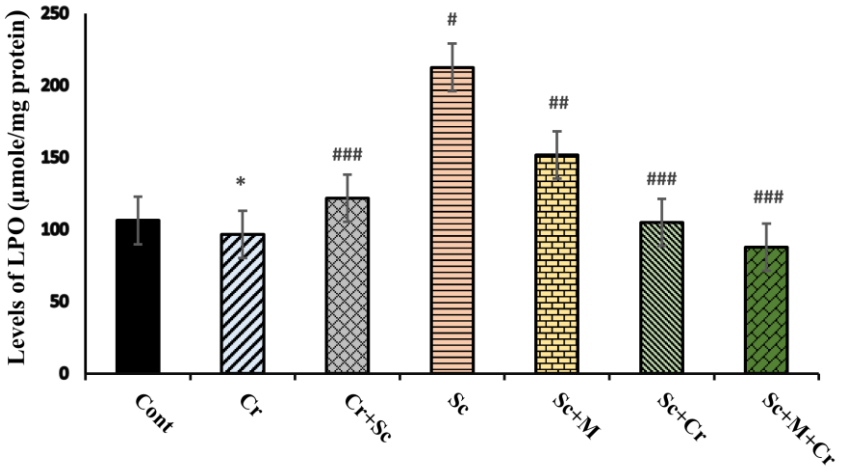

Fig.1: LPO Levels in the brain hippocampus treated with scopolamine (3 mg/kg b. wt.), crocin (50 mg/kg b. wt.), memantine (20 mg/kg b. wt.) or memantine and crocin in combination. Values are means ± S.E.M. of 8 animals in each group. *Non-significant compared to control group (P≥0.05). # Very high significant compared with control group (p<0.001). ## Highly significant compared with AD group (p<0.01). ### Very high significant compared with AD group (p<0.001).

3.2-Activity of Superoxide dismutase (SOD)

The study of SOD activity in the brain hippocampus of AD rat models revealed a significant (p<0.001) decline, showing a value of 71.39±0.083 in Sc-treated rats. On the other hand, protective and curative groups of crocin reversed the effect of scopolamine, recording an extremely significant increase (p<0.001) in SOD level, showing values of 149.6±0.28 and 141.87±0.21, respectively. Additionally, memantine treatment caused a highly significant increase (p<0.01) with a value of 114.62±0.32 compared to scopolamine treated rats. Also, the combination of memantine and crocin showed a significant improvement (p<0.001) with a value of 195.16±0.42 **(Table 1 and Fig. 2).**

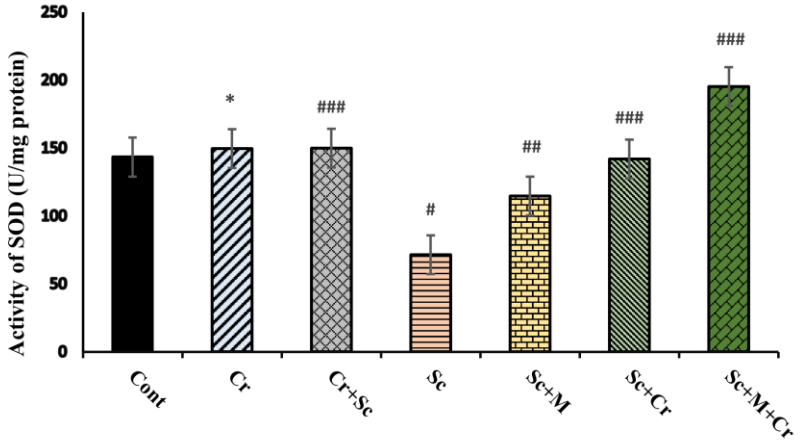

Fig. 2: Activity of SOD in the brain hippocampus treated with scopolamine (3 mg/kg b. wt.), crocin (50 mg/kg b. wt.), memantine (20 mg/kg b. wt.) or memantine and crocin in combination. Values are means ± S.E.M. of 6 animals in each group. *Non-significant compared to control group (P≥0.05). # Very high significant compared with control group (p<0.001). ## Highly significant compared with AD group (p<0.01). ### Very high significant

compared with AD group (p<0.001).

3.3-Activity of Catalase (CAT)

CAT activity displayed a very highly significant (p<0.001) reduction in Sc-treated rats (0.4789±0.04). However, preventive and curative groups of crocin in Sc-treated rats showed a highly significant increase (p<0.01) in CAT activity with values of 1.124±0.09 and 1.065±0.07 respectively, compared with scopolamine group. Also, treatment of memantine alone against scopolamine led to a significant increase (p≤0.05) with a value of 0.895±0.07. Additionally, the combination of memantine and crocin treatment caused a significant increase (p<0.001) with a value of 1.376±0.07 **(Table 1 and Fig. 3).**

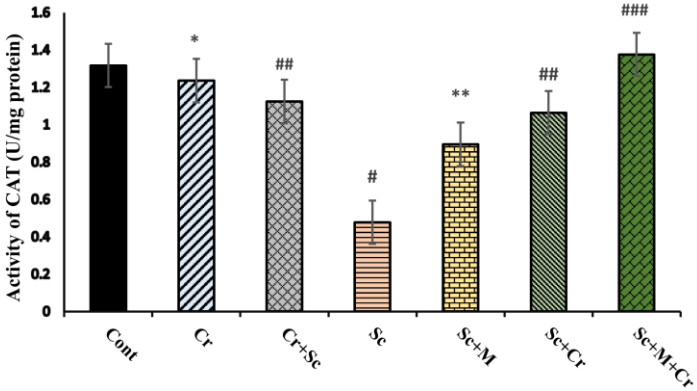

Fig. 3: Activity of CAT in the brain hippocampus treated with scopolamine (3 mg/kg b. wt.), crocin (50 mg/kg b. wt.), memantine (20 mg/kg b. wt.) or memantine and crocin in combination. Values are means ± S.E.M. of 6 animals in each group. *Non-significant compared to control group (P≥0.05). # Very high significant compared with control group (p<0.001). ## Highly significant compared with AD group (p<0.01).### Very high significant compared with AD group (p<0.001). **Significant compared with AD group (p≤0.05).

3.4-Bielschowsky silver stain examination:

The histochemical examination of the cortical sections from the scopolamine group revealed strong and intense dark brown staining, indicating a high accumulation of tau protein in the cortical neurons. In contrast, the cortical sections from the protective group (Cr + Sc) and the curative group (Sc + Cr) showed a noticeably lower intensity of brown staining compared to the induced (Sc) group. Sections from the Sc + M group, examined using the Bielschowsky technique, revealed a moderate intensity of brown staining, indicating a mild accumulation of tau tangles in the cortical tissue. In contrast, histochemical sections from the Sc + M + Cr group displayed faint brown staining in the cortical neurons, suggesting a near-normal level of tau tangles **(Fig. 4).**

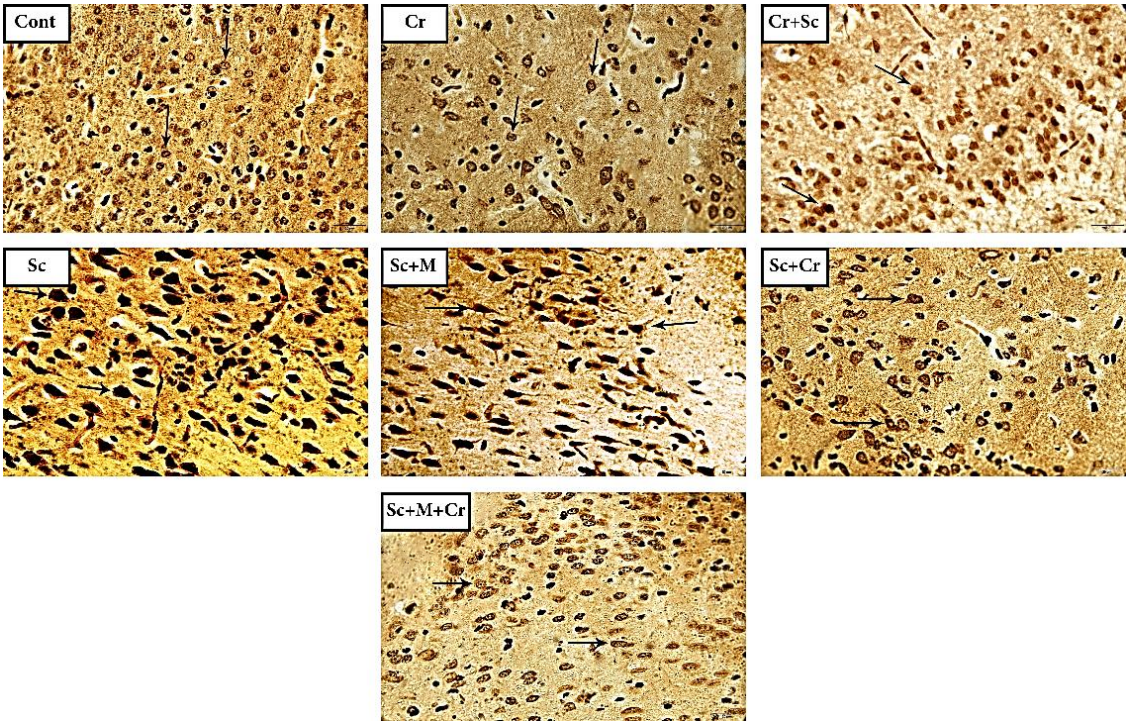

Fig. 4: Photomicrographs of male albino rat's brain cortex tissue sections stained with Bielschowsky silver stain (bar = 50 µm). Arrow refers to tau protein deposition. Cont: control; Cr: Crocin; Sc: Scopolamine, M: memantine.

Scopolamine is considered as golden standard for prompting memory deficits in healthy individuals and animals to impress the feature of muscarinic failure of dementia **(Drachman and Leavitt, 1974; Klinkenberg and Blokland, 2010).**

Excessive formation of free radicals destroys the natural antioxidant protection system, causing oxidative stress that downregulates the endogenous defense system **(Von Arnim et al., 2012; Anjum et al., 2020)**. Multiple sources of data propose that scopolamine increases oxidative brain damage and neuronal death **(Tang, 2019; Tripathi and Mitra Mazumder, 2021).** In the current study, oxidative stress investigations confirmed that LPO levels increased significantly in the Sc-treated rats contrasted with control group. This result compatible with several reports revealed that scopolamine raised MDA levels in the brain **(Pushpalatha et al., 2013; Hejazian et al., 2016; El-Marasy et al., 2018; Rahimzadegan and Soodi, 2018).**

SOD is a type of antioxidant that contributes to oxidation-reduction reactions, it keeps reactive oxygen concentration at a manageable low limit by controlling ROS **(Lihi et al., 2020).** In addition, CAT shields neural cells from oxidative harm by halting the production of lipid peroxidation and OH^- radicals **(Valko et al., 2007).**

In this recent study, scopolamine also lowered SOD and CAT activity. These findings have agree with the earlier studies showing the relation between oxidative damage and Alzheimer`s disease after scopolamine administration **(Brinza et al., 2021; Sharma et al., 2021).** For instance, researchers established a reduction of brain antioxidant capacity in scopolamine-induced AD **(Tang, 2019; Tripathi and Mitra Mazumder, 2021; Kim et al., 2022).**

Numerous studies have found that using natural products as potential treatments for neurodegeneration has health-promoting properties. **Kowalczyk et al. (2020)** confirmed that

antioxidants administration could counteract neurodegeneration induced by scopolamine through recovering the antioxidant enzymes activity. In the present study, crocin remarkably restored memory impairment, oxidative indicators, antioxidant enzymes depletion, and cellular damage in hippocampus sections induced by scopolamine.

Recent evidence established that 100 mg/kg of crocin could recover memory deficit induced by scopolamine; hence, it could be a drug applicant for anti-AD treatment **(Bharate et al., 2018)**. In harmony with this evidence, **Hadipour et al. (2018)** reported that crocin possesses neuroprotective impacts by reducing memory loss and long-term potentiation (LTP) dysfunction.

Altinoz et al. (2018) demonstrated that crocin has a protective effect against brain injury by improving the activity of catalase, superoxide dismutase, and tissue structure, and decreasing the activity of MDA because of its strong antioxidant properties. Additionally, **Yousefsani et al. (2021)** stated that crocin (30 mg) inhibited amyloid beta-42-induced memory deficits, and mitochondrial damage through the inhibition of LPO and motivation of antioxidant mechanisms in the brain.

In the current study, scopolamine increased the levels of phosphorylated tau protein leading to formation of neuro fibrillary tangles whereas crocin alone and crocin and memantine in combination alleviated the effect of scopolamine. These results are in accordance with the outcomes of **Rashedinia et al. (2015)** who reported that 25 mg/kg of crocin could protect against acrolein caused Tau hyperphosphorylation in the rat brain recommending crocin as a promising antioxidant applicant for the treatment of NDDs. In the same line, **Mohammadzadeh et al. (2019)** data showed that 10 mg/kg of crocin decreased the elevated protein and mRNA levels of Tau protein. The data published by **Ahmed et al. (2020)** explained that crocin's antioxidant effect successfully protects neurons and improved spatial cognitive abilities. Crocin regulated MAPK, which suppressed tau phosphorylation, decreasing oxidative pressure induced by acrolein.

4- Conclusion

Overall, these promising findings demonstrated the pharmacological potential and neuroprotective effects of crocin alone or in combination with other drugs making it a natural applicant for anti-neurodegenerative medication.

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