ORIGINAL ARTICLE

Anti-amnesic activity of *Citrus aurantium* flowers extract against scopolamine-induced memory impairments in rats

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Abstract Alzheimer's disease (AD) is a progressive neurological disorder that mostly affects the elderly population. Learning and memory impairment as the most characteristic manifestation of dementia could be induced chemically by scopolamine, a cholinergic antagonist. Cholinergic neurotransmission mediated brain oxidative stress. *Citrus aurantium* (CA) has traditionally been used for the treatment of insomnia, anxiety and epilepsy. The present study was designed to investigate the effect of *Citrus aurantium* on scopolamine-induced learning and memory deficit in rats. Forty-two Wistar rats were divided into six equal groups. (1) Control (received saline), (2) SCOP (scopolamine at a dose of 1 mg/kg for 15 days), (3) and (4) SCOP + CA (scopolamine and CA extract at doses of 300 and 600 mg/kg per day for 15 days), (5) and (6) intact groups (CA extract at 300 and 600 mg/kg per day for 15 days, respectively). Administration of CA flower extract significantly restored memory and learning impairments induced by scopolamine in the passive avoidance test and also reduced escape latency during trial sessions in the Morris water maze test. *Citrus aurantium* flower extract significantly decreased the serum malondialdehyde (MDA) levels. *Citrus aurantium* flower extract has repairing effects on memory and behavioral disorders produced by scopolamine and may have beneficial effects in the treatment of AD.

Introduction

Alzheimer's disease usually leads to a progressive memory loss and causes cognitive and behavioral impairments [1]. Alzheimer's disease is known to be related to excessive neuronal loss, decrease in acetylcholine level, increased inflammation and oxidative stress [2].

Cholinergic deficits are consistently associated with memory and learning loss as well as the severity of Alzheimer's disease [3].

Impairments of learning and memory, can be induced chemically in experimental animals by scopolamine [4]. Scopolamine is a blocker of muscarinic acetylcholine receptor that serves as a beneficial pharmacological tool in producing a model of amnesia [4].

There is notable evidence that scopolamine causes oxidative stress through the interference with acetylcholine in brain leading to cognitive impairment [5].

Brain oxidative stress was reported following the intra ventricular administration of ethylcholine aziridinium, a toxic analog of choline that disrupts high-affinity choline transport producing a persistent pre-synaptic cholinergic hypofunction with the induction of amnesia [5].

Decreased numbers of [3H] N-methyl scopolamine binding sites were observed in the presence of high concentrations of H_2O_2 as an inducer of lipid peroxidation in rat cerebral cortex membranes [6].

Cholinergic nerve stimulation in basal forebrain and hippocampus was reported to be excitotoxic, causing tonic–clonic convulsions due to the release of glutamate

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mediated through the production of ROS (reactive oxygen species) [7].

The brain and nervous system are considered to be more susceptible to peroxidative damage than other tissues due to their high content of polyunsaturated lipid, high oxygen utilization and low antioxidative enzymes [8].

Although the mechanisms of the anti-amnesic effects of most herbal extracts and constituents are not yet fully understood, one or more of the effective ingredients were considered to activate the central Ach(Acetylcholine) function through inhibition of AChE (acetylcholinesterase) [9].

Citrus aurantium is among the species that have been used for medicinal purposes [10]. In Iranian folk medicine, the flowers of *Citrus aurantium* are used in the treatment of neurological disorders such as hysteria, epilepsy and neurasthenia [10].

Citrus aurantium has bioactive components such as phenolics, flavonoids and vitamins. Linalool (59 %) and linalyl acetate (23 %) constitute the major composition of the essential oil of *Citrus aurantium* [11]. Previous studies showed that *Citrus aurantium* plant had inhibitory effect on acetylcholinesterase [12, 13].

In this study, we investigated whether the methanolic extract of *Citrus aurantium* flowers could inhibit the memory impairment induced by scopolamine through the inhibition of AChE or decreased the oxidative stress. The restored degree of impairment was gauged using both passive avoidance and the Morris water maze tests with or without treatment.

Methods

Preparation of the extract

Citrus aurantium flowers were milled, finely powdered and extracted by 80 % methanol. This extract was filtered and concentrated under reduced pressure on a rotary evaporator and then lyophilized. The extract was dissolved in water and stored at 4 °C until use [10].

Measurement of antioxidant activity

Briefly, various concentrations of the extract were mixed with DPPH (2,2-diphenyl-1-picrylhydrazyl) solution in methanol. After 15 min at room temperature, the absorbance was recorded at 517 nm using a UV–Vis spectrophotometer [14].

Inhibition of free radical by DPPH (%) was calculated using the following formula

$$I\ (\%) = 100 \times \left(A_{control} - A_{sample}\right)/A_{control}$$

$$\frac{A_{control} - A_{sample}}{A_{control}}$$

Total phenolic compounds measurement

The amount of phenols was obtained based on mg/g in gallic acid equivalent. The standard curve was plotted using 12.5, 25, 50, 62.5, 100, and 125 mg/L solutions of gallic acid in methanol and water (60:40, v/v) [14].

Total flavonoid and flavonol measurement

The amount of total flavonoids in the *Citrus aurantium* extract was determined using the colorimetric method. Solution with 1.5 mL of methanol (60 %), 1 mL of 2 % aluminum chloride, and 6 mL of 5 % potassium acetate was added to 1 mL of the *Citrus aurantium* extract. The mixture was left at room temperature for 40 min. The absorbance of the reaction mixture was then measured at 415 nm. The aluminum chloride colorimetric method was employed for flavonol determination, but the incubation period was 150 min and the absorbance of the reaction mixture was determined at 440 nm. Total flavonoids and flavonols were expressed in terms of rutin equivalent (mg/g), which is a common reference compound [14].

Animals

Male Wistar rats, weighing 150–250 g and aged 3–4 months, were obtained from Pasteur Institute (Tehran, Iran). Rats were housed in cage (four per cage) at 25 °C with a controlled 12 h light–dark cycle. Food and water were freely available. All experiments were executed in accordance with the Guide for the Care and Use at Laboratory Animals and were approved by Research and Ethics Committee of Medical Sciences School of Shahrekord University.

Animals were randomly divided into the following six groups of seven rats: (1) Control, received saline for 15 days via i.p injection, (2) scopolamine-treated group: rats received scopolamine at a dose of 1 mg/kg for 15 days via i.p injection (SCOP), (3) SCOP- CA 300 group, rats received *Citrus aurantium* extract at 300 mg and scopolamine at 1 mg/kg for 15 days, (4) SCOP- CA 600 group, rats received *Citrus aurantium* extract at 600 mg and scopolamine at 1 mg/kg for 15 days, (5) and (6) intact groups received *Citrus aurantium* extract at 300 and 600 mg/kg, respectively, for 15 days. Scopolamine (sigma, USA) was dissolved in saline at final concentrations of 1 mg/kg. Administration of scopolamine and extract in experimental groups was done before the start of

behavioral testing and during behavioral testing. Following the behavioral tests, under deep anesthesia the blood sample collected from heart and the brains was quickly removed. Different parts of the brains such as hippocampus, cortex and subcortex (The Subcortex is located below the cerebral cortex and completely covered by it. It can be divided into three general areas: Brainstem or hindbrain; midbrain; and forebrain) were separated.

Water maze test

A circular water pool (183 cm diameter and 60 cm deep) was used for a water maze test [15]. A black escape platform was placed into water and submerged 1 cm below the water in one quadrant water maze surface. A platform was located in the constant position, i.e., in the middle of one quadrant (in our experiment, in zone 1) equidistant from the center and edge of the pool. Rats were allowed to locate the platform in a time span of up to 60 s. Rats that could find the platform were allowed to stay on it for 15 s. Animals were given four trials daily for four consecutive days, with an inter trial interval of 10 min. On the fifth day, rats were individually subjected to a probe trial session by removing the platform and were allowed to swim for 60 s to search for the platform.

Passive avoidance test

Passive avoidance test was carried out using a shuttle box apparatus [14]. The apparatus consisted of a lighted compartment and a dark compartment with a grid floor. The two compartments were separated by a guillotine door and each had a grid floor through which a foot shock could be delivered. This test was performed for each rat during the 4 days. On the first and second days of testing, each rat was placed on the apparatus and left for 5 min to habituate to the apparatus. On the third day, an acquisition trial was performed. On the training day, the rat was placed in the lighted compartment, facing away from the dark compartment and allowed to explore for 20 s. After 20 s the guillotine door was lifted. When the rat entered the dark compartment with all four paws, the guillotine door was closed, and the latency to enter was recorded. After the door was closed, a foot shock (1 mA, 1 s duration) was delivered through the stainless steel rods. All the animals that could enter in the dark compartment within 60 s as in the training session received a foot shock. On the fourth day (24 h after training), the rat was returned to the lighted compartment. After 5 s, the guillotine door was lifted. When the rat entered the dark compartment, the guillotine door was closed, and the step-through latency for animals was recorded.

Ferric reducing/antioxidant power (FRAP) assay

The antioxidant power of plasma was determined by measuring its ability to reduce Fe^{3+} to Fe^{2+} with FRAP (ferric reducing antioxidant power) test. $FeSO_4$ (100–1,000 μM concentration range) was used as a standard in FRAP assay [14].

Measurement of plasma MDA

The optimized assay was carried out as follows: 50 μl plasma or the standard was treated with 50 μl (0.05 %) BHT (butylated hydroxytoluene), followed by the addition of 400 μl H_3PO_4 (0.44 M) and 100 μl TBA (thiobarbituric acid) (42 mM), vortexed and then incubated for 60 min at 100 °C. The reaction was stopped by cooling at 4 °C, then 250 μl of n-butanol was added for extraction of MDA–TBA complex. The solution centrifuged for 5 min at 14,000 rpm to separate two phases. The supernatant (20 μl) was injected into the HPLC system [14].

MDA levels of brain

Brain tissues were homogenized in ice-cold Tris–HCl buffer (50 mM, pH 7.4) for 2 min at 5,000 rpm. The homogenized solution was then centrifuged for 60 min at 5,000g.

The sample was mixed with two volumes of cold 10 % (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of 0.67 % (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm using spectrophotometer [14].

AchE assay

Animals were sacrificed by decapitation and brain tissue was immediately removed and placed in a solution of 250 mM sucrose, 100 mM Tris–HCl, pH 7.5, in an ice bath. Brain tissue was then homogenized in a glass potter in the same sucrose Tris–HCl solution (1:4 (w/v)). Aliquots of resulting brain homogenates were stored at −80 °C until utilization. Acetylcholinesterase activity was measured by the method of Ellman [16]. A reaction mixture contained 470 μL sodium phosphate (0.1 mM, pH = 8), 167 μL (2-nitro benzoic acid) DTNB and 33 μL homogenate was incubated for 5 min at 37 °C. The acetylcholine iodide (1 mM) 280 μL was added to reaction mixture. The reaction was terminated by 50 μL neostigmine (2 mM).

Table 1 DPPH radical scavenging activities of CA extract

Sample	Concentration (μg/ml)	DPPH radical scavenging activity inhibition (%) IC50 (μg/ml)
CA flowers extract	100	13.4
	150	24.9
	200	33.5
	250	41.55
	300	45
	350	56.5
	400	64.7
	450	70.56
	500	75.7

Statistical analysis

Data were expressed as mean $\pm$ SEM (standard error of mean) and processed by commercially available software SPSS 11.0. All results were compared using one-way ANOVA and post hoc Tukey test. p value less than 0.05 was considered to be statistically significant.

Results

Standardization of *Citrus aurantium* flowers extract

Total amount of phenolic compounds in *Citrus aurantium* flowers extract was 89 mg/g gallic acid equivalent per one gram of dried extract. The total amounts of flavonoid and flavonol compounds were 12.5 and 6 mg/g, respectively.

Radical scavenging activity of *Citrus aurantium* flowers extract

IC_{50} values for radical scavenging activity of CA extract are shown in Table 1. *Citrus aurantium* flower extract showed free radical scavenging activity against DPPH radicals, with an IC50 value of 320 μg/ml.

Passive avoidance response

The step-through latency (T2) of SCOP-treated rats was significantly shorter than that of the control rats ($p = 0.001$). CA extract administration at a dose of 300 mg/kg reversed the shorter step-through latency induced by SCOP ($p = 0.043$). Furthermore, the step-through latency (T2) time of intact rats treated with 300 mg/kg of *Citrus aurantium* flowers extract significantly increased compared to SCOP rats ($p = 0.001$) (Fig. 1).

T2 significantly increased in Intact300CA group compared to the SCOP + 600 CA ($p = 0.031$). T2 in Intact
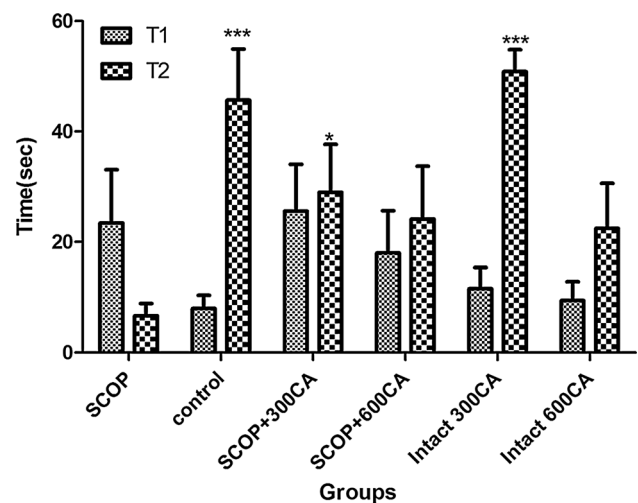

Fig. 1 The initial latency and step-through latency in the passive avoidance response. *T1* initial latency, *T2* step-through latency. *$p < 0.05$; ***$p < 0.01$. SCOP *vs.* control, SCOP + 300CA, SCOP + 600CA, Intact 300CA, Intact 600CA groups ($n = 7$). CA *Citrus aurantium*, SCOP scopolamine
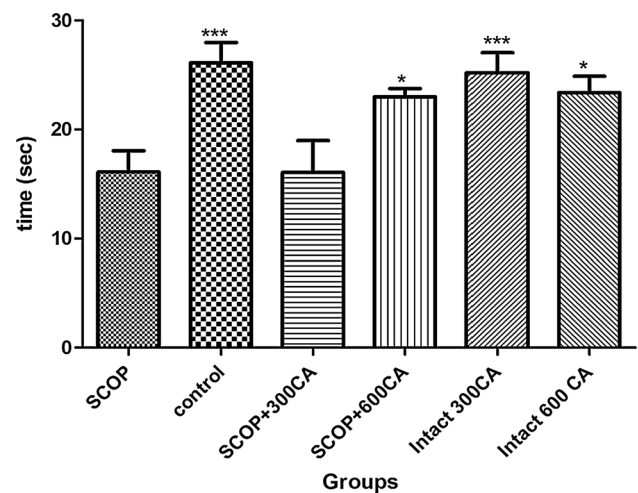

Fig. 2 The time spent in zone 1 during the probe trial. *$p < 0.05$; ***$p < 0.01$

300CA group significantly increased when compared with Intact600CA group ($p = 0.012$).

T2 of intact rats that received extract at dose of 600 mg significantly increased compared with control group ($p = 0.037$).

Spatial memory and learning

SCOP-treated group spent significantly less time in the correct quadrant (zone1) compared with control group in the probe trail ($p = 0.001$). In the probe test SCOP + 600CA, intact 300CA and intact 600CA groups
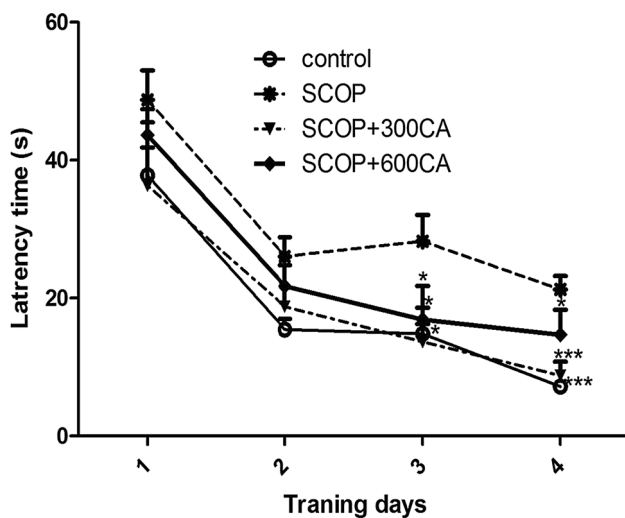
Fig. 3 Spatial learning in experimental groups $*p < 0.05$; $***p < 0.01$

Fig. 4 Plasma antioxidant levels (FRAP) in experimental groups. $*p < 0.05$; $***p < 0.01$

demonstrated a significant preference for the quadrant in which the platform was located on the preceding day (zone 1) when compared with SCOP-treated group ($p = 0.015$, $p = 0.003$, $p = 0.011$, respectively) (Fig. 2).

The escape latency of the SCOP group was significantly longer by means of memory impairment than that of the control group during trial sessions ($p < 0.05$). The decrease in the escape latency improved significantly on the third day in control, SCOP + 300CA and SCOP + 600CA when compared with SCOP group ($p = 0.031$, $p = 0.021$, $p = 0.048$, respectively). The latency before reaching platform on the fourth day significantly decreased in control, SCOP + 300CA and SCOP + 600CA when compared with SCOP group ($p = 0.001$, $p = 0.003$, $p = 0.049$, respectively) (Fig. 3).

The latency to reach platform in days 2, 3 and 4 significantly decreased compared with day 1 in experimental groups ($p < 0.05$). The latency to reach platform significantly decreased in day 4 compared with day 2 ($p = 0.033$) in experimental groups (data not shown).

Citrus aurantium flowers extract at doses of 300 and 600 mg/kg/day in intact rats caused a slight reduction (not significant, $p > 0.05$) in the latency compared with the control group (data not shown).

Plasma antioxidant level

Scopolamine treatment significantly decreased the plasma antioxidant level compared to the control group ($p = 0.043$). CA treatment significantly increased the plasma antioxidant level in SCOP + 600CA, intact 300CA and intact 600CA groups when compared with SCOP group ($p = 0.002$, $p = 0.016$, $p = 0.009$, respectively).
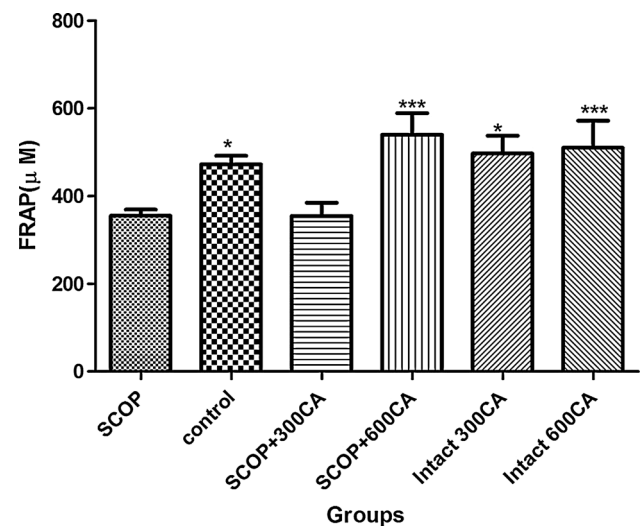
Citrus aurantium flowers extract at a dose of 300 mg had no effect on SCOP-treated group (Fig. 4). Plasma antioxidant level in SCOP + 600CA group significantly increased compared to SCOP + 300CA group ($p = 0.025$). Data not shown.

Brain MDA levels

Scopolamine treatment lead to a significant increase in MDA content of hippocampus, cortex and subcortex in SCOP group ($p = 0.048$, $p = 0.005$, $p = 0.001$, respectively). Treatment with 300 mg/kg of CA extract in SCOP + 300CA group significantly reduced MDA content in hippocampus, cortex and subcortex when compared with SCOP group ($p = 0.007$, $p = 0.004$, $p = 0.001$, respectively). CA extract significantly reduced MDA level of hippocampus, cortex and subcortex in SCOP + 600CA group when compared with SCOP group ($p = 0.008$, $p = 0.005$, $p = 0.001$, respectively). CA extract treatment at doses of 300 and 600 mg significantly decreased the MDA levels of hippocampus, cortex and subcortex in intact groups when compared with SCOP group ($p < 0.05$) (Fig. 5).

Plasma MDA level

In SCOP group, scopolamine treatment significantly increased plasma MDA level when compared with control group ($p = 0.006$). CA extract administration at doses of 300 and 600 mg/kg in SCOP + 300CA and SCOP + 600CA groups significantly reduced plasma MDA levels when compared with SCOP group ($p = 0.003$, $p = 0.005$, respectively) (Fig. 6).
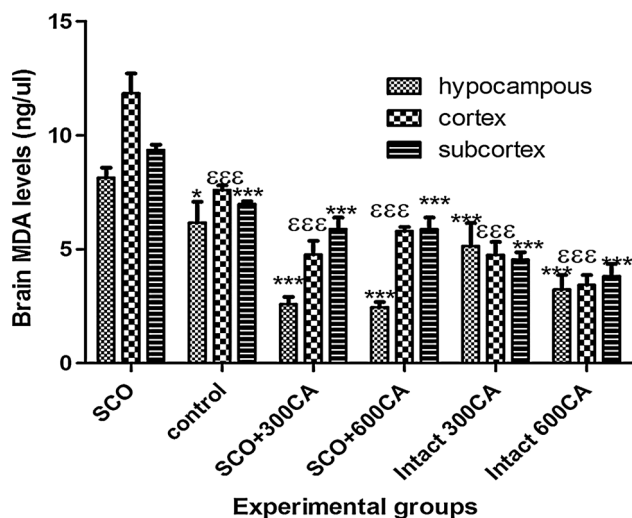
Fig. 5 Brain malondialdehyde level (MDA) in experimental groups.
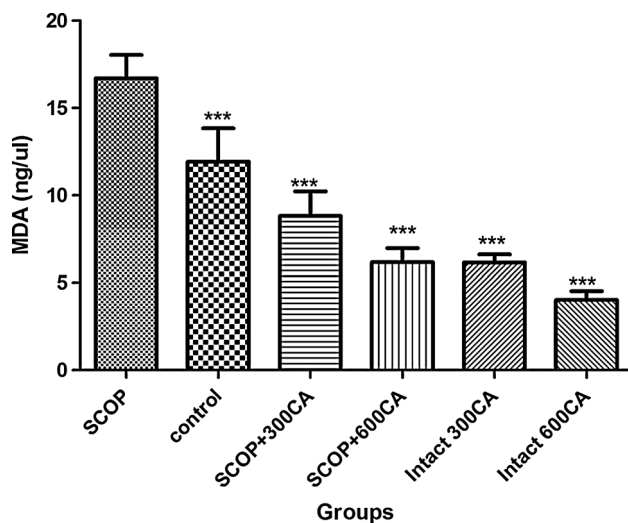$*p < 0.05$; $***$, $^{\epsilon\epsilon\epsilon}p < 0.01$

Fig. 6 Plasma MDA level in experimental groups. $***p < 0.01$

In the intact groups, administration of CA extract resulted in a significant reduction in plasma MDA levels at doses of 300 and 600 mg/kg/day when compared with SCOP group ($p = 0.001$, $p = 0.005$, respectively) and control group ($p = 0.001$, $p = 0.003$, respectively). There were no significant differences between intact groups and groups received extract and scopolamine.

Effect of *Citrus aurantium* extract on the AChE activity

Citrus aurantium flower extract at doses of 300 and 600 mg/kg significantly inhibited acetylcholinesterase activity in hippocampus ($p < 0.05$) as compared to control rats (Table 2).

Table 2 Effects of CA extract on AChE activity in hippocampus of the brain

Groups of rats	Hippocampus (OD value/mg protein)
Control	0.1090 ± 0.010
300 mg CA	$0.0823 \pm 0.012*$
600 mg CA	$0.0608 \pm 0.027*$

OD optical density, *CA Citrus aurantium*

$* p < 0.05$

Discussion

AD is one of the most ordinary neurodegenerative diseases, resulting in progressive dysfunction in the brain, which proceeds from mild and moderate to severe stages and gradually destroys the brain [1]. Brain aging is known to be related to decrease in acetylcholine level, neuronal loss, increased inflammation, and oxidative stress [1]. Hippocampal cholinergic neurotransmission possesses an important function in the process of learning and memory, which is influenced by effective drugs in this system [17]. Acetylcholinesterase modulates acetylcholine to proper levels by degradation, thus, excessive AChE activity leads to constant acetylcholine deficiency, causing memory and cognitive impairments [18].

Acetylcholinesterase inhibitors are the most effective pharmacotherapy for AD [17]. These compounds indirectly elevate acetylcholine concentrations in the AD-affected brain [17]. Scopolamine significantly increases AChE and malondialdehyde (MDA) levels in the cortex and hippocampus. Scopolamine causes oxidative stress through the interference with acetylcholine in brain leading to cognitive impairment [5]. The cognitive-enhancing activity of *Citrus aurantium* flowers extract on the scopolamine-induced memory impairments in rat was investigated using passive avoidance test, Morris water maze test and biochemical assessments. The passive avoidance test is generally used to evaluate memory in three steps (learning acquisition, memory retention, and the retrieval process) [19]. *Citrus aurantium* flowers extract administration reversed the shorter step-through latency induced by scopolamine. To confirm the effects of *Citrus aurantium* flowers extract on other types of memory, we performed the Morris water maze test on spatial learning and memory. Rats treated with scopolamine showed more prolonged escape latency than rats treated with saline from the control group. *Citrus aurantium* flowers extract treatment significantly reduced escape latency in experimental groups which suggested that long-term and working memory were impaired by scopolamine. In addition, during the probe trial session, the scopolamine-induced reduction in swimming times within the platform quadrant was significantly

ameliorated by *Citrus aurantium* flowers extract, indicating a positive effect on spatial learning and memory. In our study, to further elucidate the mechanism of anti-amnesic activity of *Citrus aurantium* flowers extract, lipid peroxidation and AchE in the brain tissue were measured following the behavioral test. *Citrus aurantium* flowers extract has potent inhibitory effect on AchE in brain tissue. Our results showed that treatment with *Citrus aurantium* flowers extract significantly decreased MDA levels of serum and brain tissue in intact and scopolamine-treated rats compared to the scopolamine-treated group.

Besides reducing cholinergic activity, oxidative stress also plays an important role and is one of the major causes for memory loss in AD [1]. Oxidative stress is produced by free radicals, i.e., reactive oxygen species (ROS) generated by oxygen and nitrogen-based molecules that have unpaired electrons. Oxidative damage to the cellular components results in alteration of the membrane properties such as fluidity, ion transport, enzyme activities, and protein cross-linking [9]. Excessive oxidative damage eventually results in cell death. Normally, biological effects of free radicals in the body are controlled with adequate antioxidants and antioxidant enzymes [9]. Our results suggest that the *Citrus aurantium* flowers extract reduced oxidative stress by reducing lipid peroxidation.

Nobiletin, a citrus poly methoxylated flavone that can pass through the blood–brain-barrier, has a potential therapeutic benefit for dementia including Alzheimer's disease. Nobiletin reverses the N-Methyl-D-aspartate receptor antagonist dizocilpine-impaired memory by activating extracellular signal-regulated kinase signaling in the hippocampus of mice [20].

Naringin, a well-known flavanone glycoside of *Citrus*, possesses antioxidant, anti-inflammatory, anti-apoptotic, anti-osteoporosis and anti-carcinogenic properties [21].

Citrus aurantium at dose of 1 g/kg was able to enhance the sleeping-time duration induced by pentobarbital in adult male Swiss mice [22]. The cognition-enhancing agents activate cholinergic transmission via an agonistic or antagonistic effect on GABA$_A$/benzodiazepine receptor and GABA$_A$/benzodiazepine receptor complex controls acetylcholine release [23].

This study evaluated whether such impaired cognition by scopolamine is associated with altered oxidative stress indices. Our study indicated that improved learning and memory in scopolamine-treated memory impairments in rats may be related to decrease in oxidative stress in brain.

Our results suggested that the anti-amnesic effect of *Citrus aurantium* flowers extract on scopolamine-induced memory impairment may be related to the antioxidant activity of extract or mediation of the cholinergic nervous system.

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