Neuroprotective Effects of *Citrus reticulata* in Scopolamine-Induced Dementia Oxidative Stress in Rats

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Abstract: The purpose of the study was to evaluate the potential effects of *Citrus reticulate* (mandarin) peel methanolic extract (MPME) on memory dysfunction in rats. Memory impairment was produced by scopolamine (1.4 mg/kg, intraperitoneally injected). Brain acetylcholinesterase enzyme (AChE) activity was measured to assess the central cholinergic activity. This study also investigated the effect of scopolamine on norepinephrine, dopamine and serotonin content in rat hippocampus, striatum and cerebral cortex. In addition, the levels of brain lipid peroxidation (LPO), nitric oxide (NO) and glutathione (GSH) were estimated to assess the degree of oxidative stress. Scopolamine administration induced a significant impairment of central cholinergic activity in rats, as indicated by a marked increase in AChE activity. The impairment of the cholinergic system was associated with a significant alternation in brain monoamines. Scopolamine administration also caused oxidant damage (elevation in LPO and NO and reduction in GSH levels). Pre-treatment of MPME (250 mg/kg, orally administered) significantly reduced scopolamine-induced alternation in brain monoamines with an attenuation of scopolamine-induced rise in brain AChE activity and brain oxidative stress. It is concluded that administration of mandarin peel extract, demonstrating antioxidant activity, may be of value for dementia exhibiting elevated brain oxidative status.

Keywords: *Citrus reticulate,* scopolamine, dementia, neuroprotection.

INTRODUCTION

Based on experimental and clinical evidence, acetyl-choline (ACh) is considered the most important neuro-transmitter involved in regulation of cognitive functions [1]. Alzheimer's disease (AD) is the most common age related neurodegenerative disorder characterized by cognitive dys-function with memory impairment and behavioral disturb-ances [2]. The therapeutic strategies to combat cognitive disorders have been aimed at improving ACh activity. Therefore, the cholinergic receptor agonists (musca-rinic and nicotinic) and enhancers of endogenous level of ACh (synthesis promoters and inhibitors of its metabolizing enzyme) have been tested to treat Alzheimer type senile dementia. Among the various approaches attempted to increase cholinergic activity, the inhibition of acetyl-choliesterase (AChE) is the most successful [1]. Cholin-esterase inhibitors are the only class of compounds consis-tently proven to be efficacious in treating the cognitive and functional symptoms of AD [3].

Scopolamine is a muscarinic cholinergic receptor antagonist that impairs memory performance and has been proposed for use in an animal model of dementia [4]. Some similarities between Alzheimer patients and scopolamine treated animals in memory deficiencies have been reported [5]. In addition, along with cholinergic atrophy, monoamines are reduced in AD and the possibility exists that enhancement of monoaminergic functions may elicit beneficial effects on behavior and cortical activity [6].

It has been reported that memory impairment induced by scopolamine in rats is associated with altered brain oxidative stress status [7]. Therefore, rats with scopolamine-induced memory deficits have been used as an animal model for screening antidementia drugs [8]. Oxidative stress is also one of the affecting factors in AD, so several antioxidants have been studied for the reduction of oxidative stress occurring during AD [9].

El-Sherbiny *et al.* [10] reported that memory impairment in the scopolamine-induced animal model is associated with increased oxidative stress within the rat brain. Jimenez-Jimenez *et al.* [11] demonstrated increased oxidation of lipids, proteins and deoxyribonucleic acid, alterations in mitochondrial function and a possible role of amyloid beta and its precursor protein in oxidative reactions in experimental models of AD. Moreover, strong evidence supporting the involvement of oxidative damage in neurodegenerative disease has been suggested by various clinical studies [12, 13]. It is known that oxidative damage may serve as an early event initiating cognitive disturbances and the pathological features observed in AD [14].

Large numbers of compounds from natural resources have provided novel lead compounds for drug development as well as useful pharmacological tools [15]. Citrus is an important crop mainly used in food industries for fresh juice production and peel is the main by-product of its processing. In general, fruit skin contains a higher concentration of antioxidant substances than the flesh of the fruit. Citrus peel, which represents roughly one half of the fruit mass, is a rich

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source of bioactive compounds including natural antioxidants such as phenolic acids and flavonoids [16]. The primary active biological constituents of them are adrenergic amines (such as synephrine, octopamine, and tyramine) [17] and flavonoids (flavanones, flavones, and flavonols) and phenolic acids [18, 19].

Xu *et al*. [20] determined four citrus flavonoids: narirutin, hesperidin, nobiletin and tangeretin, and seven phenolic acids, including four hydroxycinnamics (caffeic, *p*-coumaric, sinapic, and ferulic) and three hydroxybenzoics (protocatechuic, *p*-hydroxybenzoic, and vanillic), in mandarin peel extracted with hot water, ferulic acid being the most dominant one. Citric peel components were found to provide many health benefits. The experimental studies have demonstrated its analgesic, antibacterial, antimicrobial, antiviral, antiyeast antifungal, antidiarrheal, antiinflammatory, uricosuric activity, antimutagenic, antisplasmodic, antiatherogenic, antiperoxidative activity, anticarcinogenic activity, and radical scavenging activity [21].

Therefore, the objective of the current study was to evaluate the effect of scopolamine at amnestic doses on brain monoamine content, AChE activity, LPO, NO and GSH levels in rats and *Citrus reticulate* (mandarin) peel was used to protect against scopolamine-induced oxidative stress and memory impairment.

2. MATERIALS AND METHODS

2.1. Chemicals

Scopolamine hydrobromide (Winlab Laboratory Chemicals, Leicestershire, UK) was used. Nitro blue tetrazolium, N-(1–naphthyl) ethylenediamine and Tris-HCl were purchased from Sigma (St. Louis, MO, USA). Ellman's reagent [(5′, 5′-Dithiobis (2-nitrobenzoic acid), DTNB] was obtained from Alfa Aesar (GmbH & Co KG, Germany). Perchloric acid, thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Merck. All other chemicals and reagents used in this study were of analytical grade. Double-distilled water was used as the solvent.

2.2. Extraction

Fresh fruit peels of *Citrus reticulata* (*C. reticulate*; mandarin) were taken and grounded, and about 500 g of the plant material was consecutively macerated for one day in petroleum ether, ethyl acetate, chloroform, and methanol, respectively. On basis of the preliminary phytochemical tests conducted, the methanol extract was found to be rich in terms of chemical constituents and therefore was selected for the experiment. The methanol was removed under reduced pressure to obtain a semisolid mass of MPME. The MPME was then stored in -20 °C until used. The plant extract was subjected to screening for various phytochemicals. The quantities of flavonoids and polyphenols were established in the MPME (Table **1**).

2.3. Animals

Adult male Wistar albino rats weighing 200–250 g were obtained from the Holding Company for Biological Products and Vaccines (VACSERA, Cairo, Egypt). After an acclimatization period of one week, the animals were divided into five groups (7 rats per group) and housed in wire bottomed cages in a room under standard conditions of illumination with a 12-hours light-dark cycle at 25±1ºC. They were provided with water and a balanced diet *ad libitum*. All animals received care in compliance with the Egyptian rules for animal protection.

Table 1. Quantitative Analysis of Flavonoids and Polyphenols in the MPME

Parameter	Flavonoids	Polyphenols
(wt%)*	98.3	154.7

*: Flavonoids are expressed as µg/ mg querestin equivalents of flavonoids and polyphenols are expressed as µg/ mg gallic acid equivalent of polyphenols.

2.3.1. Experimental Design

The rats were divided into 5 groups: (1) control group received saline injection (0.9% NaCl); (2) scopolamine (Sco)-treated group injected intraperitoneally with scopolamine at a dose of 1.4 mg/kg bwt; (3) scopolamine-treated group pre-treated with mandarin peel methanolic extract for one day (MPME 1); (4) scopolamine-treated group pre-treated with mandarin peel methanolic extract for two days (MPME II); and (5) scopolamine-treated group pre-treated with mandarin peel methanolic extract for three days (MPME III).

MPME was orally administered at the present dose according to preliminary results conducted in our lab, while scopolamine was intraperitoneally injected at a dose of 1.4 mg/kg bwt, this latter dosage follows the dose reported by El-Sherbiny *et al.* [10] as producing amnestic in rats.

After 1 hr of scopolamine injection, the animals in each group were sacrificed by decapitation. The brains of the rats were carefully removed and dissection of the brains was performed on an ice-cold glass plate for the separation of hippocampus, striatum and cerebral cortex regions according to the method described by Glowinski *et al.* [22]. The three regions were equally divided in two portions; the first portion of each region was blotted and frozen for further determination of monoamines. The second portion of each region was weighed and homogenized immediately to give 50% (w/v) homogenate in ice-cold medium containing 50 mM Tris-HCl, pH, 7.4. The homogenate was centrifuged at 3000 rpm for 10 min in cooling centrifuge at 4°C. The supernatant (10%) was used for the various biochemical determinations.

2.4. Biochemical Measurements

2.4.1. Determination of Acetylcholineasterase Activity in Brain Regions

Acetylcholineasterase activity assay is based on an improved Ellman method [23], in which thiocholine produced by the action of acetylcholinesterase forms a yellow color with DTNB. The intensity of the product color, measured at 412 nm, is proportionate to the enzyme activity in the sample.

2.4.2. Monoamines Analysis by HPLC

Norepinephrine (NE), dopamine (DA) and serotonin (5-HT) of the hippocampus, striatum and cerebral cortex were assayed by means of HPLC with UV detection. The tissue samples were weighed and homogenized in ice-cold 0.1 M trichloroacetic acid containing 0.05 mM vitamin C. After centrifugation (14000 rpm, 5 min), the supernatants were filtered through RC58 0.2 AM cellulose membranes. The chromatograph (Hewlett-Packard 1050) was equipped with C18 columns. The mobile phase consisted of 0.05 M citrate–phosphate buffer, pH 3.5, 0.1 mM EDTA, 1 mM sodium octyl sulfonate and 3.5% methanol. The flow rate was maintained at 0.8 ml/min. monoamines were quantified by peak height comparisons with standards run on the day of analysis.

2.4.3. Determination of Lipid Peroxidation

Lipid peroxidation was assayed trough colourimetric tests according to the method of Ohkawa *et al.* [24]. In this method, thiobarbituric acid reactive substances (TBARS) determined by using 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67% which were then heated together in a boiling water bath for 30 min. TBARS were then determined by the absorbance at 535 nm and expressed as malondialdehyde (MDA) formed.

2.4.4. Determination of Nitrite/Nitrate

The assay of nitrite/nitrate, as an indirect measure of NO production, content in brain homogenates was done according to the method of Green *et al.* [25]. In an acid medium and in the presence of nitrite the formed nitrous acid diazotise sulphanilamide was coupled with N-(1–naphthyl) ethylenediamine. The resulting azo-dye had a bright reddish–purple colour which could be measured through spectrophotometry at 540 nm.

2.4.5. Determination of Glutathione

The neuronal glutathione (GSH) was determined by the methods of Ellman [26]. The method based on the reduction of Elman's reagent (5,5` dithiobis (2-nitrobenzoic acid) "DTNB") with GSH to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm.

2.5. Statistical Analysis

Results were expressed as the mean ± standard error of the mean (SEM). Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Duncan's test was used as a *post hoc* test according to the statistical package program (SPSS version 17.0).

3. RESULTS

Table **2** summarizes the effect of scopolamine injection on the levels of NE, DA and 5-HT in different rat brain regions. Scopolamine injection significantly decreased the levels of NE (-51.6%) and 5-HT (-46.9%) in the rat hippocampus. These decreases in NE and 5-HT were associated with a significant increase in DA contents (42.3%). The alternation in the levels of neurotransmitters due to the injection of scopolamine was ameliorated by MPME pre-treatment, however, MPME failed to lower the reduction in 5-HT in the hippocampus of rats compared to the control group. Although, the neurotransmitters in striatum were significantly (NE: -42.9%, DA: 26.3% and 5-HT: -48.2%; $p < 0.05$) reduced by scopolamine injection when compared with the control values. MPME pre-treatment prevented these changes when compared to the scopolamine group, moreover, the levels of monoamines were returned to the control level after 3 days of mandarin treatment. Moreover, levels of NE (-44.9%) and 5-HT (-31.4%) in the cerebral cortex were significantly diminished, but DA levels were significantly (34.0%; $p < 0.05$) elevated by scopolamine injection. Pre-treatment with MPME significantly attenuated the changed in NE, DA and 5-HT levels as compared to scopolamine. Pre-treatment with MPME for 3 days caused a significant elevation in the norepinephrine content of cerebral cortex as compared to the control group. Additionally, MPME didn't cause changes in monoamines levels after 1, 2 and 3 days of treatment in the tested regions (supplementary data: Table **S1**).

Table 2. Effect of Pre-Treatment with Mandarin Peels Methanolic Extract (MPME) for 1, 2 and 3 Days on Scopolamine-Induced Alternation on the Levels of DA, NE and 5-HT (µg/g Tissue) in Hippocampus, Striatum and Cerebral Cortex of Adult Rats

Groups	NE	DA	5-HT
Hippocampus			
Control	0.523±0.024	0.764±0.036	0.652±0.031
Sco	0.253±0.012[a]	1.087±0.037[a]	0.346±0.033[a]
MPME I	0.282±0.042[a]	0.934±0.024[ab]	0.383±0.043[a]
MPME II	0.373±0.026[ab]	0.892±0.037[ab]	0.485±0.039[ab]
MPME III	0.484±0.017[b]	0.810±0.048[b]	0.568±0.046[ab]
Striatum			
Control	0.357±0.011	1.214±0.074	0.496±0.028
Sco	0.204±0.009[a]	1.547±0.096[a]	0.257±0.015[a]
MPME I	0.287±0.011[ab]	1.475±0.039[ab]	0.314±0.023[ab]
MPME II	0.349±0.012[b]	1.398±0.078[ab]	0.387±0.019[ab]
MPME III	0.387±0.017[b]	1.287±0.087[b]	0.478±0.022[b]
Cerebral Cortex			
Control	0.247±0.012	0.736±0.034	0.624±0.024
Sco	0.136±0.011[a]	0.986±0.058[a]	0.428±0.036[a]
MPME I	0.179±0.009[ab]	0.913±0.046[ab]	0.487±0.019[ab]
MPME II	0.209±0.013[ab]	0.834±0.053[ab]	0.567±0.029[ab]
MPME III	0.283±0.017[ab]	0.793±0.042[b]	0.603±0.041[b]

Values are means ± SEM (n=6).
[a]$p<0.05$, significant change with respect to **Control**; [b]$p<0.05$, significant change with respect to **Sco** for Duncan's post hoc test.

The effects of scopolamine and MPME on acetylcholin-esterase activity in brain homogenates are presented in Fig.

(**1**). Scopolamine injection caused a significant ($p < 0.05$) increase in AChE activity in the brain homogenates. Pre-treatment of MPME for 2 and 3 days in rats injected with scopolamine suppressed the AChE activity, but AChE activity after one day with MPME was still significantly higher than in the control group.

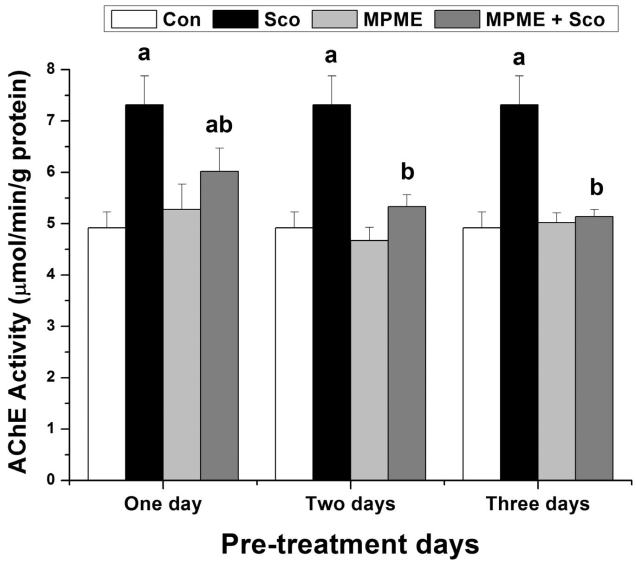

Fig. (1). Effects of MPME treatment for 1, 2 or 3 days at a dose of 250 mg/kg on scopolamine (1.4 mg/kg)-induced acetylcholinesterase activation in brain of rats treated. Values are means ± SD. a: Significant change at $P < 0.05$ with respect to the control group. b: Significant change at $P < 0.05$ with respect to scopolamine group.

The effects of MPME in lipid peroxidation and nitric oxide levels during dementia induced by scopolamine are presented in Figs. (**2**, **3**). LPO was markedly increased in the scopolamine group as compared to the corresponding values of the control group (Fig. **2**). In addition, dementia induced by scopolamine produced a significant increase in neuronal NO content of 41.2% ($p < 0.05$) when compared to the control group (Fig. **3**). Post hoc comparison of means indicated significant decreases in rats pre-treated with MPME in LPO levels and NO contents, when compared with the scopolamine group. However, the administration of mandarin peels alone for 3 days appeared to cause a significant increase in nitric oxide content in brain tissue.

Dementia in rats causes overproduction of cellular oxidants and modulation of the antioxidant defense system. As observed during the study, scopolamine injection led to modulation of several parameters of oxidative stress relative to control animals. After 1 hr of scopolamine infection, GSH content in the brain homogenate decreased significantly ($p < 0.05$) compared to the control group (Fig. **4**). On the other hand; MPME pre-treatment elevated the content of GSH and returned it to the control value throughout the experiment. Also, MPME administration in normal rats for 3 days (in other words, rats that had received no scopolamine) induced 28.6% higher content of GSH compared to the control group.

4. DISCUSSION

Many clinical studies have reported strong evidence that oxidative stress is involved in the pathogenesis of AD [27]. The oxygen-free radicals are implicated in the process of age related decline in the cognitive performance and may be responsible for the development of AD in elderly persons [28]. It has been reported that memory impairment in the scopolamine-induced animal model of dementia is associated with the increased oxidative stress within the rat brain [10].

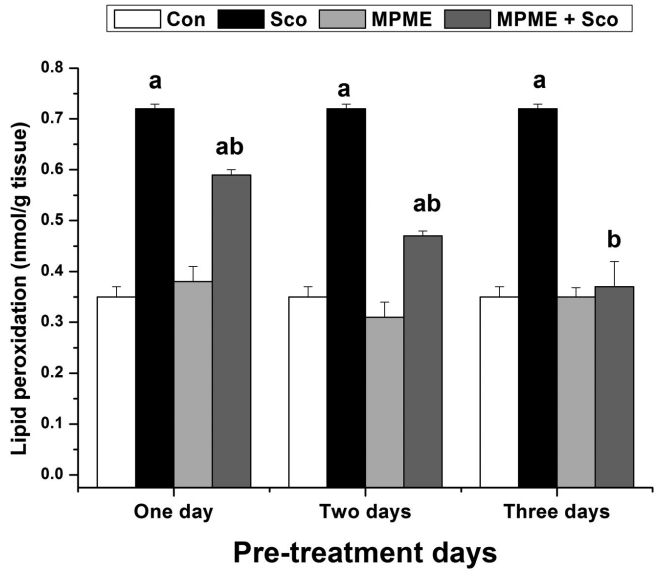

Fig. (2). Effects of MPME treatment for 1, 2 or 3 days at a dose of 250 mg/kg on lipid peroxidation elevation in brain of rats treated with scopolamine (1.4 mg/kg)-induced oxidative stress. Values are means ± SD. a: Significant change at $P < 0.05$ with respect to the control group. b: Significant change at $P < 0.05$ with respect to scopolamine group.

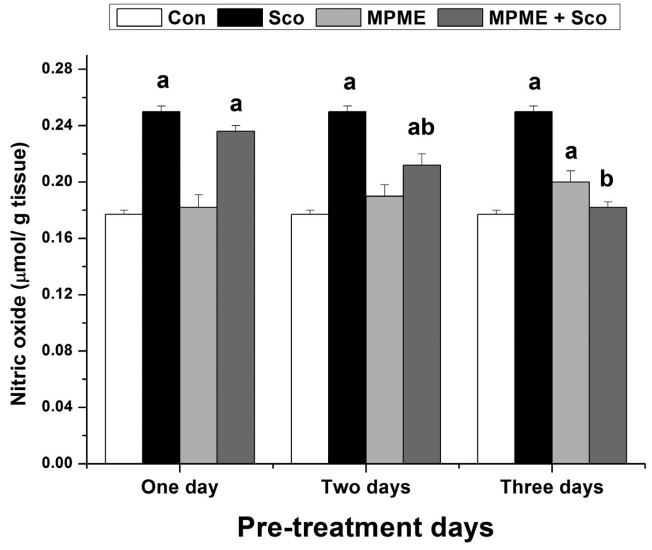

Fig. (3). Effects of MPME treatment for 1, 2 or 3 days at a dose of 250 mg/kg on nitric oxide production in brain of rats treated with scopolamine (1.4 mg/kg)-induced oxidative stress. Values are means ± SD. a: Significant change at $P < 0.05$ with respect to the control group. b: Significant change at $P < 0.05$ with respect to scopolamine group.

In the present study, AChE activity vigorously increased in the brain as a result of injection with scopolamine which is in line with Lee *et al.* [29] and Al-Hazmi *et al.* [30] study

results. Acetylcholine is the most important neurotransmitter involved in the regulation of cognitive functions [31]. Cholinergic transmission is terminated mainly by acetylcholine hydrolysis through the enzyme AChE which is responsible for degradation of acetylcholine to acetate and choline in the synaptic cleft [32]. Our study is in agreement with different studies that provide insight into the molecular basis and the involvement of cholinergic (muscarinic and nicotinic), GABAergic and NMDA receptors of scopolamine-induced memory impairment especially at high doses through the reduction of monoamines and GABA levels in cortex, hippocampus and striatum [33, 34] in addition to the reported increased stress in the brain [29]. Imbalance between cholinergic–serotonergic systems may be responsible for the cognitive impairment associated with AD in cortex [35]. Loss of cholinergic neurons, and subsequent deficits in cholinergic neurotransmission in the hippocampus and cerebral cortex, is also strongly correlated with clinical signs of cognitive impairment and dementia in AD patients [36]. The cholinergic component, along with other neurotransmitters is integrated into the neural circuitry of the dorsal hippocampus that modulates learning and memory functions [37].

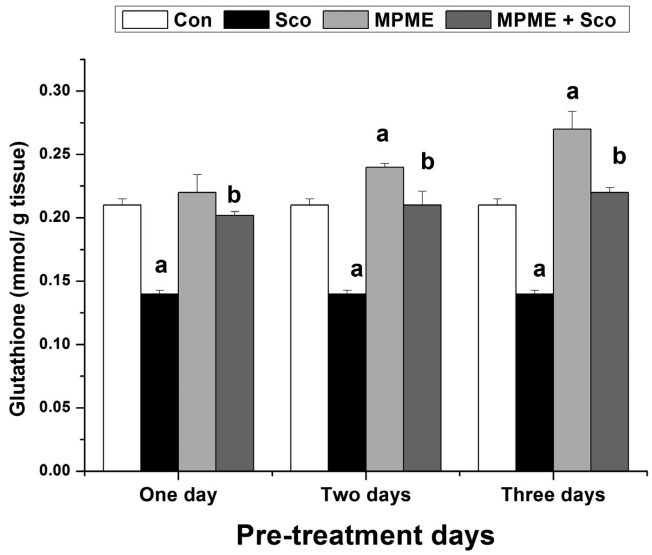

Fig. (4). Effects of MPME treatment for 1, 2 or 3 days at a dose of 250 mg/kg on glutathione depletion in brain of rats treated with scopolamine (1.4 mg/kg)-induced oxidative stress. Values are means ± SD. a: Significant change at $P < 0.05$ with respect to the control group. b: Significant change at $P < 0.05$ with respect to scopolamine group.

Chapman *et al*. [38] showed that, the systemic injection of scopolamine resulted in a robust increase in dopamine levels. Also, scopolamine produced rapid and robust antidepressant and antianxiety effects in patients with unipolar and bipolar depression [39]. Catecholaminergic neurotransmitter systems have been implicated in the pathophysiology of mood disorders. The muscarinic cholinergic system interacts with catecholaminergic neurotransmitter function such that alterations in the balance between systems may have major roles in the pathophysiology of mood disorders [40, 41]. Scopolamine is a 5-HT uptake inhibitor causing a decrease in 5-HT content [42].

Scopolamine treatment induced pathological changes represented as vacuolation and diffuse gliosis in the cerebral cortex (Supplementary data: Fig. **S1**). In the present study, the scopolamine groups had graded neuronal damage. In all the examined sections, the characteristic morphological changes of apoptosis were recognized. Research has demonstrated that patients suffering from AD and various other memory disorders have neuronal cell and synapses loss [43, 44]. Other studies have shown pyknotic, shrunken, tangle-like neurons in cortex, hippocampus and striatum regions [30, 45].

In animals exposed to scopolamine (1.4 mg/kg) injection, an elevation in brain LPO and NO while a reduction in GSH level were observed. Elevation of brain oxidative status in rats resembled the clinical situation where a considerable number have reported the incidence of oxidative stress and membrane lipid peroxidation in demented patients and many studies have reported that memory impairment in the scopolamine-induced animal model is associated with increased oxidative stress within the brain [9, 27]. In addition, the overall peroxidation activity in brains of AD patients was significantly elevated compared to normal subjects [46]. Such peroxidation processes and the overproduction of free radicals may lead to consumption of detoxifying endogenous antioxidants such as GSH.

An amnestic dose of scopolamine was reliably demonstrated in this study to elevate rat brain oxidative status through affecting LPO, NO and GSH levels. This association of oxidative stress with amnesia could be substantiated by the findings of other studies. The resultant effect of scopolamine on oxidative stress indices may not be fully interpreted since the role of cholinergic neurotransmission in mediation of brain oxidative stress has yet to be defined.

In the present study, there was a marked increase in LPO and NO levels on scopolamine injection which was significantly reduced by MPME administration. The preventive effects of MPME have been related to the inhibition of lipid peroxide formation, NO production or free radicals scavenging property as evident from the decreased LPO and NO levels.

Flavonoids were found to prevent GSH depletion by scavenging reactive oxygen species (ROS) [47, 48], therefore, they are inhibiting the oxidative damage of cellular macromolecules. Ishige *et al.* [49] found that flavonoids could protect at three stages of the cell death pathway initiated by GSH depletion: maintaining GSH levels, inhibiting the accumulation of ROS, and blocking calcium influx. Therefore, the increased intracellular GSH level in brain tissues in response to MPME treatment in the present study is indicative of increased free radical scavenging and enhanced detoxification of lipid hydroperoxides. Inhibition of lipid peroxidation by MPME may, at least partially, suppress the injury cascade induced by scopolamine in brain.

In summary, the present study indicates that multiple administrations of mandarin peel could effectively restore antioxidant brain status and may confer neuroprotection due to alleviation of oxidative damage induced by scopolamine. Moreover, mandarin peel might offer a useful therapeutic

choice in either the prevention or the treatment of dementia conditions.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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