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Beneficial Effects of *Citrus aurantium* Extract in Animal Model of Alzheimer's Disease

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ABSTRACT

The well-known cause of dementia is Alzheimer's disease (AD), in which β-Amyloid (Aβ) deposition results in neuronal death. In Iran, Citrus aurantium (CA) flowers are used in the treatment of different neurological disorders. In this study, we examined the protective effects of CA flowers extract (CAFE) against memory impairments induced by Aβ. Adult male Sprague-Dawely rats assigned to three groups (n=8) of: control (Aβ, 3 µl intracerebroventricular, ICV), vehicle (normal saline, 3 µl ICV) and CAFE pre-treated groups (300 mg/kg, IP, for 21 days). Twelve days after Alzheimer induction, behavioral analysis Morris Water Maze (MWM), as well as, western blot and morphological studies were carried out to explore CAFE effect on male rats of Alzheimer. Administration of CAFE significantly restored memory and learning impairments induced by Aβ in the MWM test. CAFE significantly decreased the cytochrome-c expression level in pre-treated group ($p<0.05$). In addition, the used extract reduced the number of degenerated neurons in the hippocampal CA1 area of the pre-treated rats ($P<0.001$). These results demonstrate that CAFE has some beneficial effects in animal model of Alzheimer's diseases.

Key words: β-Amyloid, *Citrus aurantium*, Rat, Neuroprotection

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INTRODUCTION

Alzheimer's disease (AD) as a neurodegenerative disease is the most common form of dementia, that usually starts in old age and results in progressive memory loss, impaired thinking, disorientation, and changes in personality and mood [1]. There is an urgent need for more efficient drugs against AD because available treatments for AD only offer limited symptomatic improvement [2]. The pathological characteristics of AD are accumulation of β-Amyloid (Aβ) and neurofibrillary tangles in the brain [3]. Aβ25–35 is a toxic and biologically active fragment of Aβ [4] that widely used in animal model of AD. Injection of Aβ25–35 resulted in neuronal degeneration in rat cerebral cortex and hippocampus [5].

Recently, considerable data have shown that the brain in AD is under increased oxidative stress and this may have a key role in the pathogenesis of neurodegeneration and death in AD [6], where diverse macromolecules including proteins, DNA, lipids and RNA are modified [7].

Citrus aurantium (CE) flowers are used in the treatment of different neurological diseases including epilepsy, hysteria and neurasthenia [8]. CE has bioactive components with antioxidant properties such as phenolics, flavonoids and vitamins [9]. Previous

studies showed that CE had neuroprotective effects *via* antioxidative and anti-inflammatory actions [10,11]. In the current research, the effects of CAFE on Alzheimer model rat were investigated.

MATERIALS AND METHODS

Animals

Adult male healthy Sprague–Dawley rats (weighing 220–250 g) purchased from neuroscience research animal center, Kerman University of medical sciences (KUMS) and were kept in a controlled environment at room temperature of 21°C ± 1.0°C and automatic day–night program. The rats were divided randomly into three equal groups (n=8): (1) control group, animals which was ICV injected with β-Amyloid 25-35; (2) CAFÉ vehicle group that undertook the same surgical procedures as control, treated with vehicle (distilled water) for 12 consecutive days; and (3) CAFE group that pre-treated by CAFE for 12 consecutive days before β-Amyloid 25-35 administration (300 mg/kg, IP). The CAFE dose was selected based on a pilot study. This study was approved by Kerman University Ethics Committee (EC/KNRC/93/20).

Chemicals

β-amyloid Protein Fragment 25-35 (A4559 Sigma), thionin, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, 2-mercaptoe- thanol, SDS, and benzamidine were obtained from Sigma (St. Louis, MO). An enhanced chemiluminescence (ECL) kit was obtained from Amersham (Little Chalfont, Bucks, United Kingdom). Polyvinylidene difluoride (PVDF) membrane was obtained from Bio-Rad (Hercules, CA). Anti-cytochrome-c rabbit polycolonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Surgery

For ICV injection of β-amyloid 25-35, the animals were anesthetized with ketamine and xylasine (80 and 10 mg/kg, IP, respectively) and a stainless steel cannula was inserted stereotaxically (1.5 mm from the middle, 0.9 mm posterior to the bregma and 3.5 mm from the surface of skull) into the right ventricle. Methylene blue staining was performed for confirmation of the injection site.

Preparation of CAF extract

CE fruits were picked from Jiroft city in April. One flask containing 500 g of grinded flowers was extracted with 80% methanol on a water bath adjusted to 40°C for 24 h. The extract was evaporated to dryness under reduced pressure to give 7 g methanolic extract. The obtained extract was lyophilized and store at -20°C until use.

Total flavonoids content

Method described by Park's [12] used to measure total flavonoid content in this project. 0.3 ml of the extract or different concentrations of quercetin (0 to 100 mg/l) were added to 30% methanol, 0.15 ml of $NaNO_2$ (0.5 M) and 0.15 ml of AlCl3.6 H_2O (0.3 M) in a volumetric flask. At the 5th minute, NaOH (1 M) was added to the solutes and mixed by vortex. The absorbance at 506 nm was measured against blank. Total flavonoid content of CAFE was expressed as mg quercetin equivalents per g dry mass.

Estimation of total phenolic content (TPC)

Folin-Ciocalteu reagent was used for determination of total phenolic compounds content in CAFE. In brief, 1 ml of crude extract were made up to 20 ml with 7% Na_2CO_3, 1 ml Folin–Ciocalteu reagent and distilled water and then mixed thoroughly. The mixture incubated for a further 90 min in the dark. The absorbance of solutes was read at 765 nm against blank and TPC in one gram of the plant extract measured using gallic acid equivalents as standard compound.

Total antioxidant activity

Phosphomolybdenum method used to measure total antioxidant capacity of 1 gram of plant fractions based on ascorbic acid equivalents. Briefly, .1 ml of extract (100 µg/ml) and ascorbic acid added to a solution made by 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The aliquots then incubated in a water bath at 95°C for further 90 min. the absorbance of samples cooled down to room temperature measured at 765 nm. Following formula used to determine the percentage of antioxidant capacity of the extract against control:

Antioxidant effect% = [(control absorbance-sample absorbance)/control absorbance] × 100

EC50 value which describes the effective concentration of sample required to reduce Mo by 50% was also measured. For this purpose, we used a linear regression analysis of dose-response curve plotting between % reduction and concentrations.

Morris water maze test

An assessment of learning and memory through a modified MWM known as Morris Water Maze was done. In this approach, rats learn to escape the water and stand on top of a hidden platform. The lighting of the room in this set up is dim and spatial cues are attached to the walls surrounding the maze. Noldus Ethovision system version 7.1 is a smart video tracing system which was recording the experiment for monitoring the rat's performance.

The maze is a black circular pool made of iron with 1.36 m diameter and 60 cm in height. The pool was filled with water with a temperature of 20°C ± 1°C. The animals learned to find the platform hidden 1.5 cm beneath the water (10 cm diameter and 25 cm height). Platforms location was maintained intact through the experimental trials.

Platforms location with respect to the pool was 30 cm beyond the rim of the pool in the center of one quadrant, considering visual distal cues. In a period of 4 consecutive days, the rats were tested. They were released into the water while facing the rim from four different starting

points in the pool. Platforms location was fixed and an interval was considered between each trial.

The number of training trials 16, 4 sessions of 4 consecutive days, except the final day. The rats had to swim with the aim of finding the hidden platform in 60 seconds. Also, they had to stay on the platform for 30 second. if the animals failed to find the platform they were placed on it for 10 second so that they could observe their surroundings. Traveled distance to find the hidden platform in cm was one of the factors analyzed. Escape latency, known as the time spent to find the hidden platform was another factor as well as swimming speed (cm/s). One day after the last learning trial the rats were tested for spatial memory. In order to achieve that the percentage of the time, distance, and frequency spent in target quadrant was assessed.

Immunoblot analysis

The brain tissues of rats were homogenized by an ice-cold buffer containing 0.1% Na deoxycholate, 0.1% SDS, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1% NP-40 with protease inhibitors (2.5 µg/ml of leupeptin, 1 m Mphenylmethylsulfonyl fluoride, 10 µg/ml of aprotinin), and 1 mM sodium orthovanadate to measure the cytochrome-c. The homogenate was centrifuged at 12000 rpm at 4°C for 20 min. The supernatant, as the whole cell fraction, was retained. The measurement of protein concentrations (Bio-Rad Laboratories, Munchen, Germany) was performed using Bradford method. The same quantities of protein were resolved electrophoretically on a 9% SDS-PAGE gel and transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). After blocking with 5% non-fat dried milk in Tris buffered saline with Tween 20 (blocking buffer, TBS-T, 150 mM NaCl, 20 m M Tris–HCl, pH 7.5, 0.1% Tween 20) (overnight at 4°C), the membranes were explored with 1: 1000 for 1.5 h at 20°C. After washing with TBS-T (three times, 5 min), the blots were incubated at room temperature for 1 h with a horseradish peroxidase-conjugated secondary antibody (1:15000, GE Healthcare Bio-Sciences Corp). All antibodies were diluted in blocking buffer. The antibody–antigen complexes were discovered by the ECL system and exposed to Lumi-Film chemiluminescent detection film (Roche Applied Science, Mannheim, Germany). The Lab Work analyzing software (UVP, Cambridge, UK) was used for determining the intensity of expression. B-actin immunoblotting (antibody from Cell Signaling Technology, Inc.; 1:1000) was used as a loading control.

Histology

The rat brains' were processed based on standard histological methods for quantative study. Paraffinized brains were cut into 6 µm sections on an automatic microtome (Leica RM2145, Austria) and the sections were stained with thionin (Nissl method). Neuronal damage was then approximated for each animal as the rate of the number of degenerated pyramidal neurons to that of both surviving and degenerated in three separate fields (medial, middle and lateral) of the hippocampal CA1 sector.

Statistical analysis

One-way analysis of variance (ANOVA) followed by the Tukey's post hoc test were used for analysis of data. The data were presented as mean ± SEM. The P- values <0.05 were considered statistically significant.

RESULTS

Measurement of total flavonoids contents and total phenolics

Flavonoids content of CAFE was 40 mg as quercetin equivalents/g fraction. Total phenolic compounds content as recorded in next table in CAFE equals to 602.6 mg gallic acid (GAE) per gram of dried extract (Table 1).

Antioxidant activity of CAFE

The potential antioxidant activity of CAFE extract was evaluated based on phosphomolybdenum method. The assay is a quantitative method and it works based on the reduction of Mo (VI) to Mo (V) in presence of antioxidant compound and subsequent formation of a green phosphate/Mo (V) complex at acidic pH and at higher temperature. The value of total antioxidant capacity of extract was as 27.70 µg AE g^{-1} extract (Table 1). CAFE extract exhibited antioxidant capacity in a concentration-dependent manner (Table 2).

IC50 value was also calculated from a calibration curve for the extract. IC50 value of the extract was determined as 70.84 µg/ml (Table 1).

Table 1: Total phenolics and flavonoids content of CAFÉ

Sample	Total phenolics content mg (GAE)/ gram extract	Total Flavonoids content mg (quercetin)/gram extract	Antioxidant capacity (µg AE g^{-1} extract)	EC50 µg/ml
CAFE	602.6	83.7	27.7	70.84

Table 2: Antioxidant capacity of CAFE based on phosphomolybdenum assay

Citrus extract conc. (µg/ml)	mg/g ASC equivalent	Antioxidant activity (%)
50	11.3	36.68
100	22.2	50.59
200	32.5	65.9
400	52.3	76.52

Learning and memory

There was a significant difference in distance traveled to find the hidden platform (p>0.05) (Figure 1A), and escape latency (p>0.001) (Figure 1B) for control compared with CAFÉ vehicle. Pre-treatment with CAFE significantly improved the aforementioned parameters (p>0.05, p>0.001 respectively). There was no significant difference in swimming speed among all groups.

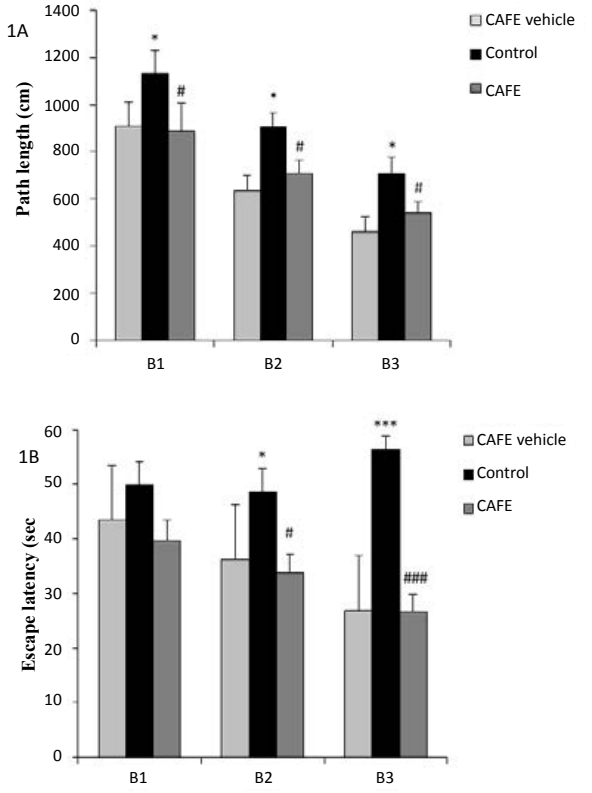

Figure 1: Effects of β-amyloid injection on learning parameters in male rats. β-amyloid administration resulted in significant increase of the traveled distance (A) and escape latency (B) to find the hidden platform in control group at all three blocks compared to CAFÉ vehicle (Pre-treatment with CAFE significantly decreased the distance and time to find the hidden platform in blocks compared to control group. Data are shown as mean ± SEM [*p<0.05 and ***p<0.001 compared to CAFÉ vehicle. #p<0.05 and ###p<0.001 compared to control group; in all groups n=7; CAFÉ: *Citrus aurantium* flowers extract)

There was a significant difference in percent of distance, time spent, and frequency in target quadrant for control compared with CAFÉ vehicle (p>0.001, 0.001, 0.01 respectively). Administration of CAFE ameliorated the above mentioned parameters (p>0.001, p>0.001, 0.001 respectively) (Figure 2).

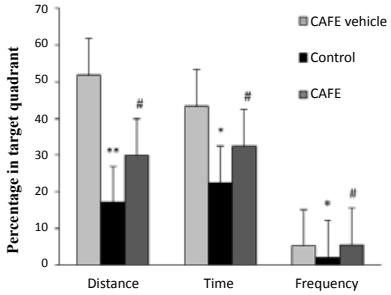

Figure 2: The effects of β-amyloid and CAFE on spatial memory in the MWM test (Percentage distance spent in target quadrant demonstrates the animals treated with β-amyloid (control) presented less distance compared to vehicle (p< 0.001). CAFE markedly ameliorated this parameter (#p<0.001). In percentage time spent in target quadrant, the animals treated with β-amyloid (control) presented less distance compared to vehicle (*p< 0.001). CAFE significantly improved this parameter (#p<0.01). In frequency in target quadrant, the animals treated with β-amyloid presented less frequency compared to sham (*p<0.001). CAFE significantly ameliorated this parameter (#p<0.001); Data are expressed as mean ± S.E.M. CAFÉ: *Citrus aurantium* flowers extract)**

Histological assessment

In the histological examination, the neuronal morphology in the CAFÉ-vehicle group was intact including light pink cytoplasm; round or oval light nuclei with prominent nucleolus. On the contrary, severe degenerative alterations were observed in most pyramidal neurons in the control group including an eosinophilic cytoplasm and dark piknotic nuclei. The severity of the degenerative changes observed in the cytoplasm and nucleus of CA1 neurons was less in the CAFE-treated group compared to control. Neuronal counting (the percentage of degenerated neurons) in CA1 area of hippocampus showed significant differences between the vehicle-treated (5.39%) and control group (29.11%) (p<0.001). Administration of CAFE markedly decreased the percentage of degenerated neurons in CA1 area of pre-treated animals (11.84%) (p<0.001) (Figure 3).

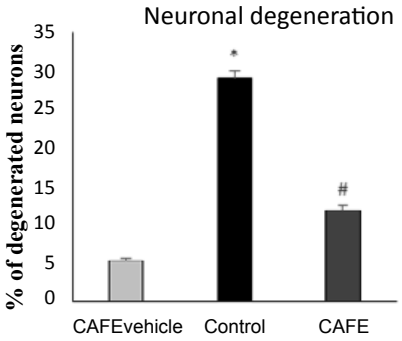

Figure 3: The effects of β-amyloid and CAFE on neuronal injury in male rats (Neuronal injury in hippocampal CA1 area was assessed using Nissl staining; β-amyloid markedly increased (29.11%) degenerated neurons comparing to CAFE vehicle (5.39%) (*p<0.001); CAFE significantly decreased (11.84%) the detrimental effect of β-amyloid induced damages (#p<0.001). Data are expressed as mean ± S.E.M; CAFÉ: *Citrus aurantium* flowers extract)

Cytochrome-c expression

Cytochrome-c immunoreactivity was evident in the hippocampus of male rats 12 days after β-amyloid administration (Figure 4A, upper panel). However, it was detected in the normal hippocampus. Obstinately, a consistent amount of β-actin immunoreactivity is seen in Figure 4A, bottom panel, suggesting that the amount of the loaded protein was consistent. Amyloid 25-35 activated cytochrome-c in hippocampus and administration of CAFE inhibited the amyloid-induced cytochrome-c activation in hippocampus of male rats (Figure 4B).

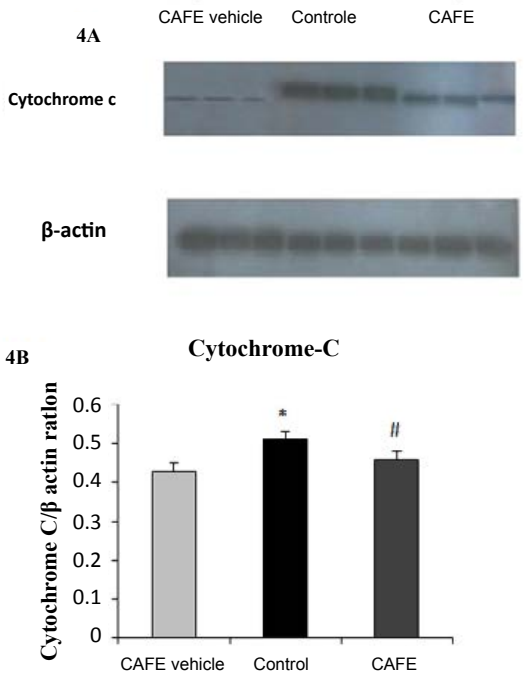

Figure 4: (A) Shows a representative western blot of cytochrome-c expression in the hippocampus obtained from CAFÉ-vehicle, control and CAFÉ-treated rats (upper panel). (B) Statistical analysis verified the significant increase ($p<0.05$) of cytochrome-c in 12 days after β-amyloid injection compared with CAFÉ-vehicle group. (A) Pre-treatment with CAFÉ significantly diminished cytochrome-c expression in hippocampus of male rats (Bottom panel) ($p<0.05$)

DISCUSSION

In the present study, CAFE at the dose of 300 mg/kg markedly ameliorated spatial memory in MWM test compared to control group. β-amyloid disturbed learning and spatial memory in MWM test and CAFE pre-treatment preserved the effect of it. In addition, CAFE pre-treatment markedly diminished neuronal injury in hippocampus of male rats.

The cognitive-augmenting activity of CAFE on the Aβ 25-35 induced memory deficits in rat was investigated using MWM test. Rats treated with Aβ demonstrated more prolonged escape latency than rats in the vehicle group. CAFE pre-treatment significantly reduced escape latency in pre-treated group which suggested that long-term memory was impaired by Aβ. Besides, during the probe trial session, the Aβ induced lessening in swimming times within the target quadrant that was obviously improved by CAFE, indicating a positive effect on spatial memory probably due to its anti-oxidant and anti-inflammatory actions. In agreement with our results, Ono et al. confirmed that phenolic compounds can block early assembly processes of Aβ and these compounds are valuable therapeutic factors for AD [13,14]. Additionally, different studies have revealed that the plant biophenols can decrease fibril formation and toxicity of Aβ [15,16]. Phenolic compounds exact mechanism against Aβ toxicity is unknown, while studies have recommended the amyloid-binding affinity of these compounds affecting Aβ on various levels, e.g. by direct inhibiting fibril formation, inhibiting Aβ accumulation, and promoting nontoxic pathways. Moreover, biophenols are involved in the inhibition of oxidative stress and neuroinflammation induced by Aβ progression [17]. Our results showed that improved learning and memory in Aβ-treated memory impairments in rats may be due to decrease in oxidative stress in the hippocampus of rats. It has been reported that CAFE has repairing effects on memory impairments and behavioral abnormalities induced by scopolamine and may have useful effects in the treatment of AD [10].

Numerous studies have shown that neuronal death in the cerebral cortex and hippocampus is the main reason for cognitive decline in AD [18] and it has been proposed that at least some part of this neuronal death is due to apoptosis [19]. Many studies have demonstrated that cytochrome-c plays a key role in apoptosis [20,21].

Stimulation of the intrinsic mitochondrial apoptotic pathway by ROS and mitochondrial DNA damage promote outer membrane permeabilization and mitochondria to cytosol translocation of cytochrome-c which triggers caspase-dependent cytosolic signaling events [19]. Cytochrome-c forms the apoptosome complex with apoptotic protease-activating factor-1 (Apaf-1) and recruited procaspase-9, which induces cleavage of caspases-3 and -7 in caspase-dependent pathway. Activation of these factors results in nuclear chromatin condensation and DNA fragmentation [22]. In our study, results showed that Aβ increased cytochrome-c expression level in the treated group and CAFE significantly decreased its level. On the other hand, neuronal degeneration study proved that CAFE could protect CA1 pyramidal neurons against the toxic effect of Aβ. These results recommend that the advantageous effects of CAFE may be due to potent antioxidant and anti-apoptotic activities of the used extract. In accordance with our results, Elyasi et al. reported that CAFÉ has protective effects against neurotoxicity, induced by 6-hydroxydopamine (6-OHDA); these protective properties were along with antiapoptotic features [23].

CAFÉ is a rich source of flavonoids including nobiletin,

naringin and hesperidin. These flavonoids have various pharmacological activities such as anti-inflammatory and anti-oxidant.

Naringin, a prominent flavonoid of CAFE, has antioxidant, anti-inflammatory, and anti-apoptotic activities [9]. Nobiletin can pass through the blood–brain-barrier. It has a potential therapeutic advantage of dementia including Alzheimer's disease. It has been shown that Nobiletin can improve dizocilpine-memory impairments by activating extracellular signal-regulated kinase signaling in mice hippocampus [24].

Based on the obtained data, Aβ can cause severe neuronal injury in hippocampus of male rats and CAFE-treatment diminished neuronal death. Our results suggest that the positive effects of CAFE may be due to strong antioxidant and anti-inflammatory properties of its components.

CONCLUSION

Although the used extract demonstrated some protective effects, further studies need to be done to identify the exact mechanism of it.

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CONFLICT OF INTERESTS

All authors declare that there is no conflict of interest.

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