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Investigation of anti-Alzheimer's activity of aqueous extract of areca nuts (*Areca catechu* L.): *In vitro* and *in vivo* studies

[Investigación de la actividad anti-Alzheimer del extracto acuoso de nueces de areca (*Areca catechu* L.): estudios *in vitro* e *in vivo*]

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Abstract: Alzheimer's disease (AD) is an age-related neurodegenerative disorder. Sever cognitive and memory impairments, huge increase in the prevalence of the disease, and lacking definite cure have absorbed worldwide efforts to develop therapeutic approaches. Since many drugs have failed in the clinical trials due to multifactorial nature of AD, symptomatic treatments are still in the center attention and now, nootropic medicinal plants have been found as versatile ameliorators to reverse memory disorders. In this work, anti-Alzheimer's activity of aqueous extract of areca nuts (*Areca catechu* L.) was investigated via in vitro and in vivo studies. It depicted good amyloid β (Aβ) aggregation inhibitory activity, 82% at 100 µg/mL. In addition, it inhibited beta-secretase 1 (BACE1) with IC50 value of 19.03 µg/mL. Evaluation of neuroprotectivity of the aqueous extract of the plant against H2O2-induced cell death in PC12 neurons revealed 84.5% protection at 1 µg/mL. It should be noted that according to our results obtained from Morris Water Maze (MWM) test, the extract reversed scopolamine-induced memory deficit in rats at concentrations of 1.5 and 3 mg/kg.

Keywords: *Areca catechu*; Alzheimer's disease; Amyloid beta; BACE1; Iranian traditional medicine.

Resumen: La enfermedad de Alzheimer (EA) es un trastorno neurodegenerativo relacionado con la edad. Los severos deterioros cognitivos y de la memoria, el enorme aumento de la prevalencia de la enfermedad y la falta de una cura definitiva han absorbido los esfuerzos mundiales para desarrollar enfoques terapéuticos. Dado que muchos fármacos han fallado en los ensayos clínicos debido a la naturaleza multifactorial de la EA, los tratamientos sintomáticos siguen siendo el centro de atención y ahora, las plantas medicinales nootrópicas se han encontrado como mejoradores versátiles para revertir los trastornos de la memoria. En este trabajo, se investigó la actividad anti-Alzheimer del extracto acuoso de nueces de areca (*Areca catechu* L.) mediante estudios in vitro e in vivo. Representaba una buena actividad inhibidora de la agregación de amiloide β (Aβ), 82% a 100 µg/mL. Además, inhibió la beta-secretasa 1 (BACE1) con un valor de CI50 de 19,03 µg/mL. La evaluación de la neuroprotección del extracto acuoso de la planta contra la muerte celular inducida por H2O2 en neuronas PC12 reveló una protección del 84,5% a 1 µg/mL. Cabe señalar que, de acuerdo con nuestros resultados obtenidos de la prueba Morris Water Maze (MWM), el extracto revirtió el déficit de memoria inducido por escopolamina en ratas a concentraciones de 1,5 y 3 mg/kg.

Palabras clave: *Areca catechu*; Enfermedad de Alzheimer; Beta amiloide; BACE1; Medicina tradicional iraní.

INTRODUCTION

Alzheimer's disease (AD) is a progressive and age-associated neurodegenerative disorder. It is usually characterized by the cognitive and functional deficiency as well as behavioral changes in functional abilities. AD is the most common type of dementia (60-70% of all cases) and the prevalence of disease is increasing with increasing age. It has been also known as one of the five main causes of death worldwide affecting 10% of people over the age of 65 and approximately 50% of people over the age of 85. It is estimated that the number of people with AD will reach 131 million in 2050. Another issue comes back to the cost imposed by AD which is comparable to cardiovascular disorders (Zvěřová, 2019; Taylor & Sloan, 2000).

Etiology of AD has not been completely recognized. Various complex mechanisms have been identified to be involved in the pathogenesis of AD. They usually include accumulation of extracellular Aβ42 plaques (Takahashi *et al*., 2017), intracellular hyper-phosphorylated tau neurofibrillary tangles (Naseri *et al*., 2019), mitochondrial dysfunction leading to oxidative stress (Bhat *et al*., 2015), and reduction of acetylcholine in the brain (Chen & Mobley, 2019). Currently, it has been revealed that treatment of AD based on the single-target drug therapies has failed due to multifactorial nature of the disease (Cummings *et al*., 2019). In this regard, design and development of therapeutic agents based on the various mechanisms involved in AD has attracted a great deal of attention in drug discovery research (Cummings *et al*., 2018; González *et al*., 2019; Ibrahim & Gabr, 2019; Wang *et al*. 2019). Although design and synthesis of selective ligands pointing single disease targets, usually has been the main tool in drug discovery research, this approach has not been always successful owing to the multifactorial pathogenesis of many diseases such as AD. In this regard, phytotherapy which profits from the action of a mixture of constituents, offers a new and efficient approach to the treatment of many diseases (Efferth & Koch, 2011).

Potential of herbal plants in the treatment of AD has been widely discussed in the literature (Perry *et al*., 1998; Howes & Houghton, 2003; Houghton & Howes, 2005; Perry, 2007; Rao *et al*., 2012; Vyoma, 2015; Sahoo *et al*., 2018). In continuation of our research program on the efficacy and versatile biological activities of medicinal plants, we previously investigated *in vitro* cholinesterase (ChE) inhibitory activity of different extracts of *Areca catechu* L. (Saeedi *et al*., 2017) (Table No. 1) which was recommended in Iranian traditional medicine (ITM) for improving the memory.

A. catechu belongs to Arecaceae family and grows in Asia, the tropical Pacific and some regions in east Africa (Lechner *et al*., 2019). The plant is famous for its seeds, which are generally called betel nuts or areca nuts. It contains different types of phytochemicals like alkaloids, tannins and polyphenols (Peng *et al*., 2015) and its efficacy in decreasing AD symptoms has been discussed by Bhat et al. (Bhat *et al*., 2017).

In this work, we developed anti-AD activity of aqueous extract of *A. catechu* using various assays. On the one hand, the plant was frequently recommended in ITM for the memory improvement. On the other hand, its efficient cholinesterase (ChE) inhibitory activity both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities (IC_{50}s = 32.0 and 48.8 µg/mL, respectively. Saeedi *et al*., 2017) was confirmed in our previous study. In this respect, we decided to expand our study to the evaluation of aqueous extract of *A. catechu* for its multi-target activity against AD including inhibitory activity against accumulation of Aβ and BACE1 as well as neuroprotectivity and memory improvement *via in vivo* Morris Water Maze (MWM) which are reported for the first time.

MATERIAL AND METHODS

Plant material and extraction

Areca nuts were obtained from Tehran market, Iran, identified and deposited in the herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran using the following code PMP-691. The powdered nuts (10 g) was boiled in water (200 mL) for 15 min, the resulting suspension was centrifuged for 5 min at 3000 rpm, dried under vacuum, and the lyophilized to afford the aqueous extract.

Self-induced Aβ (1-42) aggregation assay

Inhibition of self-induced Aβ (1-42) aggregation was measured using a Thioflavin T (ThT) binding assay comparing with curcumin as descried in the literature (Bartolini *et al*., 2007; Rahmani-Nezhad *et al*., 2019). HFIP pretreated Aβ (1-42) samples (Anaspec Inc) were resolubilized in a phosphate buffer (50 mM, pH 7.4) affording a solution of 20 µM. 10 µL of peptide solution (10 µM, final concentration) with or without the sample solution (10 µL, 50 µg/mL, prepared in DMSO, final concentration) was incubated at 30°C for 48 h. Blank sample containing phosphate buffer

(50 mM, pH 7.4) instead of Aβ with or without inhibitor was also involved in the assay. After incubation, samples were diluted to a final volume of 200 µM using glycine-NaOH buffer (50 Mm, pH 8.0) containing thioflavin-T (5 µM). Each measurement was run in triplicate and fluorescence was measured on a Synergy HTX Multi-Mode reader from BioTek Instruments with excitation and emission wavelengths at 440 nm and 485 nm, respectively. The percent inhibition of aggregation was calculated by the expression $(1- IF_i/IF_O)\times100\%$ in which IF_i and IF_O are the fluorescence intensities obtained for Aβ in the presence and absence of inhibitor, respectively.

BACE1 enzymatic assay

The BACE1 enzyme inhibition assay was achieved using a FRET (Forster resonance energy transfer) kit, from Invitrogen (former Pan Vera corporation, Madison, WI) comparing with OM99-2 as the reference inhibitor based on the literature (Iraji *et al*., 2017; Rastegari *et al*., 2019). BACE1 (purified baculovirus-expressed enzyme) was diluted using buffer (50 mM sodium acetate, pH 4.5) to prepare a 3X working solution of 1 Unit/mL. The peptide substrate (RhEVNLDAEFK-Quencher) was also diluted in the same buffer to obtain the 3X stock solution. DMSO stock solutions were diluted with buffer to give 3X solution of test samples at different concentrations. The 3X solution of BACE1 enzyme (10 µL) and each inhibitor sample (10 µL) were placed in 96-well plates and gently mixed. The substrate 3X solution (10 µL) was then added to this mixture in each well to initiate the reaction at the final reaction volume of 30 µL. The reaction mixtures were incubated at 25°C for 90 min in the dark and then the reaction was stopped by adding 10 µL of 2.5 mM sodium acetate. Fluorescence was monitored at 544 nm (excitation wave length) and 590 nm (emission wavelength). OM99-2 was used as the reference drug, the IC_{50} value was calculated with Curve Expert software version 1.34 for Windows, and each experiment was repeated for three to five times.

Neuroprotection assay

PC12 cell line was obtained from Pasteur institute and all culture media as well as supplements were purchased from Gibco. The neuroprotection assay against H_2O_2-induced cell death in PC12 neurons was completely performed according to our previous report (Rastegari *et al*., 2019). Briefly, cells were cultivated in DMEM supplemented with 10% fetal calf serum plus antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin). To induce neuronal differentiation, PC12 cells were re-suspended using trypsin/EDTA (0.25%) and seeded in 96 well culture plate (4000 cells/well) and cultured for 1 week in differentiation medium (DMEM + 2% horse serum + NGF (100 ng/mL) + penicillin & streptomycin). To evaluate the effect of aqueous extract *A. catechu* on the survival rate of neurons, the culture medium was changed to NGF free medium and different concentrations of the above-mentioned extract (1, 10, 100 µg/mL) were applied on cells. Quercetin (3 µg/mL) was applied as a positive control. The extract was diluted in DMEM and added to each well in the volume of 10 µL. Then, after 3 h, induction of ROS mediated apoptosis was initiated by adding the H_2O_2 (400 µM) to their medium. After 12 h, MTT assay was performed (29). The plates were allowed to stand overnight in the incubator in a humidified atmosphere. Absorbance was measured at 570 nm with a reference wavelength of 630 nm using a plate reading spectrophotometer (BioTek ELx808, USA).

Morris water maze

The animals were randomly divided into the following groups containing 7 rats in each group. (*i*) The normal saline-treated group which received normal saline intra-peritoneally (ip) as extract vehicle 1 h before training (control group). (*ii*) The extract groups which received three different doses of the extract (10, 20, and 40 mg/kg, ip, dissolved in normal saline) with 7 rats in each dose group 1 h before training. (*iii*) The scopolamine group with 7 rats which received scopolamine (4 mg/kg, ip, dissolved in normal saline) 30 min before training. (*iv*) The extract + scopolamine groups which were administered at four different doses of extract (1.5, 3, 6, and 10 mg/kg) with 7 rats in each dose group 1 h before training and scopolamine (4 mg/kg) 30 min later and they were trained in the Morris water maze (MWM) 30 min after scopolamine administration.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test was used for the comparison of test groups, first 4 days of trials (training days), and post training probe trial test, respectively. All the data were expressed as Mean ± SD.

Results and discussion

To develop herbal strategies in the treatment of AD,

we searched credible sources of Iranian traditional medicine (ITM) and found *A. catechu* beneficial for the improvement of memory and in this respect, ChE inhibitory activity of various extracts of the betel nuts were studied (Table No. 1, Saeedi *et al*., 2017). The aqueous extract demonstrated good anti-AChE and anti-BChE activity with IC_{50} values of 32.0 and 48.8 µg/mL, respectively. The multi-factorial nature of AD and good ChEI activity encouraged us to investigate further biological activities associated with the creation and progress of AD.

Table No. 1
The IC_{50} values of aqueous extract of against AChE and BChE[a]

Plants	Name	Aqueous extract AChEI [IC50 (µg)/mL]
Betel nuts	*Areca catechu*	32.00 ± 0.84
Rivastigmine		2.77 ± 0.09

[a]Data are expressed as Mean ± SE (three independent experiments)

Various biological properties such as anti-malarial (Boniface *et al*., 2014), antioxidant (Bhandare *et al*., 2010), anti-allergic (Wang *et al*., 2013), and anti-migraine (Bhandare *et al*., 2011) have been reported for areca nuts. Also, it has been known to possess beneficial effects on digestive and nervous systems (Bhat *et al*., 2017; Peng *et al*., 2015). Bhat *et al*. (2017) have reviewed different studies, endorsing anti-AD activity of *A. catechu*. Although a wide range of compounds have been isolated from *A. catechu*, pyridine-type alkaloids and condensed tannins are the main components and among them arecoline has been identified as the primary and key bioactive constituent probably responsible for the desired biological activities. The role of arecoline in increasing the level of acetylcholine in the Central Nervous System (CNS) of animals and AD patients have been fully discussed in the literature (Peng *et al*., 2015; Volgin *et al*., 2019). Also, significant neuroprotective activity of hydroalcoholic extract of betel nuts at a dose of 200 mg/kg on Aβ-induced cognitive dysfunction in mice has been reported by Kannan *et al*. (2013). However, other biological activities involved in AD have not been investigated and in this study, they were evaluated.

Inhibition of self-induced Aβ (1-42) aggregation

The role of self-assembly of amyloid β-protein is noticeably recognized in the pathogenesis of AD (Sikanyika *et al*., 2019). In this respect, aqueous extract of *A. catechu* was evaluated for its Aβ aggregation inhibitory activity. It showed very good inhibitory activity (82.00 ± 0.53% at 100 µg/mL) comparing with curcumin (36.15 ± 2.88% at 10 µM).

BACE1 enzymatic assay

Inhibition of β-site amyloid-precursor-protein-cleaving enzyme 1 (BACE-1) which cleaves amyloid precursor protein (APP) at the β-secretase site currently has attracted lots attention even more than Aβ aggregation (Prati *et al*., 2018). In this study, aqueous extract of *A. catechu* was tested against BACE-1 and showed good inhibition with IC_{50} value of 19.03 ± 7.44 µg/mL comparing with OM99-2 (IC_{50} = 0.014 ± 0.003 µM) as the reference drug.

Neuroprotective effect against H_2O_2-induced cell death in PC12 neurons

Neuroprotective effect of aqueous extract of *A. catechu* at different concentrations (0.1, 1, and 10 µg/mL) was investigated against oxidative damage induced by H_2O_2 in PC12 neurons in comparison to intact (normal, no intervention), quercetin+H_2O_2-treated (positive control) and H_2O_2-treated (negative control) cells (Figure No. 1). It showed good neuroprotectivity at the above mentioned concentrations by 74.4, 84.5, and 64.9%, respectively comparing with quercetin (cell viability = 69.3%) (p<0.0001vs. H_2O_2-treated).

Morris water maze

The effects of aqueous extract of A. catechu on learning and memory for all training days and post training probe trial test in healthy rats

The effect of the aqueous extract of *A. catechu* on spatial memory was assessed using the MWM test

(Figure No. 2). Figure No. 2A represents the mean escape latency during 4 consecutive day training. The groups that received the extract at the dose of 10 mg/kg showed significant improvement in mean escape latency in comparison to control group (p<0.05) while groups treated at the doses of 20 and 40 mg/kg did not display significant difference comparing with the control group. There were no significant difference in swimming speed between the groups during 4 consecutive day training (Figure No. 2B).

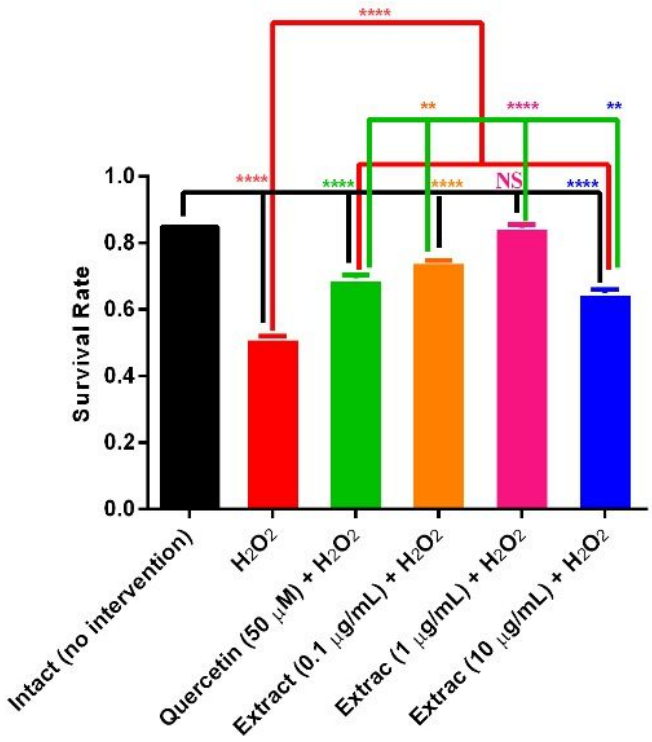

Figure No. 1
Neuroprotective effect of aqueous extract of *A. catechu* on survival of H_2O_2-treated neurons. Data are expressed as mean ± SD and one-way analysis of variance ANOVA followed by Dunnett's multiple comparisons test was used to determine the level of significance (**p<0.0001, (**p<0.01)**

Also, animals in the10- and 20-mg/kg-treated groups did not show significantly different time spent in the target quadrant comparing with the control group in the probe test whereas 40-mg/kg-treated group spent significantly less time in the target quadrant than the control group with p<0.001 (Figure No. 2C). These results indicated that the extract may lead to memory damage at higher doses and lower doses were selected for the study of memory impairment with scopolamine.

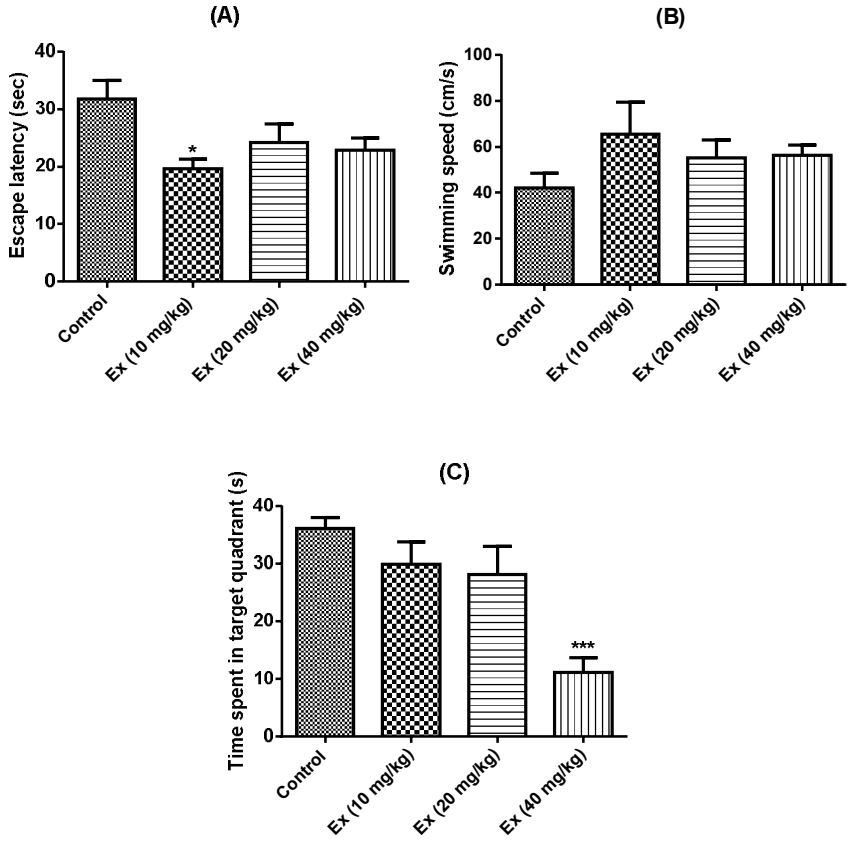

Figure No. 2
The effect of aqueous extract of *A. catechu* on escape latency (A), escape latency (B) swimming speed during training sessions and (C) time spent in target quadrant during post training probe trial test of healthy rats in Morris water maze task. Each animal received extract or normal saline (Control) intra-peritoneally 1 h before training. Each column represents mean ± SEM for 6 animals. *Significantly different from control group (ANOVA,*$p<0.05$, **$p<0.01$)

The effects of aqueous extract of A. catechu on scopolamine-induced learning and memory impairment in the Morris water maze task

As shown in Figure No. 3, the scopolamine treated group showed significant difference comparing with the normal saline-treated group in all training days in escape latency ($p<0.01$) (Figure No. 3A). These animals spent less time in the target quadrant comparing with the normal saline-treated group in the probe test ($p<0.01$) (Figure No. 3C) which implies that the scopolamine treatment impaired the learning ability of the animals. It is clear that the escape latency was improved in groups that received extract at the doses of 1.5 and 3 mg/kg plus scopolamine in comparison to scopolamine administered group ($p<0.05$). It can be observed that administration of the extract at the doses of 6 and 10 mg/kg could not reverse scopolamine-induced memory impairment in escape latency (Figure No. 3A).

Also in the probe test, animals pretreated with extract doses of 1.5 and 3 mg/kg spent significantly more time in the target quadrant comparing with the scopolamine-treated group (Figure No. 3C) ($p<0.05$) whereas groups pretreated with extract at the doses of 6 and 10 mg/kg did not show significant difference comparing with the scopolamine group. Also, there were no significant difference in speed between the groups (Figure No. 3B).

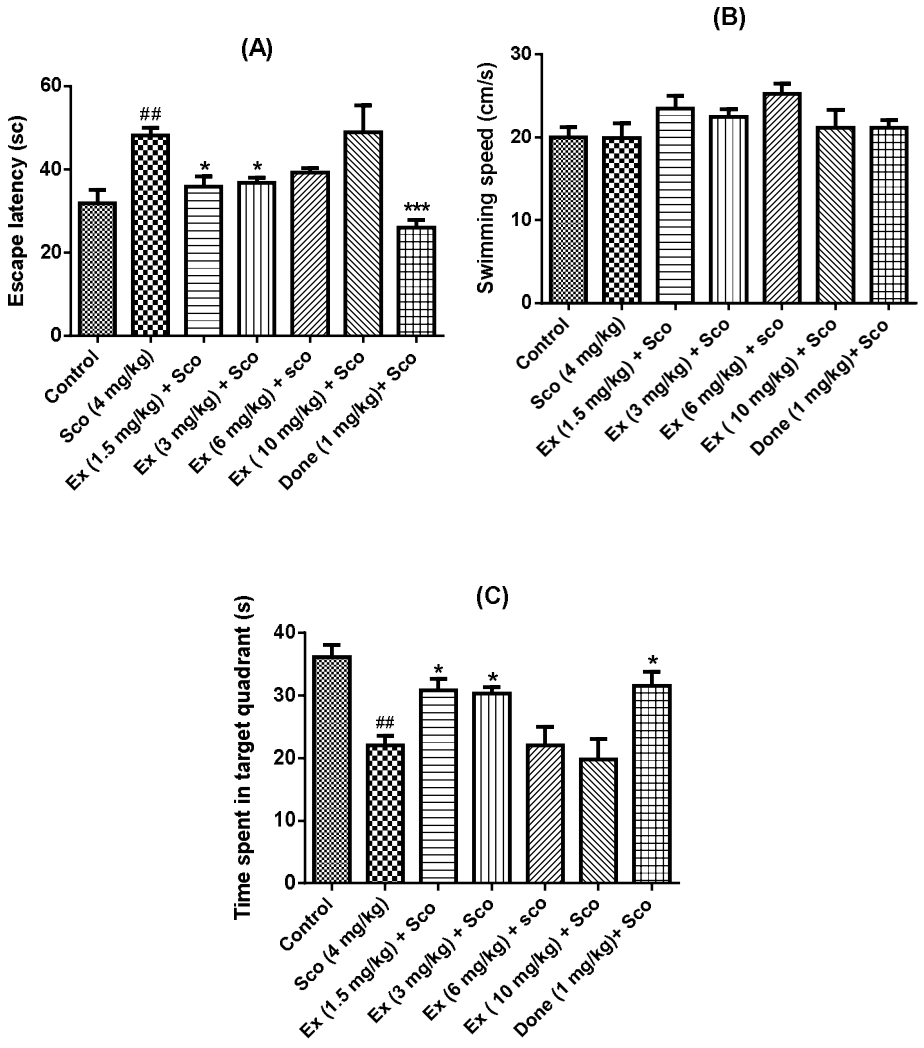

Figure No. 3

The effect of aqueous extract of *A. catechu* on escape latency (A) and swimming speed (B) on scopolamine-induced memory impairment in Morris water maze task during training sessions and time spent in target quadrant during post training probe trial test (C). Each animal administrated intra-peritoneally extract 1 h and scopolamine 30 min before training in training days and the probe trail was performed 24 h later than last training day. Each column represents mean ± SEM for 6 animals. Sco: Scopolamine. *Versus scopolamine -treated group (ANOVA, *p<0.05). #Versus normal saline -treated group (ANOVA, ##p<0.01)

CONCLUSION

In conclusion, aqueous extract of *A. catechu* L. depicted good and satisfactory biological activities against AD. It possessed AChE and BChE inhibitory activity with IC_{50}s = 32.0 and 48.8 µg/mL, respectively. Inhibitory activity against accumulation of Aβ and BACE1 were also evaluated and it showed 82% (at 100 µg/mL) and IC_{50} = 19.03 µg/mL, respectively. Also, good neuroprotectivity against H_2O_2-induced cell death in PC12 neurons was observed. Our results from MWM test revealed that the extract at the doses of 1.5 and 3 mg/kg could reverse scopolamine-induced memory deficit in rats. Considering the fact that LD_{50} of the aqueous extract of betel nuts was found to be ≥15.000 mg/kg body weight (Sari *et al*., 2014; Kim *et al*., 2018), using very low toxic dose of extract would be beneficial to afford an herbal remedy possessing multi-target activity for the treatment of AD.

Ethical approval

All *in vivo* experiments were achieved according to ethical principles approved by Animal Ethics Committee of Tehran University of Medical Sciences with ID IR.TUMS.VCR.REC.1395.1123.

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