Effects of *Cyperus rotundus* Extract on Spatial Memory Impairment and Neuronal Differentiation in Rat Model of Alzheimer's Disease

Abstract

Background: Alzheimer's disease (AD) is one of the most common neurodegenerative diseases in the older population and characterized by progressive memory and cognitive impairment. *Cyperus rotundus*, a traditional medicinal herb, has analgesic, sedative, and anti-inflammatory effects and also used to increase memory in Islamic traditional medicine. This study was designed to consider the effects of *C. rotundus* extract on memory impairment and neurogenesis in the Beta-Amyloid rats' model. **Materials and Methods:** Forty-two male Wistar rats were randomly divided into six groups ($n = 7$) for the evaluation of baseline training performance in the Morris water maze test. Then, amyloid-beta (Aβ1-42) was injected in animal hippocampal CA1 bilaterally in four groups. The first probe trial was performed 21 days after Aβ injection. Then, 250, 500, and 750 mg/kg of *C. rotundus* extract were administered to three Aβ-injected groups for 1 month; after that, the second probe trial was performed, and rats were sacrificed after 28 days of the second probe trial. The neurogenesis was detected in the hippocampus, by immunohistochemical staining. **Results:** This study showed that spatial memory increased in the behavioral test in AD treated group with *C. rotundus* extract, compared with the AD group ($P = 0.02$). Immunohistochemical staining revealed that neuronal differentiation has been occurred in the hippocampus in the AD-treated group with *C. rotundus* extract compared with the AD group ($P = 0.01$). **Conclusions:** This study showed that *C. rotundus* extract, repaired spatial memory impairment in the Aβ rats, through increased neurogenesis in the hippocampus, which could be related to the flavonoid components in the extract.

Keywords: *Cyperus rotundus*, neuronal differentiation, spatial memory

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Introduction

Alzheimer's disease (AD) is the most popular form of dementia in elderly people, affecting more than 30 million people in the world since it was discovered.[1] This disease is a progressive neurodegenerative disorder[2] and has become a main medical and social problem in developing countries.[3] The major neuropathological features of AD are extracellular deposition of amyloid-beta (Aβ) plaque, intracellular formation of neurofibrillary tangles,[4] degeneration, and loss of neurons and synaptic dysfunction in the hippocampus and brain cortex.[5]

It was reported that the administration of Aβ causes long-term potentiation to be changed in the hippocampus, which leads to impairment of cognitive function and memory loss in rodents.[6] Deposition of Aβ leads to oxidative stress, formation of reactive oxygen species (ROS), reduction in activity of antioxidant enzymes, and consequently neuronal death.

It was shown that neurogenesis is remarkably decreased during aging in the rodent brain.[7] Furthermore, most studies using transgenic animal models of AD reported a reduction in hippocampal neurogenesis.[8] This reduction has an important role during the onset and progression of the disease. The formation of toxic Aβ peptides associated with a decrease in neurogenesis.[9] However, some other works on transgenic animals have reported that amyloid deposition increases neurogenesis.[10] However, the most general view in the field is that AD associated with

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a decrease in hippocampal neurogenesis and consequence impairment of cognitive function.[11]

Evidence suggests that consuming external antioxidants can improve brain damages and related cognitive performance.[12] Several therapeutic strategies have been developed to treat AD, including anti-inflammatory, anti-oxidant, and anti-amyloid approaches. Recently, herbal treatments have been tested in animals[13] and cellular[14] models of AD and clinical trials with AD subjects.[15]

Cyperus rotundus, a sedge of the *Cyperaceae* family, is used widely in traditional medicine as nervine tonic, sedative, antispasmodic, antimalarial, and to relieve diarrhea.[16] *C. rotundus* rhizome extracts revealed the presence of β-sitosterol, cyperene, cyperol, flavonoids, sesquiterpenoids, ascorbic acid, and polyphenols.[17] Several studies have reported that flavonoids may cause to modulate the immune cell functions, against inflammation, and stress oxidative.[18]

Lots of evidence showed that dietary flavonoids have significant neuroprotective effects such as neurogenesis and neuronal regeneration that lead to improve learning and memory performance.[19-22]

The purpose of this study is to consider and assess the therapeutic effects of *C. rotundus* rhizome extract on memory impairment and neurogenesis in the hippocampus in the Aβ rats' model.

Materials and Methods

Herbal extract preparation

Fresh rhizomes of *C. rotundus* (10 kg) were prepared from Iraq. The rhizomes were confirmed by Dr. Ghanadian, Pharmacognosy Department, Isfahan University of Medical Sciences, Iran, according to the voucher specimen number 2262 deposited there and was shade, dried, and powdered. After sieving, ethanol was added and the mixture was poured into a percolator. After 3 days, the extract was discharged with a flow rate of 4 mL/min from the bottom tap through cotton wool. The obtained extract was vaporized by a rotary vacuum evaporator (Büchi-Labortechnik, Germany) at 45°C and 10 mbar. After that, the extract was suspended in a mixture of water (1000 mL) and chloroform (500 mL), and then the chloroform and aqueous parts were detached. The chloroform part rich in oil, fat, and nonpolar compounds. The defatted aqueous part was filtered, and evaporated under reduced pressure and stored at 4°C until use.

Extract standardization through measurement of total flavonoid compounds

A volume of 100 μl of the extract was poured into the tested tube (three replications were performed). After that, 100 μl AlCl3 20% solution and 50 μl of acetic acid glacial were added to each tested tube, and finally, methanol was added up to 3 ml. They were left, then, at room temperature for 40 min and absorbance was read at 415 nm.

Animals

Forty-two male Wistar rats weighing 230–250 g were obtained from Isfahan University of Medical Sciences and housed in a 12-h light-dark cycle (22°C–24°C) with food and water available *ad libitum*. The Ethics Committee for Animal experiments (IR. MUI. REC.393476, revised 1985) at Isfahan University of Medical Sciences approved the study and all experiments were conducted with the international guiding principles for animals. Animals were randomly divided into six groups ($n = 7$) for baseline training performance measurement in Morris test,[23] after which, animals were grouped as:

Control: Without any surgery and treatment

Sham: operated group, received 5 μL normal saline into CA1 bilaterally and administered 1 mL distill water orally for 30 days after the surgery.

AD: received 5 μg/μL Aβ1-42 into CA1 bilaterally, and 1 mL distilled water orally for 30 days after the surgery.

AD+CR (250, 500, and 750 mg/kg) groups, received 5 μg/μL Aβ1-42 into CA1 bilaterally, and oral administration of extract in three different doses of 250, 500, and 750 mg/kg for 30 days after the surgery. The protocol of the experimental design is summarized in Figure 1.

Morris water maze test

Spatial learning and memory were evaluated through Morris test modified for use in rats.[24] This test was run in a circular pool (diameter, 183 cm; height, 60 cm), was filled with water (22°C ± 1°C). This pool was separated into four quadrants of North West, North East, South West, and South East (SE). An invisible platform (diameter, 10 cm) was located 2 cm below the water surface in the SE quadrant (target quadrant). There was a video camera at 1.4 m above the center of the pool, which recorded the swimming trials. Rats were trained for four trials per day (with an intertrial interval of 5 min). The training was performed for 4 days. During each trial, rats were placed in a different quadrant of the pool and allowed to find the platform within 60 s. All rats were allowed to spend 30 s on the platform for training. When rats failed to find the platform within the set time, they were conducted to the platform. In the probe trial, the platform was removed, and the rats were allowed to swim for 60 s. The average time rats spent in the target quadrant for searching the platform was recorded to measure spatial memory ability with Neurovision 1.1 software. (Tajhizgostar Iranian, Iran)

Stereotaxic surgery

Rat Aβ1-42 (Sigma, St. Louis, MO, USA) was solubilized in sterile normal saline (0.9%), at a concentration of 1 μg/μl, and were stored at −20°C until use. Before surgery, the Aβ
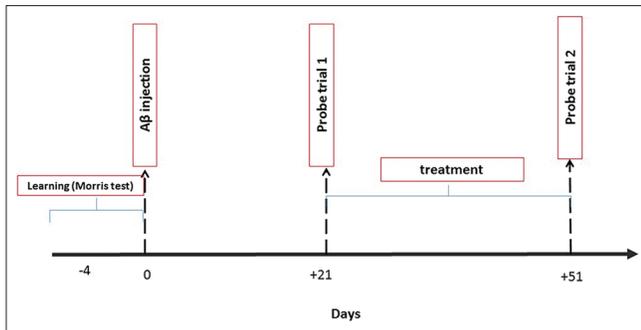
Figure 1: Diagram of the experimental timeline during the study

Figure 2: Coronal photomicrograph of lateral microinjection site in the CA1 area. Arrow: Needle path, CO: Cortex, D: Dentate gyrus (*x* = 25)

solution was incubated at 37°C for 5 days.[25,26] Rats were anesthetized with chloral hydrate (350 mg/kg) and placed in a stereotaxic device (Stoelting, Kiel, WI, USA). The scalp was incised in the midline, and the area surrounding bregma was cleaned and dried. Two small holes were drilled into the skull above the CA1. The Aβ (5 μg/5 μl) was injected using microinjection and 10 Hamilton syringe in CA1 region bilaterally (anterior-posterior = −3.36 mm, medial-lateral = ±1.6 mm from the bregma and dorsal-ventral = 3.2 mm from top of the skull according to Paxinos atlas [Figure 2].[27]

After injection, the needle was kept in CA1 for 5 min to allow complete diffusion of the drug. Sham group, was received normal saline. After surgery, each animal was allowed to recover for 5–7 days. Moreover, the histological evaluation was run to assess the correct injected area. Twenty-one days after surgery, the first probe trial of Morris test was run for finding memory impairment.[23]

Treatment

After the first probe trial of the Morris test, *C. rotundus* was solubilized in distilled water and administered orally at 250, 500, and 750 mg/kg/day doses for 30 days. The sham and AD groups received only distilled water.

For selection safe doses, acute toxicity and LD_{50} study were done. The extract was found to be nontoxic up to 1000 mg/kg and in the limited dose of 5000 mg/kg. It showed that LD_{50} was >5000 mg/kg. Therefore, according to experience in our laboratory and similar studies, 5%, 10%, and 15% lethal doses equal to 250, 500, and 750 mg/kg were selected for the main study.

Histological study

Twenty-eight days after probe trial 2, all rats were sacrificed transcardially with 4% paraformaldehyde. The brain tissue including the hippocampus was removed and postfixed in the same fixative solution overnight at 4°C. After that, the hippocampus was separated, dehydrated with an ascending ethanol series, cleared with xylene, and embedded in paraffin for 3–6 h and then blocked. For histological studies, a series of coronal serial sections with 3-μm thickness and 500 μm intervals were prepared from the hippocampus by a rotary
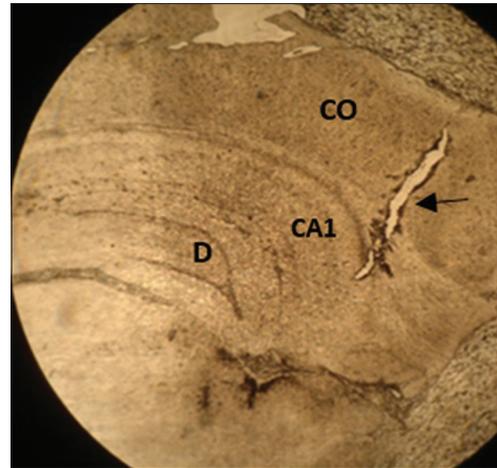
microtome (Leica, Vienna, Austria). After deparaffinization, the sections were incubated in 2XSSC formamide (for DNA denaturation), were rinsed with 2XSSC 10 min, incubated in H2O2 and methanol for 20 min, incubated with 2N HCl for 30 min, rinsed in 0.1 M boric acid, pH 8.5, and washed in TBS. Then, the sections were incubated with TBS 3% goat serum and 0.3% Triton X (TBS++) and incubated overnight at 4°C for staining with a primary antibody, mouse anti-neuron-specific nuclear protein (NeuN) (UK) Catalog No. MAB377 [2:200]. The next day, the sections were rinsed with TBS and incubated with a secondary antibody, goat anti-mouse Alexa Fluor 488 (catalog No. A-11001 (green; 2:150)) for 1 h and washed with TBS. Nuclear counterstaining was performed with 4',6'-diamidino-2-phenylindole dihydrochloride hydrate (Sigma) for 3 min. The sections were considered with a fluorescence microscope and photographed digitally (Zeiss, Germany).[23]

Statistical analysis

All data were expressed as mean ± standard error of the mean and analyzed in SPSS 24 software (Chicago: SPSS Inc. IBM Corp.). The one-way ANOVA *post hoc* Least significant difference and Kruskal–Wallis test were run for a one-way comparison. For observing frequencies in alternative times, the generalized linear models (GLM) tests were conducted. The statistical significance in all run tests is $P < 0.05$.

Results

Result of measurement of total flavonoid compounds

The calibration curve was plotted by linear regression in the concentration of 0.13, 0.66, 3.33, and 16.66 μg/mL. The regression equation was expressed as: $Y = 0.0709x − 0.0086$. y-axis shows absorption and x-axis shows flavonoid concentration in sample (μg/mL) with the correlation factor of $r^2 = 0.999$. The mean percentage of flavonoid in total extract is 0.17% ± 0.02% quercetin equivalent in dry weight [Figure 3].

Effect of *Cyperus rotundus* extract on memory

The GLM test showed no significant difference in the mean of traveled distance for finding invisible platform in all animal groups during 4 days spatial acquisition phase before the surgery ($P = 0.81$), but this statistical analysis indicated significant differences in traveled distance between days 1 and 4 in all groups ($P < 0.001$), revealing that the improvement of learning performance in all groups. The Kruskal–Wallis showed no significant difference between the measured groups on the first day ($P = 0.6$), second day ($P = 0.18$), third day ($P = 0.61$), and the last day ($P = 0.19$) [Figure 4].

The LSD analysis of probe trial showed a significant difference in the mean percentage of time in probe1 between all groups ($P = 0.003$) as: control with AD ($P = 0.004$), control with AD + CR 250 ($P = 0.004$), control with AD + CR 500 ($P = 0.003$), and control with AD + CR 750 ($P = 0.004$). Furthermore, the statistical analysis showed a significant difference in the mean percentage of time in probe1 between sham with AD ($P = 0.01$), sham with AD + CR 250 ($P = 0.01$), sham with AD + CR500 ($P = 0.008$), and sham with AD + CR 750 ($P = 0.009$).

In second probe trial (probe2) that was done after treatment, statistical analysis indicated a significant increase in the mean percentage of spending time in target quadrant among six groups ($P = 0.02$) as: AD with control ($P = 0.008$), AD with sham ($P = 0.01$), AD with AD + CR 500 ($P = 0.004$), AD with AD 250 ($P = 0.4$), and AD with AD 750 ($P = 0.06$) and also significant difference between AD + CR 250 with AD + CR 500 groups ($P = 0.03$) [Figure 5].

Effect of *Cyperus rotundus* extract on neuron-specific nuclear protein expression

The Kruskal–Wallis analysis for assessing NeuN-positive cells in dentate gyrus indicated that there existed significant differences between six groups ($P = 0.01$). These significant differences existed among the AD + CR500 group with AD and AD + CR250 groups and also among control groups with AD and AD + CR250 groups ($P < 0.05$) [Figure 6].

Discussion

The propose of this experimental study was the evaluation of the neuroprotective effect of *C. rotundus* on memory impairment in Aβ-injected rats. Aggregation and deposition of Aβ in the hippocampus and brain lead to an increase in acetylcholinesterase activity, induction of oxidative stress, inflammation, and impairment of learning and memory.[28]

Aβ accumulation is toxic to neurons and inhibits neuronal differentiation and also causes neuroinflammation in the brain.[9] Neuroinflammation leads to suppression of neurogenesis in the hippocampus.[29] Furthermore, Aβ causes oxidative stress and an increase of ROS activity. ROS activity stimulates cell death and leads to alteration
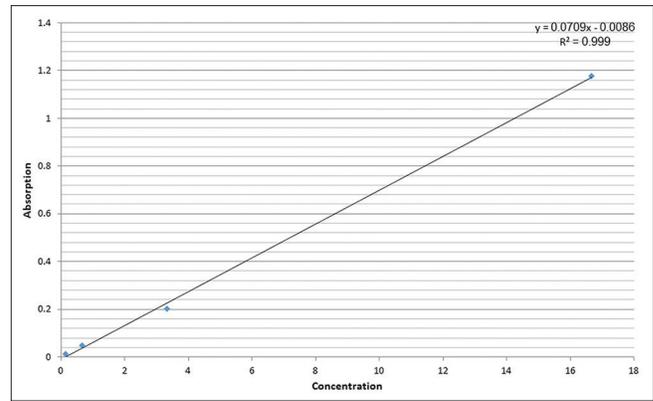

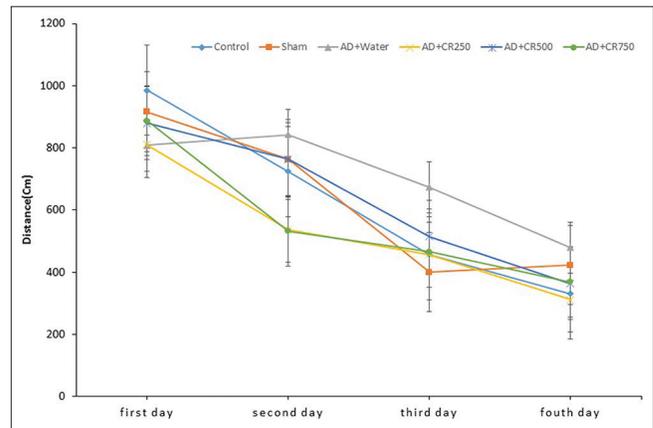**Figure 3:** Calibration curve of total flavonoid for *Cyperus rotundus* extract

Figure 4: Travelled distance for finding platform in Morris test in experimental groups in four training days before surgery
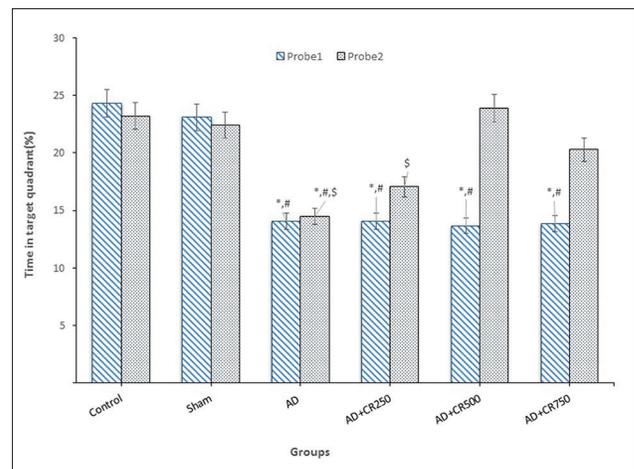

Figure 5: Probe 1 and probe 2. Blue bar shows animal swimming in the target quadrant 21 days after amyloid-beta injection. Gray bar shows animal swimming in target quadrant after treatment. *Significant difference with the control group, #Significant difference with the sham group, $Significant difference with AD + CR500 group ($P < 0.05$)

in adult neurogenesis. In oxidative stress conditions, deficiency of antioxidant enzymes like SOD induces a reduction in the production of new neurons in the SGZ of the hippocampal dentate gyrus.[30]
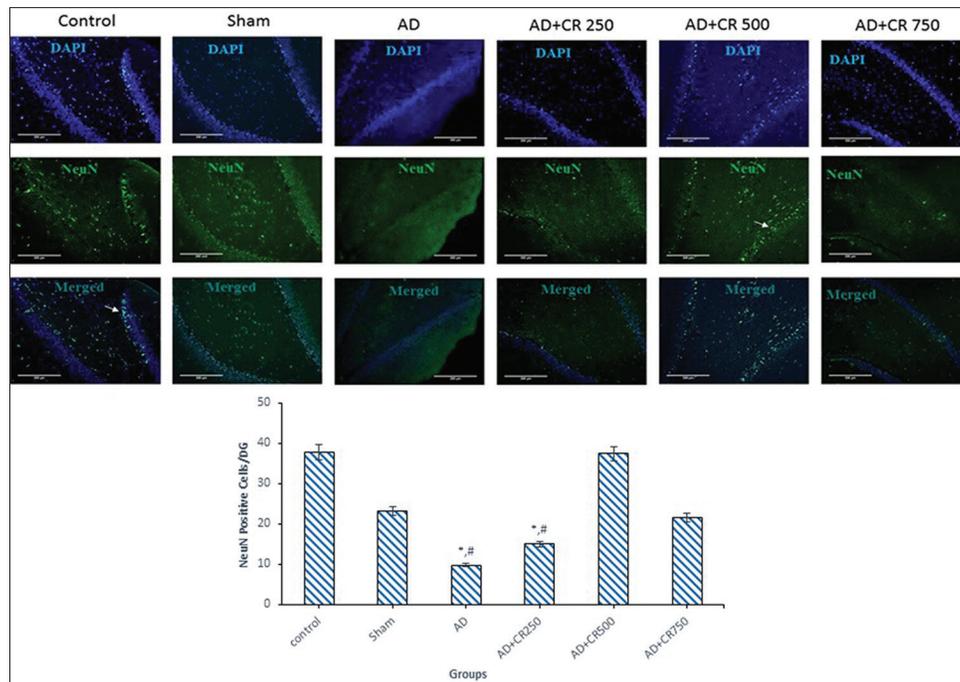
Figure 6: Immunofluorescence staining for neuron-specific nuclear protein in experimental groups, arrow new differentiated neurons (x = 200). *Significant difference with the Alzheimer's disease + CR500 group, #Significant difference with control group (P < 0.05)

In a previous study, it was reported that Aβ plaque aggregate in synaptic spaces, 21 days after Aβ injection into the hippocampus.[23] Therefore, in this study, memory impairment was detected in the first probe trial, by decreasing in mean percentage of time spent in the target quadrant between AD groups with control and sham groups. *C. rotundus* is a traditional medicinal herb that has anti-inflammatory and antioxidant effects.

Mehdizadeh *et al*. showed the protective effect of *C. rotundus* on memory impairment in Aβ injected rat only in the behavioral test.[31] Our finding in Morris test was confirmed with this study. Ardestani *et al*. showed decrease oxidative damage, antioxidant, and metal chelating effects of *C. rotundus*.[32] Antioxidant and lipid peroxidation effects of this plant also were considered during *in vitro* studies.[32] Dang *et al*. investigated the anti-inflammatory effect of this plant in animal models.[33] There were not many studies about the effect of *C. rotundus* on neurogenesis. Therefore in this study, we considered the effect of this plant on the loss of memory impairment through increasing neurogenesis by expression of NeuN. The generation of new neurons is the main property of the adult brain that was occurred in the hippocampus and subventricular zone.[34]

NeuN is localized in nuclei and perinuclear cytoplasm of the neurons in the central nervous system of mammals that marks post-mitotic and mature neurons.[35] Monoclonal antibodies to the NeuN protein have been actively used in the immunohistochemical staining of neuronal differentiation to evaluate the functional status of neurons in normal and pathological states for more than 20 years.

This protein has not been detected in glial cells and tissues other than nervous, which suggests it is a specific neuronal marker.[36]

Behavioral studies indicated that the hippocampus plays a critical role in learning and memory and hippocampal neurogenesis play an important role in the memory performance.[37] In this study, dose 500 mg/kg *C. rotundus* significantly increased neurogenesis and consequently increased the percentage of time spent in the target quadrant in the Morris test.

According to the anti-inflammatory and anti-oxidant effects of this plant in the previous studies, *C. rotundus* extract may be through the reduction of neuroinflammation and oxidative stress lead to increase of neurogenesis.

Aggregation and deposition of Aβ in the brain lead to a degeneration process in neurons, which interfere with the electrolyte barrier function of the nerve cell membrane, which ultimately causes brain edema and inflammation. The treatment with *C. rotundus* extract may through Aβ depletion from the brain tissue causing a regeneration process in which neurons refind their normal function during 4 weeks and this leads to brain recovery from edema and inflammation. Meanwhile, this treatment will stimulate neurogenesis, which had been stopped as a result of the toxic effect of deposition of Aβ in the brain, especially in the hippocampus, later on.

The analysis of *C. rotundus* extract revealed the presence of flavonoid compound in this extract. Flavonoid compounds represent different biological activities through interaction

with specific signaling pathways[38] and also cause increased neurotrophins such as Brain Drive Neurotrophic Factors[39] and Nerve Growth Factors.[40] Expression of these factors stimulates the production of new neurons in the brain.[41] Lee *et al.* investigated the neuroprotective role of *C. rotundus* in an *in vitro* model of Parkinsonism.[42] Sunil *et al.* demonstrated total oligomeric flavonoids of *C. rotundus* extract have neuroprotective effects against the ischemic-reperfusion-induced neurodegeneration in rats.[43] The present study showed useful effects of *C. rotundus* extract on neurogenesis and behavioral disturbance in Alzheimer rats model.

Conclusions

This experimental study showed that *C. rotundus* extract, repaired spatial memory impairment in Aβ rat model, through increased neurogenesis in the hippocampus. This effect most probably is related to the flavonoid components in the extract.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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