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ORIGINAL ARTICLE

Pretreatment effect of *Potentilla reptans* L. extract on anxiety injury, neuronal density, and Aβ plaque in the rat brain

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Abstract: The protective effects of *Potentilla reptans* L. (*P. reptans*) include cardioprotective, neuroprotective, and anti-stroke effects. In this study, the protective effects of the methanolic extract of *P. reptans* root on neuronal density and beta-amyloid were investigated in the hippocampal and prefrontal regions of the brain in the rat model of anxiety. Twenty-four male Wistar rats were randomly divided into four groups (n = 6), including the control group (received normal saline) and three groups receiving different concentrations of *P. reptans* (0.025, 0.05, and 0.1 g/kg/BW), which were intraperitoneally (IP) injected daily for 7 days. The elevated plus maze test was used to assess the anxiolytic effect and anxiety response. Meanwhile, all concentrations of *P. reptans* reduced the accumulation of β-amyloid plaques in both the hippocampus (CA1, CA3, and dentate gyrus areas) and prefrontal cortex compared to the control group. The reduction in β-amyloid plaques was statistically more significant at the higher concentrations (0.05 and 0.1 g/kg/BW). In addition, *P. reptans* increased the number of neurons at three concentrations, with the higher concentrations showing more significant effects with the effective concentration (EC_{50}) (0.05 g/kg/BW). Hence, *P. reptans* root extract shows anti-anxiety effects and a protective role in anxiety-induced neuron degeneration and amyloid accumulation.

Keywords: *Potentilla reptans* root, β-amyloid plaque neurons, hippocampus, prefrontal, anxiety

1 Introduction

Anxiety is a type of adaptive response in the face of threats or aversive stimuli, characterized by widespread fear and reactivity to prevent harm (MG, 2016). Epidemiological studies have shown that anxiety disorders such as generalized anxiety disorder (GAD), obsessive-compulsive disorder, and post-traumatic stress disorder (PTSD) are the most common mental disorders in the general population. According to a large population-based survey, up to 33.7% of people will experience an anxiety disorder during their lifetime (Bandelow & Michaelis, 2015).

The hippocampus, amygdala, hypothalamus, and prefrontal cortex (PFC) are among the major brain regions involved in anxiety (Calhoon & Tye, 2015; Canteras et al., 2009). Studies suggest that the hippocampus functions as a crucial brain area in processing contextual fear information (Bernier et al., 2017). Schoenfeld et al. observed significant hippocampal activation in mice exposed to an anxiogenic environment, while hippocampal inactivation reduced anxiety-like behaviors in mice (Schoenfeld et al., 2016). Studies have shown the role of PFC in regulating the behavioral expression of anxiety and the extinction of learned fear memories through its extensive reciprocal connection with other brain regions, including the hippocampus and amygdala (Kim & Cho, 2017, 2020). Consequently, aberrant functioning within any of the anxiety circuit components in the brain contributes to the expression of maladaptive anxiety (MG, 2016).

Previous studies demonstrated a relationship between anxiety and beta-amyloid (Aβ) plaque in the cingulate cortex,

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PFC, parietal cortex, and hippocampus; there is an association between Aβ and the worsening of anxiety symptoms in adults without cognitive impairment (Marshall et al., 2013; Palmqvist et al., 2017). Pathological accumulation of Aβ plaques can lead to neurotoxicity via oxidative effects and the production of reactive oxygen species (ROS), thereby inducing apoptosis (Huang et al., 2016). Furthermore, neurons in these regions are highly plastic and undergo dramatic structural changes and degeneration in the face of anxiety (Leuner & Shors, 2013). A reduction in hippocampal volume has generally been reported in patients with anxiety (Gould et al., 1992). Schoenfeld et al. demonstrated that chronic unpredictable stress over 4 weeks reduced neurogenesis by 33% and hippocampal volume throughout the dorsal and ventral subfields (Schoenfeld et al., 2017).

The pharmacological treatment of anxiety disorders is mainly based on benzodiazepines, Selective Serotonin Reuptake Inhibitors (SSRIs), Serotonin and Norepinephrine Reuptake Inhibitors (SNRIs), and other anxiolytic drugs; their mechanism of action is mainly based on regulating the neurotransmitter systems such as GABA, serotonin, and dopamine neurotransmitters (Liu, 2024). Side effects such as dependence, drug withdrawal syndrome, and tolerance during long-term use may be associated with these medications (Hindmarch, 1998). Studies have demonstrated the anxiolytic effects of natural compounds from a variety of plants and herbs (Fernández-Demeneghi et al., 2019; Na et al., 2016). Therefore, natural compounds with minimum side effects can be included as an appropriate treatment option for these disorders (Eisenberg et al., 1998).

The genus *Potentilla* has been used in traditional medicine for various internal and external problems, especially in the treatment of gastrointestinal disorders, mouth and throat complications, skin diseases, thyroid, wound-healing, bleeding, kidney stones, diabetes, hemorrhoid, and general strengthening (Augustynowicz et al., 2021). Morevere, this genus contains a rich array of natural compounds, including polyphenols, flavonoids, proanthocyanidins, tannins, and triterpenoids (Ahangar et al., 2021; Augustynowicz et al., 2021). A study on ethanol root extracts from four selected *Potentilla* species indicates that *P. recta* and *P. reptans* contain the highest levels of phenolics, saponins, and triterpenoids (Uysal et al., 2019). Evidence reveals that *P. reptans* has antioxidant, anti-inflammatory, and anti-apoptosis effects (Enayati et al., 2018; Tomovic et al., 2015). Studies also showed the cardioprotective, preconditioning, and postconditioning effects of *P. reptans* (Enayati et al., 2018). In a recent study, *P. reptans* demonstrated neuroprotective effects via reducing neuronal loss and β-amyloid accumulation in the hippocampus of the rat brain ischemia/reperfusion (IR) model (Noorgaldi et al., 2023).

Considering that the potential beneficial effects of *P. reptans* root on anxiety have not been investigated, the present study was designed to examine the potential anxiolytic and neuroprotective effects of the methanolic extract of *P. reptans* root by investigating congophilic Aβ plaque and neuronal density in the hippocampus and prefrontal cortex of male rats.

2 Materials and Methods

2.1 Plant Material and Identification

P. reptans roots were gathered from Golestan Province, North of Iran, in June 2015, according to the Herbal Medicinal Product Committee (HMPC) (Ref. EMEA/HMPC/246816/2005) guideline. The plant was identified by Professor Farideh Attar and deposited at the Central Herbarium of Tehran University, Iran (45815-TUH) (Enayati et al., 2019; Enayati et al., 2021; Mirzaei et al., 2023).

2.2 Preparation of Plant Extract

The preparation process of the methanolic (80%) extract of *P. reptans* root was described previously (Enayati et al., 2018). The powder of *P. reptans* extract was dissolved in distilled water.

2.3 Animals

Male Wistar rats weighing 220–270 g were maintained under standard conditions with a 12/12-hour light/dark cycle, an ambient temperature (20–23°C) and an air-conditioned environment. The rats had free access to normal chow and water. The animal experiments were conducted in accordance with the National Institutes of Health and ARRIVE guidelines (Enayati et al., 2021). All experimental protocols were approved by the Ethics Committee of Golestan University of Medical Sciences, Gorgan, Iran (IR.GOUMS.AEC.1401.001), (IR.GOUMS.AEC.1401.002), and (IR.GOUMS.AEC.1401.003).

2.4 Study Design

Rats were randomly divided into 4 groups (n = 6). The following protocols were performed: 1: control group received normal saline (7 days) and behavioral test; and 2–4: *P. reptans* methanolic extract in three concentrations (0.025, 0.05, and 0.1 g/kg/BW) administered 7 days before the final behavioral test. Before starting drug administration, rats in both groups underwent a behavioral test (elevated plus-maze). All drugs were injected intraperitoneally (IP), and the last injection was done 30 min before the behavioral test in both control and experimental groups. Furthermore, the effective anti-anxiolytic concentration of *P. reptans* methanolic extract (EC_{50}) was determined by GraphPad Prism 6 software.

2.5 Behavioral Study (Elevated Plus-Maze)

The elevated plus-maze is a reliable method to study drug-induced anxiolytic action and anxiety response in rodents. The elevated plus maze was performed 24 hours before starting the injection of normal saline or *P. reptans* extract to rats, and after 7 days of injections in both control and *P. reptans* groups. The process of the behavioral (elevated plus maze) test was described previously (Karimi et al., 2014; Nikmahzar et al., 2016). Briefly, this apparatus is elevated 50 cm above the floor and consists of two closed arms (50 × 10 × 20 cm) positioned opposite each other and perpendicular to two open arms (50 × 10 cm). Behavioral testing was conducted under dim red lighting between 09:00 AM and 15:00. Before

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the behavioral test (60 min), all rats were transferred to the behavior-testing room to accustom them to the room's conditions. To begin the experiment, the rat was placed in the center of the maze, facing an open arm, and allowed to move freely for 5 minutes. A trained experimenter recorded the entries and time spent on both arms. Rats prefer to stay in closed arms and have an aversion to high and open spaces, so they spend more time in closed arms. Increased open arm entries and/or open arm time indicate the anti-anxiety behavior of rats.

2.6 Tissue Preparation

On day 8, after performing the final behavioral test (24 h later), the rats were sacrificed using carbon dioxide (CO_2). The brains of the rats were carefully removed from the skull and fixed for seven days in a 4% paraformaldehyde solution. Tissue processing was automated (using Did Sabz tissue processor, Iran), and prefrontal and hippocampal slices were prepared using a rotary microtome (Pooyan MK 1110, Iran), each with a six µM thickness. The hippocampus and prefrontal cortex slices were divided into two groups for analyzing Aβ plaques (Congo red staining) and neuron counts (Cresyl violet staining) (Noorgaldi et al., 2023).

2.7 Cresyl Violet Staining

To determine neuronal density in the prefrontal cortex and hippocampus of a rat brain, Cresyl violet staining was performed following a previously reported method (Noorgaldi et al., 2023). First, prefrontal and hippocampus slices were deparaffinized using xylene and rehydrated through a descending ethanol concentration. Next, the brain slices were stained in a 0.02% solution of cresyl violet (Sigma, USA) for 10 min. After washing twice in distilled water, the brain sections were dehydrated in increasing ethanol concentrations. Finally, sections were cleaned with xylene for 3–5 min and covered with Entellan glue (Merck, Germany).

2.8 Congo Red Staining

Congo red staining (Aragen, Iran) (DDK) was performed on the prefrontal cortex and hippocampus sections to identify the density of Aβ plaque, as previously described (Noorgaldi et al., 2023). First, slices of the prefrontal cortex and hippocampus were deparaffinized using xylene and rehydrated through a gradient ethanol series. Next, the slices were stained in a solution of Congo red (one hour, 25°C) and then washed in distilled water and alcohol solution at room temperature. The brain slices were rinsed with running tap water (5 min), followed by five minutes of counterstaining with Mayer hematoxylin solution, and then washed in running tap water for an additional 10 minutes. Subsequently, the brain slices were rapidly immersed in alcohol (95%, three times), followed by immersion in 100% alcohol (twice). After clarification with xylene, the sections were covered and slipped using Entellan glue.

2.9 Morphometry

The stained brain sections were imaged with a light microscope (Olympus BX53, Japan) and a digital camera (Olympus DP73, Japan) at × 400 magnification. In the hippocampus sections (CA1 and CA3 areas and in the dentate gyrus (DG)) and in the prefrontal sections, the number of beta-amyloid plaques and neurons was blindly counted using the ImageJ software in a 12,000 μM^2 area.

2.10 Statistical Analyses

The data were analyzed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The normality of data was assessed with the Shapiro-Wilk test. The results of the behavioral study were evaluated using a two-way ANOVA. The Histology results for normally distributed data were evaluated using one-way ANOVA with a post hoc Tukey test, while non-normally distributed data were evaluated using the Kruskal-Wallis test. The results were presented as means ± standard deviations (SD) and statistical significance was set at $p < 0.05$.

3 Results

3.1 Anxiety in Behavioral Study

Overall, pretreatment with different concentrations of *P. reptans* demonstrated an upward trend in the number of open-arm entries, especially at 0.1 g/kg/body weight (P = 0.02), compared to the control group (Table 1). Meanwhile, the group that received a concentration of 0.1 g/kg BW showed a longer open-arm time, while the others showed a downward trend (Table 1).

Furthermore, administration of this extract reduced the number of entries into the closed arm, and the time spent in the closed arm showed a decreasing trend with increasing concentration. Notably, at a concentration of 0.1 g/kg/BW, a significant decrease was observed in both closed-arm entries (P = 0.02) (Table 1) and closed-arm times (P = 0.009) (Table 1).

3.2 The Effect of P. reptans Extract on Number of Neurons in the Hippocampus and Prefrontal Cortex of Rats

The effects of *P. reptans* on histological parameters of the hippocampal regions (CA1, CA3, and DG) and prefrontal cortex were assessed using cresyl violet staining, as depicted in Figure 1(A) and (B). This shows that pretreatment with *P. reptans* extract (all concentrations) significantly increased neuronal density in both hippocampus regions (CA1 and DG) and in the prefrontal cortex compared with the control group ($P < 0.001$). While *P. reptans* only at (0.05 g/kg/BW) increased neuronal count in the CA3, with no statistically significant difference compared with the control group.

Table 1. The results of the behavioral test

	Before (%)(mean ± SD)	After (%)(mean ± SD)	P. value
Open arm entries			
Control	41.95 ± 11.29	42.14 ± 5.370	>0.99
P. reptans (0.025 g/kg/b.w.)	41.05 ± 16.99	44.71 ± 14.53	0.98
P. reptans (0.05 g/kg/b.w.)	50.59 ± 12.10	56.15 ± 11.68	0.94
P. reptans (0.1 g/kg/b.w.)	26.15 ± 22.35	51.57 ± 18.07	0.02
Open arm time			
Control	31.80 ± 14.74	29.33 ± 9.18	0.99
P. reptans (0.025 g/kg/b.w.)	34.73 ± 17.84	33.06 ± 16.32	0.99
P. reptans (0.05 g/kg/b.w.)	37.13 ± 12.90	36.20 ± 12.15	0.99
P. reptans (0.1 g/kg/b.w.)	20.33 ± 23.31	28.26 ± 11.43	0.73
Closed arm entries			
Control	58.04 ± 11.29	57.85 ± 5.370	>0.99
P. reptans (0.025 g/kg/b.w.)	58.94 ± 16.99	55.28 ± 14.53	0.98
P. reptans (0.05 g/kg/b.w.)	49.40 ± 12.10	43.84 ± 11.68	0.94
P. reptans (0.1 g/kg/b.w.)	73.85 ± 22.35	48.42 ± 18.07	0.02
Closed arm time			
Control	30.46 ± 9.80	31.33 ± 6.69	0.99
P. reptans (0.025 g/kg/b.w.)	41.19 ± 10.63	32.33 ± 9.56	0.28
P. reptans (0.05 g/kg/b.w.)	26.66 ± 3.91	17.73 ± 7.33	0.27
P. reptans (0.1 g/kg/b.w.)	37.73 ± 14.69	20.60 ± 9.90	0.009

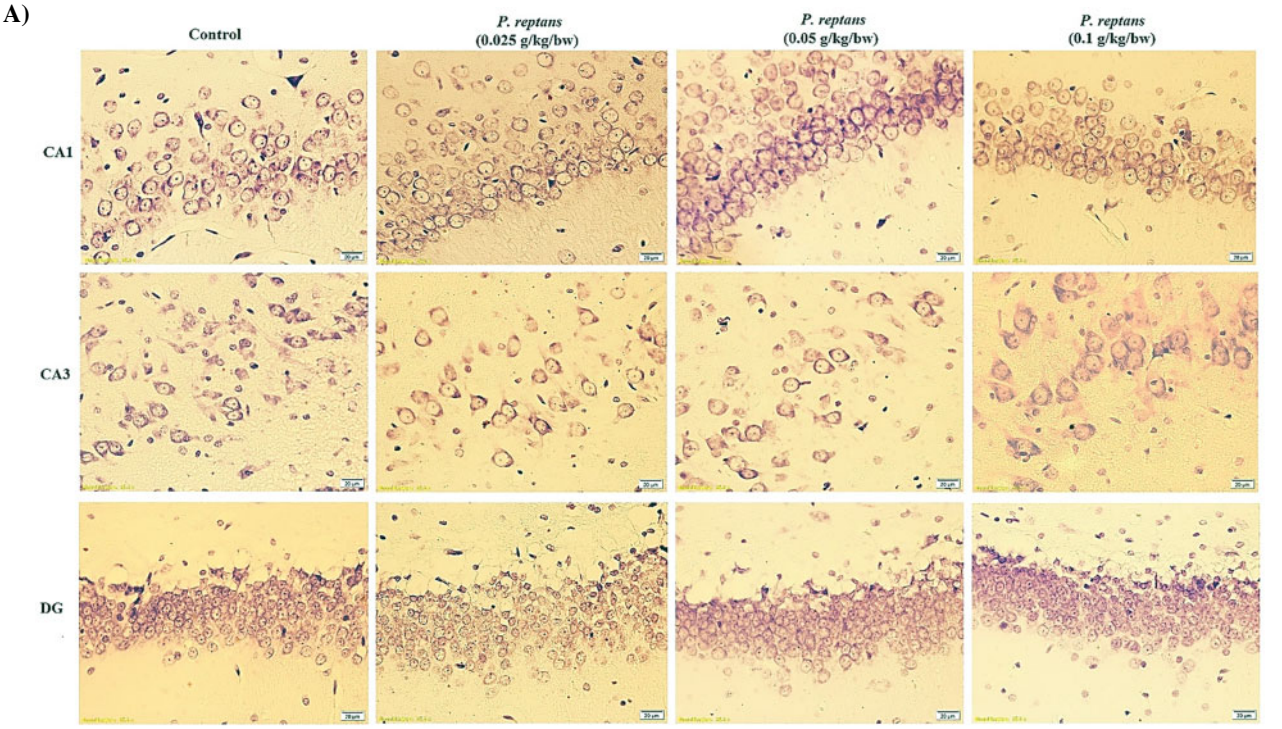

Figure 1. (Continued)

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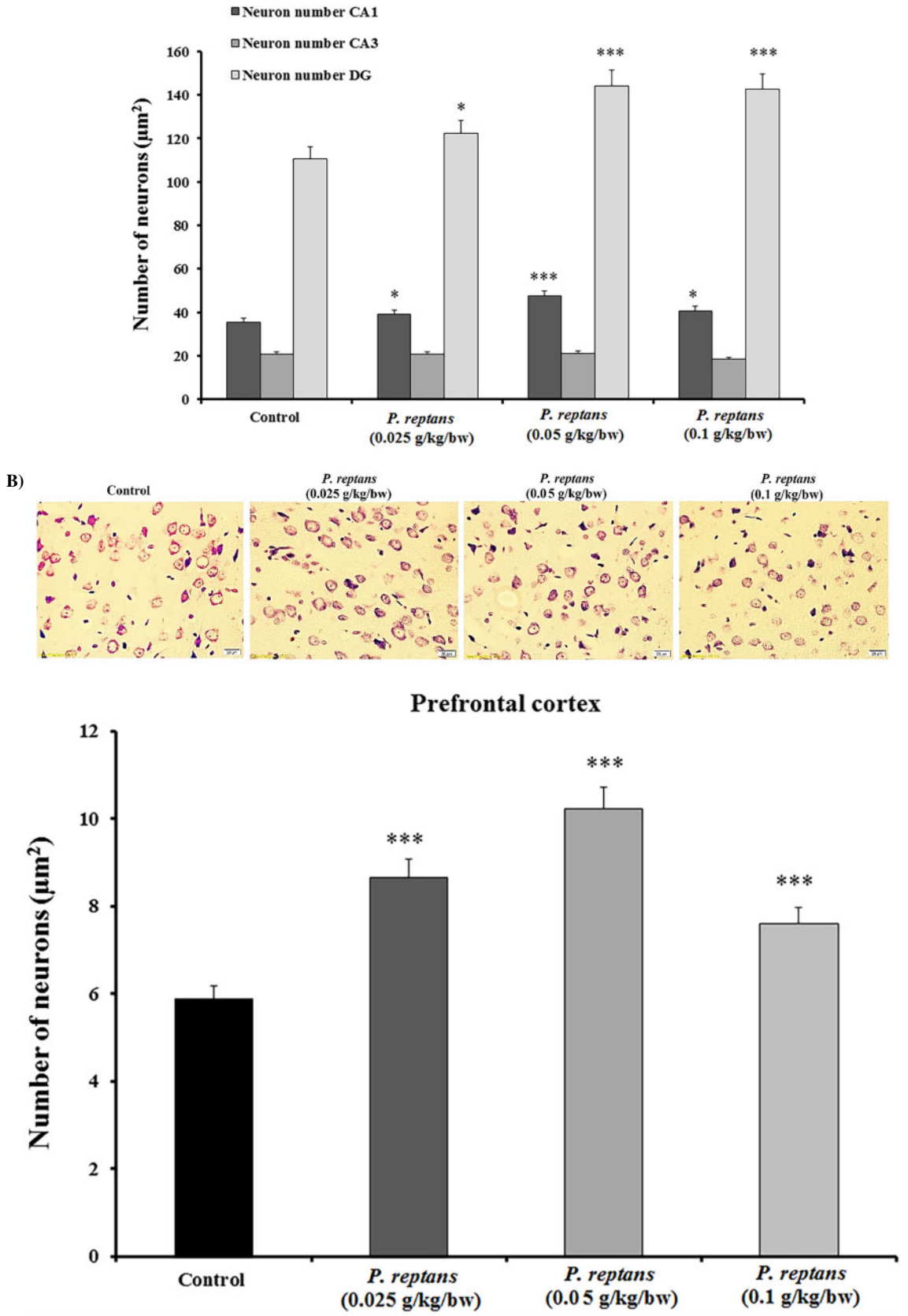

Figure 1. Effect of *Potentilla reptans* L. root extract on the neuron number in a anxity rat hippocampus (A) and prefrontal cortex (B) in the control, *P. reptans* (0.025 g/kg/BW), *P. reptans* (0.05 g/kg/BW), and *P. reptans* (0.1 g/kg/BW) groups. The bars show the means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. bw: body weight; DG: dentate gyrus. Scale bar: 20 µM

3.3 The Effect of P. reptans Extract on β-amyloid Plaque Number in Hippocampus and Prefrontal Cortex of Rats

As shown in Figure 2A, Congo red staining reveals β-amyloid plaques in the hippocampus (CA1, CA3, and DG) in the control and *P. reptans* pretreatment groups. The results indicate a significant reduction in plaque counts after administration of different concentrations of *P. reptans* extract, particularly at 0.05 and 0.1 g/kg bw, compared with the control group ($P < 0.001$).

P. reptans, in a concentration-dependent manner (0.025, 0.05, 0.1 g/kg/BW), reduced β-amyloid plaques in the prefrontal cortex of the rat compared to the control group (Figure 2B). Furthermore, *P. reptans* showed a statistically significant reduction at concentrations of 0.05 and 0.1 g/kg bw ($p < 0.001$). The maximum reduction in β-amyloid plaques was achieved with (0.1 g/kg/BW) of methanolic extract of *P. reptans* (0.38 ± 0.57) compared to the control group (3.33 ± 2.34).

3.4 EC_{50} Determination of P. reptans Extract

In the present study, *P. reptans* root extract improved the anxiety responses, the density of Aβ plaque and neuron counts in a concentration-dependent manner in the hippocampus/prefrontal cortex of anxiety rats. Based on these results, the effective concentration (EC_{50}) of *P. reptans* was determined at (0.05 g/kg/BW) concentration (Table 2).

4 Discussion

The present study utilized the plus elevated maze model to measure anxiety levels in rats. The increase in the open arm entries (OAE) and open arm time (OAT) and the decrease in the closed arm entries (CAE) and closed arm time (CAT) indicate decrease in the rats' anxiety in this maze (Walf & Frye, 2007). A 7-day pretreatment with *P. reptans* extract resulted in an increase in OAE and a decrease in CAE and CAT. The difference in results before and after the pretreatment period compared to the control group, except for the OAT parameter, demonstrated a dose-dependent trend, reaching the maximum anxiolytic effect at 0.1 g/kg/BW. These findings strongly indicate the potential anti-anxiety effects of *P. reptans* extract.

This study is the first research that has been conducted on the anxiolytic effect of *Potentilla* in rats. However, similar studies have investigated the effects of *Potentilla* relatives. Shikov et al. investigated the effect of *Potentilla alba* extract, a member of the *Potentilla* species, on adaptive activities and the central nervous system (Shikov et al., 2011). This study reported that the extract of this plant reduces anxiety-related behaviors in rats (Shikov et al., 2011). In Nemati et al.'s study (2013), *Rosa canina* extract elicited an anti-anxiety effect in rats, with potency increasing with dose (Nemati et al., 2015).

The anti-inflammatory effects of numerous plant species have been documented in ancient medicine and validated through modern scientific methods (Modaresi et al., 2016; Rezaie et al., 2010). However, the specific mechanisms behind these effects remain less understood. Related to the known anxiety mechanism, compounds such as flavonoids present in *P. reptans* may influence the GABAergic system by binding to benzodiazepine receptors or inhibiting GABA-producing enzymes (Sarris et al., 2013). Given the involvement of inflammatory and oxidative pathways in anxiety (Lee et al., 2022a), the anti-inflammatory and antioxidant properties of this plant may be another explanation for its anxiolytic effect (Avcı et al., 2006; Shikov et al., 2011; Tomovic et al., 2015; Uysal et al., 2016).

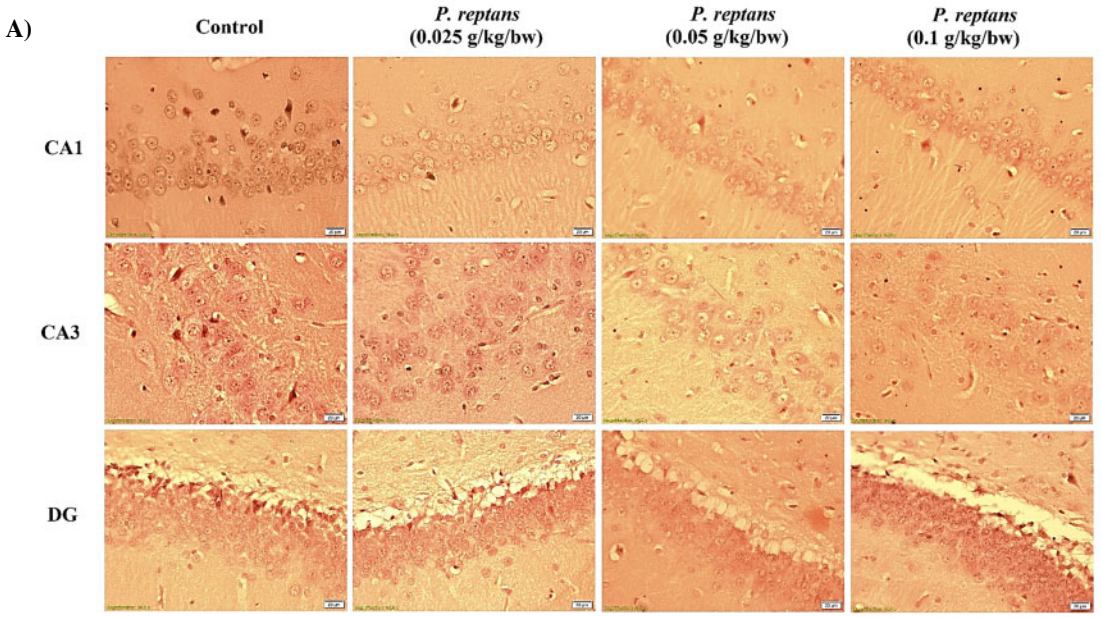

Figure 2. (Continued)

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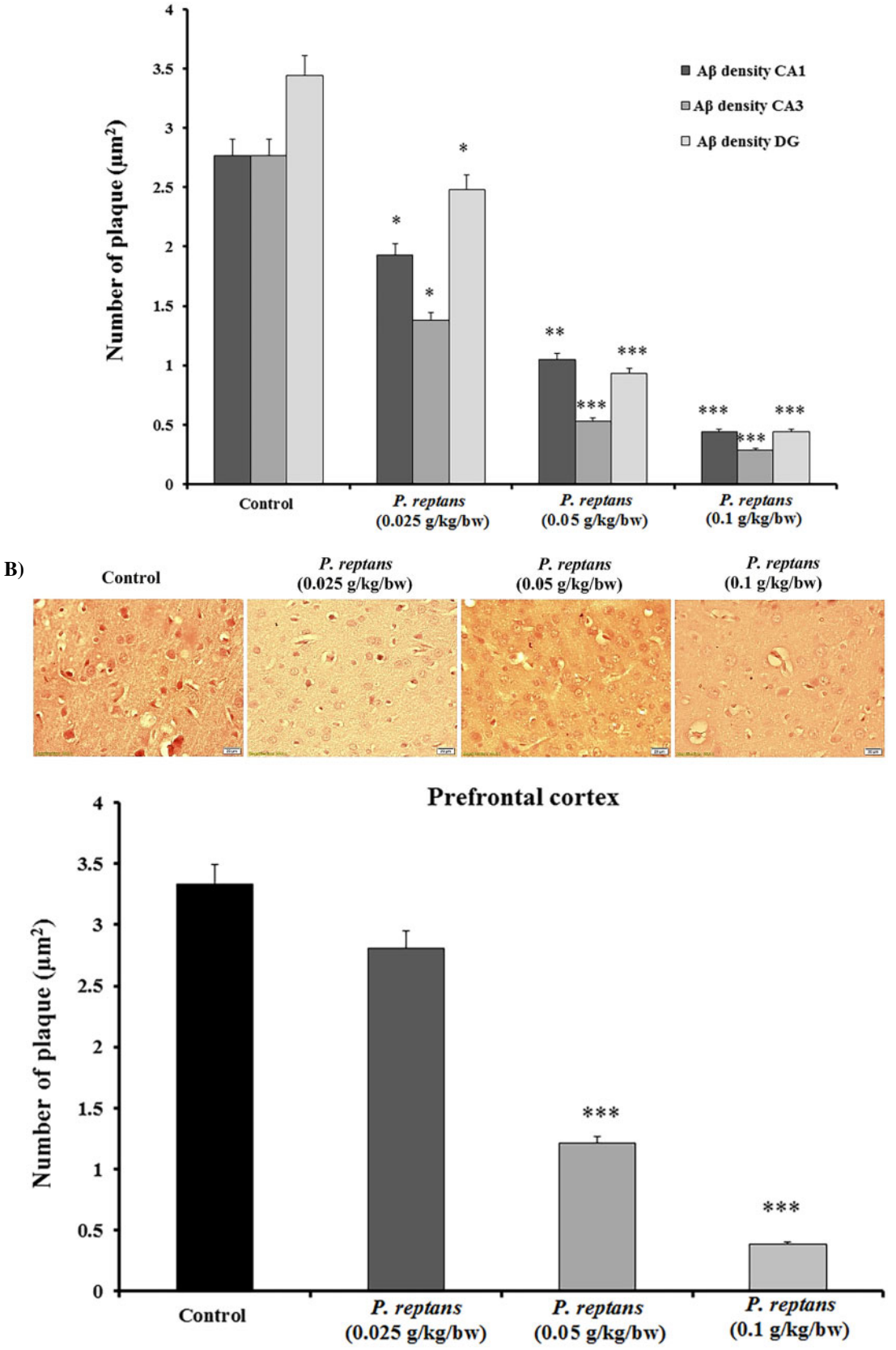

Figure 2. Effect of *Potentilla reptans* L. root extract on the β-amyloid plaque number in an anxiety rat hippocampus and prefrontal cortex in the control, *P. reptans* (0.025 g/kg/BW), *P. reptans* (0.05 g/kg/BW), and *P. reptans* (0.1 g/kg/BW) groups. The bars show the means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. bw: body weight; DG: dentate gyrus. Scale bar: 20 μM

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Table 2. Anxiety behavioral result and brain parameters (beta-amyloid (Aβ) plaques and neuron count) according to different concentrations of the methanolic extract of *P. reptans* root in the hippocampus and prefrontal cortex of male rats in the anxiety

	Congophilic Aβ density CA1	Congophilic Aβ density CA3	Congophilic Aβ density DG	Congophilic Aβ density prefrontal cortex	Neuron number CA1	Neuron number CA3	Neuron number DG	Neuron number prefrontal cortex	Behavioral test
Control	2.77 ± 1.22	2.77 ± 1.28	3.44 ± 1.18	3.33 ± 2.34	35.55 ± 9.930	20.63 ± 5.71	110.5 ± 17.6	5.87 ± 1.49	40.36 ± 9.23
P. reptans (0.025 g/kg/BW)	1.93 ± 1.14*	1.38 ± 1.10*	2.48 ± 0.75*	2.81 ± 1.75	39.15 ± 12.11*	20.76 ± 6.17	122.3 ± 34.2*	8.64 ± 2.09***	14.7 ± 42.66
P. reptans (0.05 g/kg/BW)	1.05 ± 1.31**	0.53 ± 0.71***	0.93 ± 0.94***	1.21 ± 1.17***	47.48 ± 16.52***	21.00 ± 8.14	144.3 ± 30.3***	10.21 ± 2.07***	10.5 ± 39.71
P. reptans (0.1 g/kg/BW)	0.44 ± 0.63***	0.29 ± 0.51***	0.44 ± 0.67***	0.38 ± 0.57***	40.65 ± 11.07*	18.41 ± 5.85	142.5 ± 25.4***	7.59 ± 1.63***	17.5* ± 38.36
EC_{50} (g/kg/bw)	0.045	0.036	0.037	0.041	0.039	0.056	0.033	0.048	0.055

Note: * means $p < 0.05$, ** means $p < 0.01$ and *** means $p < 0.001$; bw: body weight.

In the next step, the present study showed that *P. reptans* extract reduces the accumulation of these plaques in the hippocampus and prefrontal cortex of rats, and that this effect is directly related to the concentration of the extract.

According to previous studies, there is a relationship between anxiety symptoms and the amount of beta-amyloid deposition. Healthy people with higher beta-amyloid levels showed more anxious behavior. Additionally, increased beta-amyloid deposition has been linked to anxiety symptoms and cell destruction (Cai et al., 2021; Ramakers et al., 2013). The severity of these anxiety symptoms is related to the amount of deposition in the posterior regions of the cingulate, frontal, and medial temporal regions (Lavretsky et al., 2008). In animal model experiments, injecting glucocorticoid hormone in concentrations similar to anxiety levels increased beta-amyloid deposition in the hippocampus of Alzheimer's model rats (Green et al., 2006). Recent studies suggest an effective role of the hypothalamus-pituitary-adrenal (HPA) axis in the link between anxiety and beta-amyloid plaques (Green et al., 2006). In addition, extracts from various plants have shown plaque-reducing effects, and it has been suggested that this effect is due to the presence of polyphenols. These substances can inhibit the accumulation of amyloidogenic peptides, thus preventing the formation of beta-amyloid plaques (Frydman-Marom et al., 2011; Fujiwara et al., 2006; Ono et al., 2008). Although the exact mechanism of action of this extract is not known, its anti-anxiety and beta-amyloid-reducing effects may hold promise for the development of new treatments for various diseases such as Alzheimer's. Excessive fear may be due to increased fear acquisition or impaired fear extinction (Ghasemi et al., 2022). Studies have revealed that fear extinction is a context-dependent process (Garfinkel et al., 2014). Conflict arises when there is a discrepancy between what is expected based on the information retrieved from memory and what is happening. Human imaging studies have demonstrated the role of the hippocampus in context-dependent fear processing. The DG, CA3, and CA1 sub-regions of the hippocampus are involved in contextual fear retrieval, while DG and CA1 play a role in retrieving contextual fear memories. Furthermore, both DG and CA1 contribute to the extinction of contextual fear (Bernier et al., 2017; Morellini et al., 2017). Since stressful experiences are not completely identical, fear reactions must be assigned to stimuli with sufficient similarity to the initial event; otherwise, generalization of fear can occur (Dymond et al., 2015).

According to previous studies, exposure to anxiety causes structural changes in the brain (Leuner & Shors, 2013). The activation of the HPA axis in the face of anxiety leads to excessive amounts of glucocorticoids and glutamates in the brain, which induce neuroinflammation (Lee et al., 2022b). According to a study by R.J. Rodgers et al., exposure to an elevated plus-maze for 5 minutes significantly increases plasma cortisol levels in male rats (Rodgers et al., 1999). Additionally, stress stimulates sympathetic nerves, which activate the Renin-Angiotensin System (RAS) (Lee et al., 2022b). Angiotensin II enhances the production of ROS and inflammatory cytokines, which contribute to neuroinflammation and induce neuronal apoptosis (Cardinale et al., 2012).

Neuronal plasticity is one of the prominent features of the human brain that ensures adaptation to environmental changes. The DG of the hippocampus is one of the most plastic brain areas because it produces adult-born Granule cells (GCs) (Zhao et al., 2008). This area contains a wide range of neurons in different stages of maturity, which are assumed to be involved in anxiety processing (Anacker et al., 2018; Catavero et al., 2018). According to studies, mature GCs are mainly activated in anxiogenic environments, while adult-born GCs inhibit these stress-induced cells and protect the brain from anxiety effects (Anacker et al., 2018). It has also been noted that reduction of these neuronal populations leads to an increase in anxiety-like behaviors in mice (Deng & Gage, 2015), and functional impairment of newborn GCs has been reported in mice with higher levels of innate anxiety (Sah et al., 2012). Thus, it has been assumed that this new population of neurons contributes to the regulation of anxiety behaviors by enhancing the extinction of fear memories (Graham & Milad, 2011). In a study conducted by Ishikawa et al., enhancing hippocampal neurogenesis by memantine promoted forgetting of contextual fear memory in a PTSD rodent model (Ishikawa et al., 2016). In another study, treatment with an herbal combination improved hippocampal neurogenesis and memory and reduced fear responses in mice. Hence, compounds that can enhance neurogenesis or reduce neuronal loss in this area can be considered as a therapeutic option for anxiety (Park et al., 2023).

Pretreatment with 3 concentrations of *P. reptans* has demonstrated neuroprotective effects on hippocampal sub-regions. There is evidence of the antioxidant and anti-inflammatory effects of *P. reptans* (Tomovic et al., 2015). Moreover, Enayati et al. in 2018 demonstrated the cardioprotective preconditioning effects of *P. reptans*, which is mainly through nitric oxide (NO) release, activating the nuclear factor erythroid 2-related factor 2 (NRF2) pathway, and inhibition of ROS that led to a decrease in the apoptotic index (Enayati et al., 2018). A 2023 study has shown the neuroprotective effects of this extract, which protect hippocampal subregions from neuronal loss and β-amyloid accumulation in a global ischemia/reperfusion model (Noorgaldi et al., 2023). A recent investigation into phytochemical profiling and enzyme inhibition in *P. reptans* root revealed that ACHE inhibitors in *P. reptans* root exhibit neuroprotective properties (Mirzaei et al., 2024). Furthermore, a rich number of flavones, flavonoids, and anthocyanins exhibit antioxidant and anti-apoptotic effects through direct interaction with ROS, improving cerebral blood flow via increasing NO levels and protecting neurons against neurotoxins (Zhang et al., 2012). In addition, flavones may cause sedative, anxiolytic, or anticonvulsant effects by acting on the GABA-A receptor (Zhang et al., 2012). Studies have also demonstrated that beta-amyloid plaque deposition has a neurotoxic effect due to ROS production and the induction of cell apoptosis (Huang et al., 2016). Considering that the present study has shown a reduction in

beta-amyloid plaques in the hippocampus after pretreatment with *P. reptans*, this can be hypothesized as one of the possible mechanisms of neuroprotection in this area. Despite the possible mechanisms mentioned, further studies are needed to investigate the actual mechanisms underlying the neuroprotective effect of *P. reptans* root extract as a potential anti-anxiety candidate.

5 Conclusion

In conclusion, we found that anxiety can cause behavioral deficits, degeneration of neurons, and accumulation of β-amyloid plaque in the brain's hippocampus and prefrontal cortex. It appears that *P. reptans* root extract, through its active ingredients, shows beneficial effects in reducing anxiety and related damage through anxiolytic effects, including neuronal damage and amyloid accumulation. However, further studies with more reliable evidence are recommended to qualify the pharmaceutical and pharmacological potential of this plant as an anti-anxiety supplement.

Author Contributions

Ali Ashkbary, Farnaz Hooshmand and Hadise Heidarpour: data collection and animal model performance; Mehrdad Jahanshahi, Gokhan Zengin and Ayesheh Enayati: conceptualization, the study design and editing, clarification of data, writing-original draft and final version; Mehrdad Jahanshahi and Ayesheh Enayati: data analysis; and all authors approved the final version.

Availability of Data and Materials

The authors declare that should any raw data files be needed about the further data of the study, they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Ethics Approval

The protocol for animal and plant use were approved by the Research Ethics Committees of Golestan University of Medical Sciences with ethics codes of (IR.GOUMS.AEC.1401.001), (IR.GOUMS.AEC.1401.002), and (IR.GOUMS.AEC.1401.003). The study was carried out in compliance with the ARRIVE guidelines and also, the plant protocols were performed in accordance with the HMPC guidelines. The authors declare that all of the experiments comply with the current guidelines and legislation of the Islamic Republic of Iran and the Golestan University of Medicals Sciences. For consent to participate, not applicable.

Artificial Intelligence (AI) Statement

The authors affirm that no Artificial Intelligence (AI) tools were employed in the preparation of this manuscript, which includes language editing, graphical abstract preparation, figure generation, or image editing.

Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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