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Original Article

Bufo viridis secretions improve anxiety and depression-like behavior following intracerebroventricular injection of amyloid β

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Abstract

Background and purpose: Venenum Bufonis is a Chinese traditional medicine produced from the glandular secretions of toads that contain biogenic amines, which have anti-inflammatory properties. The present study aimed to examine the effect of *Bufo viridis* secretions (BVS) on anxiety and depression-like behavior and hippocampal senile plaques volume in an animal model of Alzheimer's disease (AD).
Experimental approach: Thirty-eight male Wistar rats were used. AD was induced by amyloid-beta ($A\beta_{1-42}$) (10 µg/2 µL, intracerebroventricular injection, icv) and then BVS at 20, 40, and 80 mg/kg were injected intraperitoneally (ip) in six equal intervals over 21 days. Anxiety and depression-like behavior were assessed using behavioral tests including open field test (OFT), elevated plus maze (EPM), and forced swimming test (FST) 21 days after the surgery. The volume of senile plaques was assessed based on the Cavalieri principle.
Findings/Results: Results of the OFT showed that the central crossing number and the time in the AD group were significantly decreased compared to the sham group ($P < 0.01$ and $P < 0.001$, respectively). Also, the values of these two parameters significantly increased in the AD + BVS80 group than the AD group ($P < 0.05$ and $P < 0.001$, respectively). The time spent in the closed arm in the EPM dramatically increased in the AD group compared to the sham group ($P < 0.05$) and significantly decreased in the AD + BVS80 group compared to the AD group ($P < 0.05$). Results of the FST indicated that immobility time had a reduction in the AD + BVS20 ($P < 0.01$), AD + BVS40, and AD + BVS80 groups compared to the AD group ($P < 0.001$). The volume of senile plaques in the hippocampus showed a reduction in the treatment groups in comparison with the AD group ($P < 0.001$ for all).
Conclusion and implications: Results revealed that BVS injection could improve symptoms of anxiety and depression and decrease senile plaques in the hippocampus in an animal model of AD.

Keywords: Amyloid-β_{1-42}; *Bufo viridis* secretion; Depression; Elevated plus-maze; Forced swimming test; Open field test.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative and inflammatory disease that causes changes in cognition, memory, and behavior and is accompanied by symptoms such as depression, anxiety, and psychosis (1). AD is the costliest disease in society, the sixth cause of mortality in the US, and the second most prevalent disease, after heart failure, among the elderly (2). In this disease, the precipitation of amyloid-beta (Aβ) peptide in certain brain regions, including the hippocampus, results in neurological disorders and disruptions in the function of synapses (3). In recent years, one of the main focus of studies is on the clearance pathways for senile plaques and also on finding a medication to alleviate cognitive disorders and controlling the precipitation of the Aβ (4).

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Evidence indicated that antidepressants may play a role in improving the symptoms of AD, suggesting a possible link between AD and anxiety and depression (5-7). Since depression is common in AD patients and has also a negative effect on their general status, its diagnosis and management are very important (8). Chronic inflammation as a basic mechanism is not only involved in the formation of senile plaques containing the Aβ and neurofibrillary tangles in AD but also in the reduction of monoamines and making disorders in the hypothalamic-pituitary-adrenal axis (HPA axis) in depression (7). These factors eventually increase the level of glucocorticoids and pro-inflammatory cytokines such as interleukin 6 (IL-6), IL-1β, IL-12, and tumor necrosis factor-alpha (TNF-α), and also decrease the level of brain-derived neurotrophic factor (BDNF) in the brain (9). The increase in pro-inflammatory cytokines changes the metabolism of serotonin and increases neurodegeneration through activating the indoleamine 2, 3 dioxygenase, and tryptophan 2, 3 dioxygenase pathways and reducing the plasticity of synapses in both diseases (10). Studies showed that the risk of increased anxiety in AD patients is higher than in others. In addition, the presence of anxiety symptoms leads to a faster reduction of verbal memory, language, and executive function (11). Moreover, anxiety reduces the patient's quality of life by causing symptoms such as changes in behaviors, limiting daily life activities, and waking up at midnight (12). Anxiety-induced stress increases amyloid precursor protein expression and Aβ peptide production, which worsens AD (13). Therefore, treatments that can reduce anxiety and depression not only improve the quality of the patient's life but also prevent the acceleration of the AD process.

For centuries, China and other Asian countries have been aware of the medicinal properties of the secretions of auricular and skin glands of Bufonidae (14). Medications extracted from *Venenum Bufonis* (VB) have been used for a long time as anti-malignant tumors, anti-diabetic, analgesic, anti-viral, anti-microbial, immunomodulatory, and contraceptive agent. Upon the discovery of biological substances in the dried secretions of the skin and parotid glands of Bufonidae toads, a new area of anti-inflammatory and anticancer drug research was introduced. Medications extracted from VB affect nuclear factor kappa B (NFκB), nitric oxide (NO), cyclooxygenase-2 (COX-2), NO synthase (iNOS), prostaglandin E2, IL-8, IL-6, IL-1, TNF-α, and their signaling (15,16). VB contains biogenic amines, steroid compounds, alkaloids, and peptides. Biogenic amines include serotonin, histamine, dopamine, adrenaline, and noradrenaline. Toad's glandular secretions have been used for diseases such as depression, inflammation, memory, learning, and neurologic diseases such as AD and Parkinson's disease (17-19).

Bufo viridis is extensively distributed in the northeast of Iran (20). Numerous studies have been conducted on various types of extracts and the crude drug form of the secretions of different species of the *Bufo*. However, few studies have been conducted on the effects of the *Bufo viridis* species secretion (BVS) on anxiety and depression. The present study examined the effect of BVS on anxiety and depression-like behavior using behavioral tests including open field test (OFT), elevated plus maze (EPM), and forced swimming test (FST) and measurement the left hippocampal senile plaques volume through histopathological methods in an animal AD model.

METHODS AND MATERIALS

Animals

Thirty-eight male Wistar rats (220-280 g) were kept in the animal house of the Islamic Azad University- Mashhad branch with a 12/12-light/dark cycle (light from 8:00 to 20:00) at 21-23 °C with free access to food and water. All experiments were performed according to the guideline of the National Institutes of Health (NIH) and the Institutional Ethics Committee at the Islamic Azad University (Ethical code: IR.IAU.MSHD.REC.1397.061) for the care and the use of laboratory animals. The animals were randomly divided into 5 groups (n = 7-8) including (1) the sham group, surgical stress is induced and received an injection of saline (2 µL; intracerebroventricular injection, icv) instead of Aβ, and also saline (0.5 mL;

intraperitoneally, ip) as a vehicle was injected instead of 6 times treatment during the next 21 days; (2) the AD group received an injection of $A\beta_{1-42}$ (10 µg/2 µL, icv) and saline as a vehicle (0.5 mL, ip) instead of 6 times treatment during the next 21 days; (3-5) the AD + BVS20, AD + BVS40, and AD + BVS80 groups received an injection of $A\beta_{1-42}$ (10 µg/2 µL, icv) and 20, 40, and 80 mg/kg of BVS (ip) 6 times during the next 21 days, respectively. The saline and BVS were injected every 4 days. Before performing the behavioral tests, the animals were handled for 5 days. On the day of the test, they were kept at the laboratory for 30 min for adapting to the environment. All the behavioral tests were performed 21 days after Aβ injection and BVS treatments (Fig. 1).

Collection of BVS

In total, 10 adult *Bufo viridis* toads (40-50 g) were collected from the suburbs of Mashhad, Razavi Khorasan Province, I.R. Iran, during summer (from July to August 2018). Crude BVS was obtained by mild electrical stimulation using a stimulator (5-10 V) under anesthesia with ether (14,21). On average, 50 mg of venom was obtained from each toad. BVS was very sticky and creamy in color at the time of collection, but after drying, it turned brown a few hours later. Based on several studies, the median lethal dose (LD_{50}) in the secretions of Bufonidae toads was 500 mg/kg for the rats. BVS was dissolved in distilled water and 20, 40, and 80 mg/kg (ip) were injected in 6 times after the induction of AD with an interval of 4 days.

Chemical analysis of the BVS extract

In the dissolving stage, the specimen with the concentration of 15 w/v% was prepared in three solvents of dimethylformamide, methanol, and ethyl acetate, with the same molar ratio (1:1:1). Then, 1 µL of the extract was injected into the gas chromatography device coupled with a mass detector (Agilent Company, USA). The chemical profile of BVS was analyzed using the gas chromatography-mass spectrometry (GC-MS) device (mode "selected ion monitoring", SIM). Each chromatographic peak represented a unique species. The compounds were identified by matching the resulting M/Z pattern with the Wiley and NIST digital library (M stands for mass and Z stands for charge number of ions).

AD induction

To prepare the Aβ solution, the $A\beta_{1-42}$ (10 µg/2 µL) peptide (Sigma-Aldrich, Germany) was dissolved in distilled water and to create neurological toxicity, the solution was kept in an incubator at 37 °C for 1 week. The $A\beta_{1-42}$ solution was kept in the freezer at -20 °C until use. To induce AD, the animals were anesthetized by the intraperitoneal injection of xylazine (10 mg/kg; Alfasan, Netherlands) and ketamine (80 mg/kg; Alfasan, Netherlands). Then, their heads were fixed in the stereotactic surgery device. The coordinates of the lateral ventricle were anterior- posterior = -0.84, medial-lateral = ±1.6, and dorsal-ventral = 4 mm (based on the Paxinos and Watson atlas) (22). Then, 10 µg/2 µL of $A\beta_{1-42}$ solution was injected gently with Hamilton syringes into the right lateral ventricle (23).

Open field test

This test is used to evaluate behavioral responses such as locomotor activity, hyperactivity, exploratory behavior, and anxiety (24). OFT is an indicator of the activity of dopaminergic and glutamate system, performed for 5 min. To perform this test, a white wooden cube (100 × 100 cm) with a 50 cm wall divided into 16 squares (25 × 25 cm each) was employed. The movement of the rats was recorded using a camera and the following parameters were measured:

(1) Central crossing, (2) central time, (3) peripheral crossing, (4) peripheral time, and (5) total crossing (25,26).

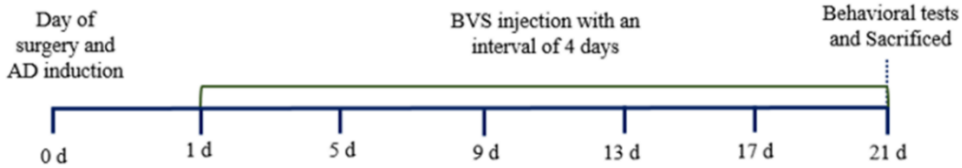

Fig. 1. Study design.

Elevated plus maze

To assess anxiety in the behavioral models, EPM was utilized. This instrument had 4 arms looking like a plus sign. The dimensions of the open and closed arms were 50 × 10 cm, and walls with a height of 50 cm were placed at the two ends of the enclosed arm. The four arms led to a central space (10 × 10 cm). The maze was elevated from the ground by 50 cm using stands. The duration of the presence of the rats in the open and enclosed arms was recorded using a digital camera for 5 min (25).

Forced swimming test

FST is one of the most common tests for examining the level of depression. A glass column with a height of 60 cm and a diameter of 38 cm was used which contained water at 24 ± 1 ℃ to the depth of 40 cm. Immobility time, active time, and climbing time were measured and recorded using a chronometer for 5 min (27).

Histological and stereological examination

After behavioral tests, under deep anesthesia using ketamine and xylazine, the animals were perfused with 4% paraformaldehyde. Finally, the brains were removed and hematoxylin and eosin (H&E) staining was performed to determine the presence of senile plaque (28). The Cavalieri principle was used to measure the volume of senile plaques in the left hippocampus. In this technique, the equation below was used to calculate the volume:

$$EstV = \Sigma p \times ap \times d$$

Where, Est V stands for the computed volume, the ΣP represents the sum of test points, the ap and the d are the planar areas related to the lattice test point, and the distance between the sampled section planes, respectively (29).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The Kolmogorov-Smirnov test was used to determine the normality of the data distribution. Data were measured based on one-way ANOVA followed by Tukey's post hoc test. Data were represented by mean ± SEM. *P* values < 0.05 were considered significant.

RESULTS

GC-MS analysis of BVS extract

Here, 66 sections of BVS extract at the SCAN mode were identified using the GC-MS device (data were not shown). Moreover, 4 different masses were selected for 3 species of biogenic amines effective in the treatment of depression, and quantitative measurement was performed at mode SIM. This value was 31.11% for serotonin, 33.32% for norepinephrine, and 35.57% for histamine (Fig.2).

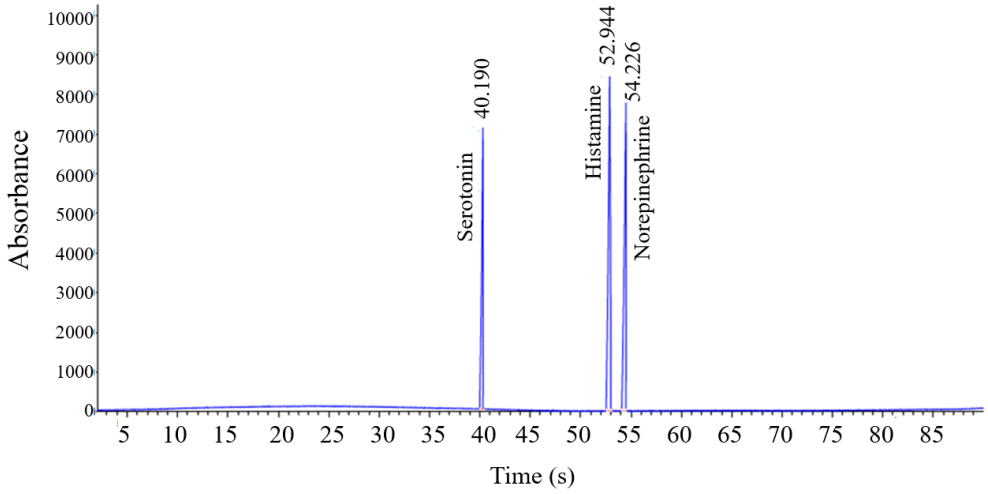

Fig. 2. SIM graph of the real sample *Bufo viridis* secretion extract in SIM mode (serotonin (30, 42, 204, **58**), histamine (**82**, 30, 54, 41), norepinephrine (111, 65, **93**, 139)). SIM, Selected ion monitoring.

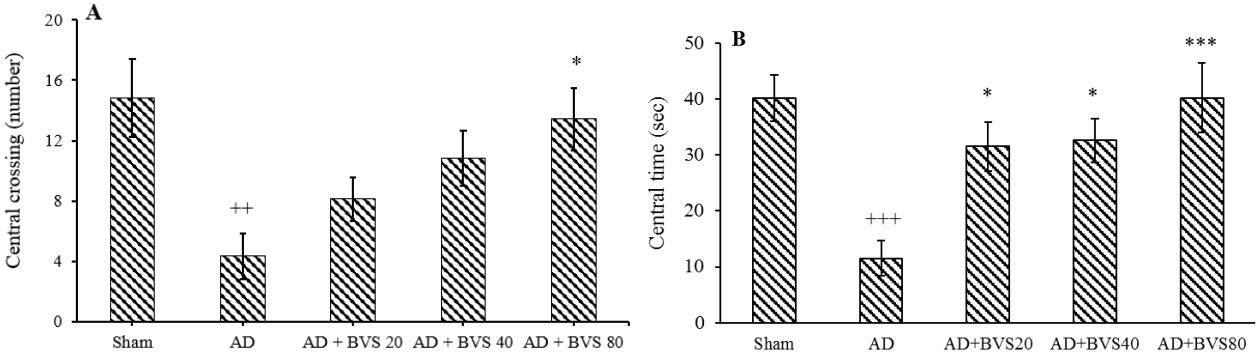

Fig. 3. BVS effects on (A) central crossing number and (B) the time spent in the central zone in the open field test between the five groups. Data are shown as mean ± SEM, n = 7-8. $^{++}P < 0.01$ and $^{+++}P < 0.001$ Indicates significant differences in comparison to the sham group; $^{*}P < 0.05$ and $^{***}P < 0.001$ *vs* the AD group. BVS, *Bufo viridis* secretion; AD, Alzheimer's disease.

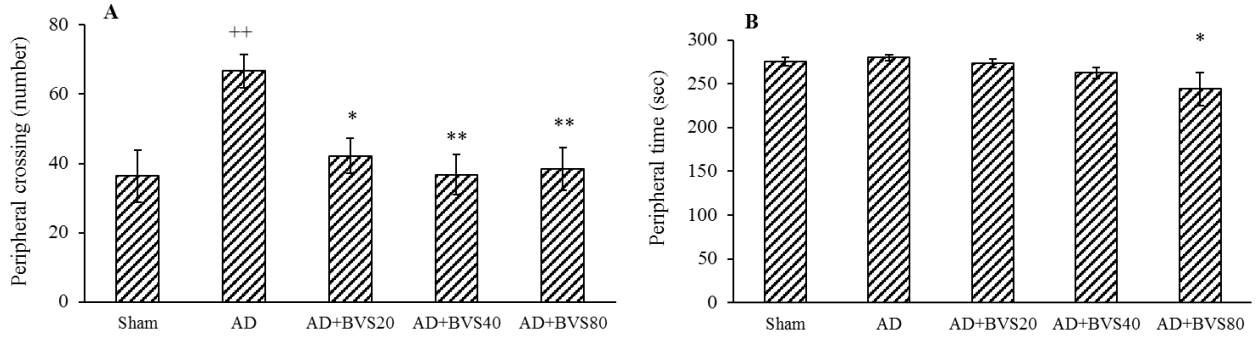

Fig. 4. BVS effects on (A) peripheral crossing number and (B) the time spent in the peripheral zone in the open field test between the five groups. Data are expressed as mean ± SEM, n = 7-8. $^{++}P < 0.01$ Indicates significant differences in comparison with the sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ in contrast with the AD group. BVS, *Bufo viridis* secretion; AD, Alzheimer's disease.

BVS increased the locomotor activity of rats in the central zone in OFT

Results of OFT showed that the central crossing number in the AD group (4.33 ± 1.49) was decreased compared to the sham group (14.83 ± 2.57; $P < 0.01$). No significant difference was found in the number of crossings in the central zone in the AD + BVS20 (8.11 ± 1.43) and AD + BVS40 (10.83 ± 1.81) groups compared to the AD group. Animals in the AD + BVS80 group (13.43± 2.069; $P < 0.05$) had a higher number of crossing times in the central zone than the AD group (Fig. 3A). Results of OFT also showed that the time spent in the central zone in the AD group (11.50 ± 3.13) was decreased significantly ($P < 0.001$) compared to the sham group (40.14 ± 4.19). Nevertheless, the animals in the AD + BVS20 (31.50 ± 4.35); AD + BVS40 (32.57 ± 3.89; $P < 0.05$), and AD + BVS80 (40.22 ± 6.15; $P < 0.001$) groups spent longer time than the AD group in the central zone (Fig. 3B).

BVS decreased the activity of rats in the peripheral zone in OFT

An increase in peripheral crossing and time can be considered as an anxiety-like behavior and entering into the central squares is used to evaluate the exploratory behavior. Peripheral crossing number in the AD group (66.66 ± 4.91) was significantly ($P < 0.01$) increased compared to the sham group (36.33 ± 7.47). The number of crossings in the peripheral zone showed a significant decrease in the AD + BVS20 (42.12 ± 5.08; $P < 0.05$), AD + BVS40 (36.71 ± 5.77; $P < 0.01$), and AD + BVS80 (38.44 ± 6.23; $P < 0.01$) groups compared to the AD group (Fig. 4A). Comparing the duration of the presence of the rats in the peripheral zone showed no significant difference between the AD (279.90 ± 3.63) and sham (275.70 ± 4.94) groups. Moreover, the results indicated no significant difference between the AD + BVS20 (273.30 ± 4.70) and AD + BVS40 (262.30 ± 6.05) groups in comparison to the AD group.

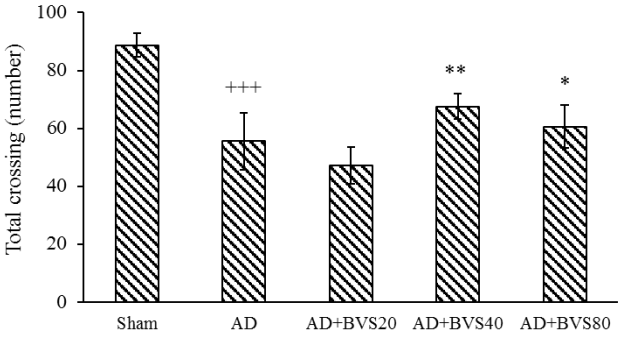

Fig. 5. BVS effects on total crossing number in the open field test among the five groups. Data are expressed as mean ± SEM, n = 7-8. $^{+++}P < 0.001$ Indicates significant differences in comparison to the sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ against the AD group. BVS, *Bufo viridis* secretion; AD, Alzheimer's disease.

The duration of the presence of the animals in the AD + BVS80 group (243.88 ± 19.16; $P < 0.05$) was remarkably decreased compared to the AD group (Fig. 4B).

BVS increased exploratory behavior in animals in OFT

Increasing the total crossing number is an indicator of locomotor activity. Results of OFT demonstrated that the total number of crossing in the AD group (55.5 ± 9.76) was significantly decreased compared to the sham group (88.66 ± 4.05; $P < 0.001$). No significant difference between the AD and AD + BVS20 (47.16 ± 6.38) groups was observed, but the number of crossing in the whole zone had a significant increase in the AD + BVS40 (67.57 ± 4.40; $P < 0.01$) and AD + BVS80 (60.60 ± 7.44; $P < 0.05$) groups compared to the AD groups (Fig.5).

BVS reduced anxiety-like behavioral symptoms and decreased the duration of the presence of rats in the closed arm in EPM

Results of the EPM test indicated that a significant difference existed between duration of presence for the rats in the AD group (59 ± 8.69; $P < 0.01$) in the open arm compared to the sham group (158.16 ± 7.42). No significant difference existed between the AD and AD + BVS20 (91.66 ± 22.75) and AD + BVS40 (97.2 ± 13.83) groups. However, the AD + BVS80 group (191.50 ± 34.02; $P < 0.001$) spent a longer duration than the AD group in the open arm (Fig. 6A). The duration of the presence of the rats in the AD group (228.85 ± 12.13) in the closed arm showed a significant increase compared to the sham group (141.83 ± 7.42; $P < 0.05$). However, the AD + BVS20 (198.37 ± 23.19 s) and AD + BVS40 (190.87 ± 14.35) groups showed no significant difference compared to the AD group. The duration of the presence of the rats of the AD + BVS80 group (126 ± 33.66; $P < 0.05$) in the closed arm was significantly decreased compared to the AD group (Fig. 6B).

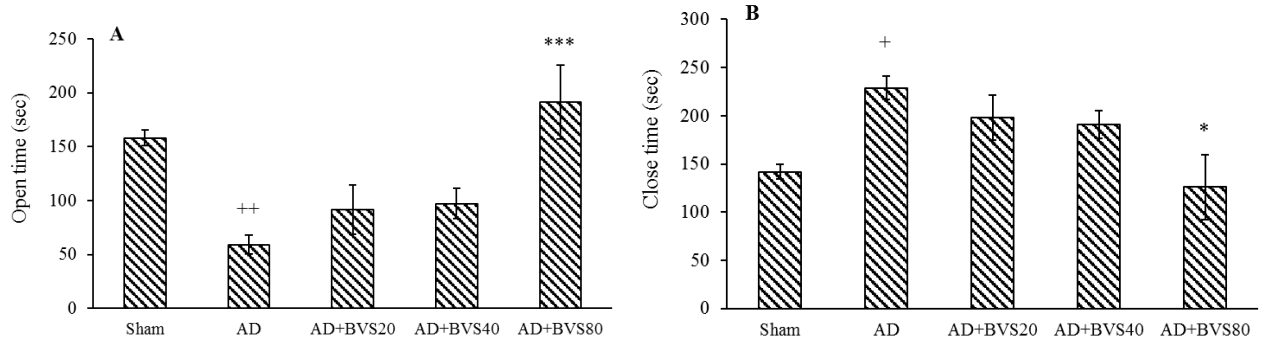

Fig. 6. BVS effects on the (A) time spent in open arm and (B) closed arm in the elevated plus maze among the five groups. Data are expressed as mean ± SEM, n = 7-8. $^{+}P < 0.05$ and $^{++}P < 0.01$ Indicates significant differences compared to the sham group; $^{*}P < 0.05$ and $^{***}P < 0.001$ in comparison with the AD group. BVS, *Bufo viridis* secretion; AD, Alzheimer's disease.

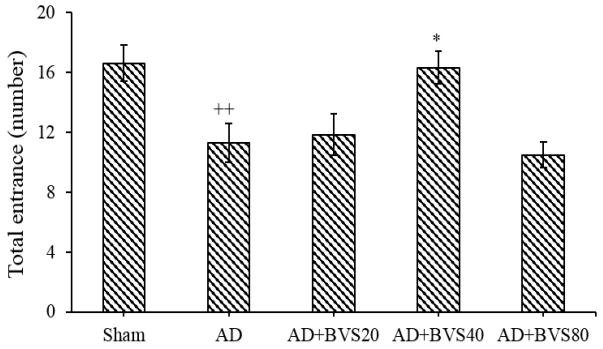

Fig. 7. BVS effects on the total entry into elevated plus maze arms. Data are expressed as mean ± SEM, n = 7-8. $^{++}P < 0.01$ Indicates significant differences compared to the sham group; $^{*}P < 0.05$ *vs* the AD group. BVS, *Bufo viridis* secretion; AD, Alzheimer's disease.

BVS increased the number of entries into open and closed arms in EPM

The total number of entries into open and closed arms was the locomotor activity index. The total number of entries in the AD (11.28 ± 1.30) group was significantly lower than the sham group (16.62 ± 1.22; $P < 0.01$). We did not observe any significant difference between the AD + BVS20 (11.85 ± 1.38) and AD + BVS80 (10.5 ± 0.84) with AD groups. However, this amount was increased in the AD + BVS40 (16.33 ± 1.08; $P < 0.05$) group compared to the AD group (Fig.7).

BVS improved depression-like behavioral symptoms in FST

FST was used to assess the level of depression in the animals. The results of this test indicated that immobility time was (89.87 ± 3.48) in the AD group, showing a significant increase in comparison to the sham group (53.66 ± 5.00; $P < 0.001$). Immobility time had a significant reduction in the AD + BVS20 (67 ± 5.88; $P < 0.01$), AD + BVS40 (51.3 ± 3.90; $P < 0.001$), and AD + BVS80 (36.11 ± 4.08; $P < 0.001$) groups compared to the AD group (Fig. 8A). Based on the results, the active time showed a significant decrease in the AD group (132.62 ± 8.28) compared to the sham group (206.77 ± 7.69; $P < 0.001$). Also, the active time had an increase in the AD + BVS20 (179.22 ± 10.55), AD + BVS40 (196.8 ± 7.75), and AD + BVS80 (227 ± 8.33) ($P < 0.001$, for all comparisons) groups compared to the AD group (Fig. 8B). Examining the results of climbing time showed a significant increase in the AD group (77.50 ± 6.83; $P < 0.001$) compared to the sham group (39.55±5.91). The AD + BVS20 (53.77 ± 4.66), AD + BVS40 (51.90 ± 4.07) ($P < 0.01$), and AD + BVS80 (36.88 ± 4.86; $P < 0.001$) groups indicated a decrease in climbing time compared to the AD group (Fig. 8C).

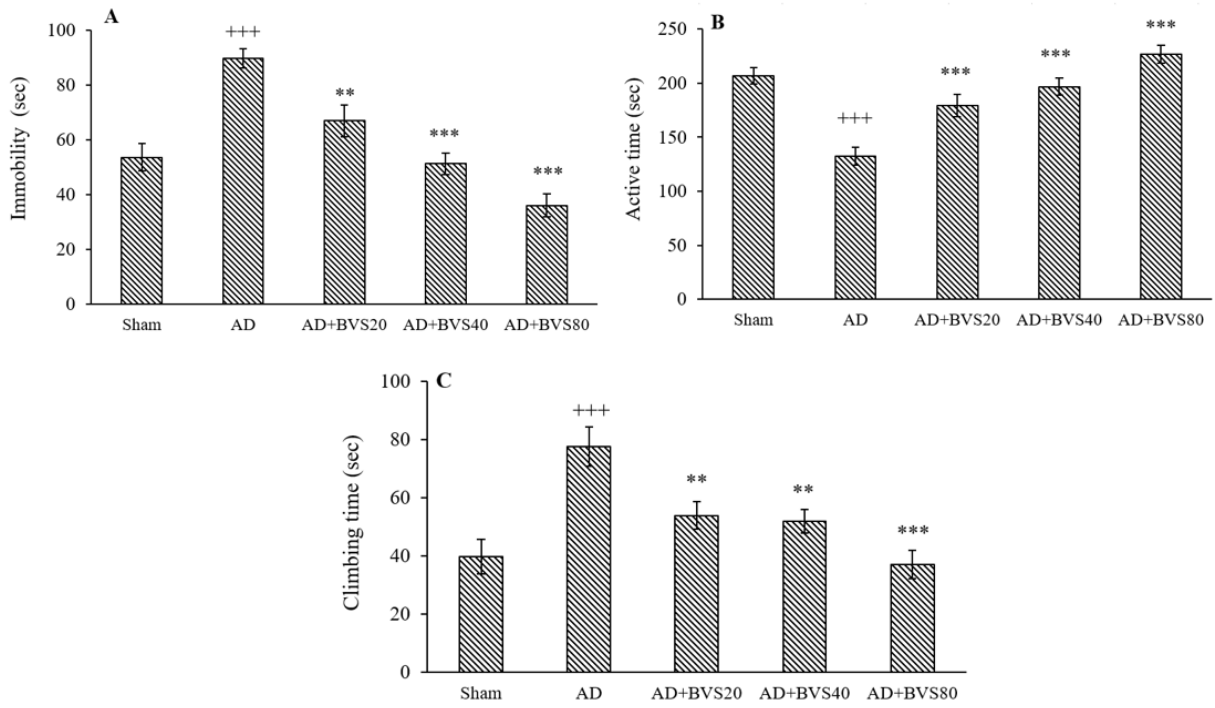

Fig. 8. BVS effects on (A) immobility time, (B) active time, and (C) climbing time in the forced swimming test among the five groups. Data are shown as mean ± SEM, n = 7-8. $^{+++}P < 0.001$ shows significant differences in comparison to the sham group; $^{**}P < 0.01$ and $^{***}P < 0.001$ in comparison to the AD group. BVS, *Bufo viridis* secretion; AD, Alzheimer's disease.

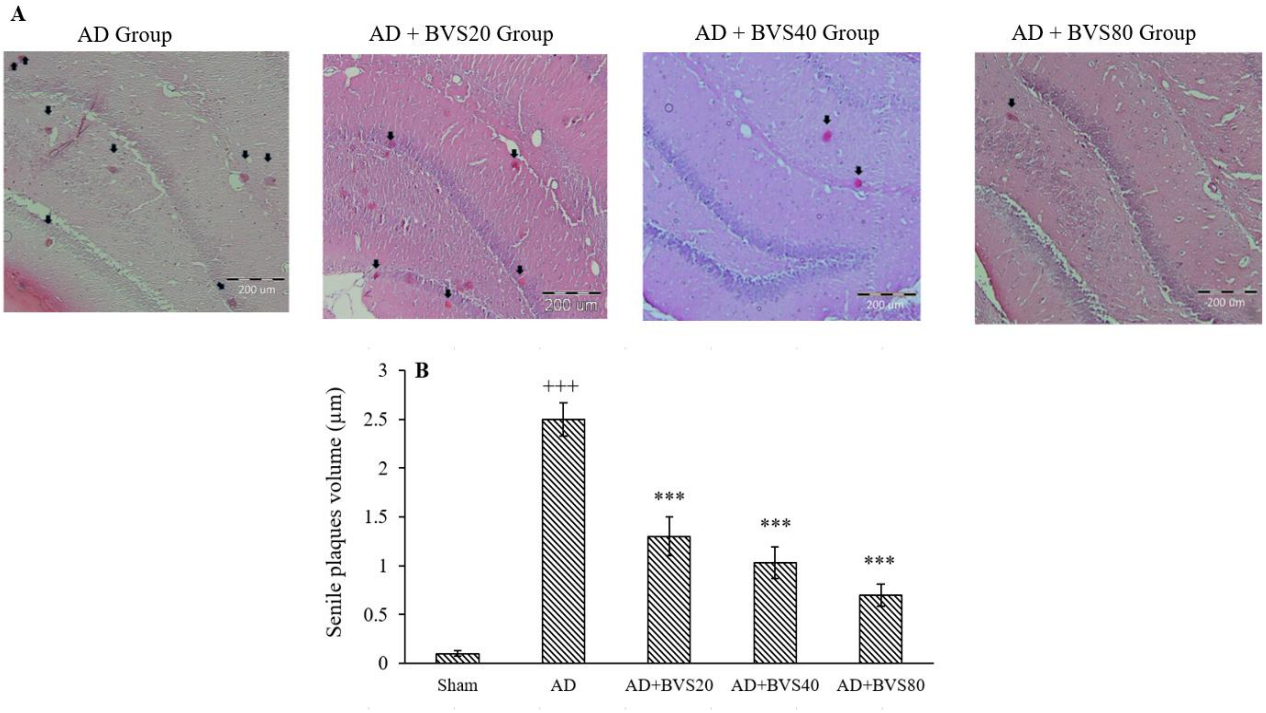

Fig. 9. (A) Representative photomicrographs of the rat hippocampus in the different groups. Black arrows show the senile plaques. (B) BVS effects on the volume occupied by the senile plaques in the left hippocampus (in µm) among the five groups. Data are expressed as mean ± SEM, n = 7-8. $^{+++}P < 0.001$ in comparison to the sham group; $^{***}P < 0.001$ against the AD group. BVS, *Bufo viridis* secretion; AD, Alzheimer's disease.

Table 1. BVS effects on primary and secondary weight differences of the samples among the five groups. Data are expressed as mean ± SEM, n = 7-8.

Groups	**Weight differences**	***P* values**
Sham	27.2 ± 5.71	-
AD	26.1 ± 5.61	NS
AD + BVS20	12.90 ± 5.25	NS
AD + BVS40	-2.44 ± 5.09	0.01
AD + BVS80	-10.55 ± 6.62	0.001

BVS, *Bufo viridis secretion;* AD, Alzheimer's disease; NS, non-significant.

Intraperitoneal injection of bufotoxin led to a decreased volume of senile plaques in the hippocampus of rats.

The results of examining the volume of senile plaques in the left hippocampus showed that the volume of plaques in the AD group (2.5 ± 0.168) had a significant increase compared to the sham group (0.1 ± 0.03) (Fig. 9). Analysis of this result showed a significant reduction in this value in the AD + BVS20 (1.3 ± 0.199), AD + BVS40 (1.03 ± 0.165), and AD + BVS80 (0.7 ± 0.115) groups compared to the AD group ($P < 0.001$ for all groups).

BVS decreased the weight of the rats in a dose-dependent manner

Table 1 compared the difference of the initial weight of rats before the induction of AD and their secondary weight after the experimental steps and injection of BVS. The results demonstrated a lack of a significant difference between the AD (26.1 ± 5.61) and the sham (27.2 ± 5.71) groups. Although the AD + BVS20 (12.90 ± 5.25) and AD groups did not have a significant difference, the weight of the AD + BVS40 (-2.44 ± 5.09; $P < 0.01$) and AD + BVS80 (-10.55 ± 6.62; $P < 0.001$) groups showed a considerable decrease compared to the AD group.

DISCUSSION

Based on the result of the present study, we observed that the skin glandular secretions of *Bufo viridis* could significantly decrease the symptoms of anxiety and depression caused by the induction of AD in a rat model. Results of OFT and EPM tests indicated an increase in anxiety behavior in AD rats compared to the sham group, which was inconsistent with the other studies (27). Also, improvement in

behavioral defects was observed following the ip injection of BVS. In OFT the crossing number in the central area is considered as criteria for anxiety which may be evidence for sickness and depression-like behaviors due to AD induction. According to the results of OFT, we observed that the central time and the crossing numbers were decreased in the AD group compared to the sham group probably due to depressive-like behaviors after Aβ injection. Central time and crossing were increased after BVS injection in OFT. Also, we observed that the open time was decreased after Aβ injection compared with the sham group in EPM. Then the open time significantly was increased and the closed time was decreased in the AD + BVS80 compared with the AD group. Injection of BVS improved locomotor activity in the OFT and EPM. Brureau *et al*. reported that Aβ injection into the brain ventricles can increase anxiety-related behaviors in AD rats. Aβ injection has been reported to lead to memory impairment, HPA axis hyperactivity, and an increase in glucocorticoid and mineralocorticoid receptors in areas of the brain that are related to the function of the HPA axis (30,31). These effects are similar to those that happen during anxiety which might be considered as an explanation for anxiety-like behaviors after Aβ injection as shown in the present study. Injection of Aβ (icv) induces a spectrum of behavioral responses such as sickness behaviors, which are along with the reduction of locomotor activity (32). Following depression and anxiety, serotonin and norepinephrine levels are greatly reduced, which indirectly modulates dopamine. Because of the disorder in monoaminergic neurotransmitters due to depression and anxiety, it turns out that modulating these substances at synapses can be one of the most important treatments (33). The open field and elevated plus maze tests are used to measure anxiety and stress. The findings of this research showed that the locomotor activity in AD samples was decreased, which is an indicator that the dopaminergic system and glutamate are responsible for depression and anxiety caused by AD. On the other hand, we demonstrated a significant decrease in anxiety symptoms in the experimental specimens receiving BVS in a dose-dependent manner. Therefore, These secretions may be able to control these symptoms *via* synaptic modulation of some neurotransmitters such as glutamate and dopamine. Excretions produced by toad skin glands include monoamines (indole alkyl amines and biogenic amines), steroids (bufogenine and bufotoxin), alkaloids (tetrodotoxin, batrachotoxin, and epibatidine), and peptides (anionic antimicrobial peptides, cationic antimicrobial peptides, combined cationic antimicrobial peptides, and antimicrobial peptides with cysteine sequence) (34). Due to the high levels of biogenic amines in the skin of toads, including serotonin, histamine, tyramine, dopamine, adrenaline, and norepinephrine derivatives, these substances are likely to improve locomotor activity by modulating the synaptic amines. The precise mechanism of action of BVS on depression and anxiety is not clear but, it may be due to the neutralization of various stress parameters and the return of levels of monoaminergic and neurotransmitters to normal, and also, increasing the level of monoamine neurotransmitters in areas of the cerebral cortex. Choi *et al*. considered the effects of a VB extract on the improvement of depressive symptoms as the result of the presence of serotonin in its compounds. One of the main ingredients in BVS is serotonin, which is associated with mood, appetite, and sleep, as well as with cognitive functions such as memory and learning. The modulation of serotonin at synapses is thought to be a major mechanism of action in several classes of pharmacological antidepressants (19). In the present study, we found the serotonin, norepinephrine, and histamine in BVS through the GC-MS device. The authors suggest that in the present study the serotonergic system reduced the symptoms of depression in a rat model of AD. Whether BVS affected depression by inhibiting the re-absorption or preventing the degradation of monoamines requires further studies.

The level of BDNF and neurogenesis after the prescription of antidepressants are increased, but the use of a very high dose of VB led to epileptical seizures and reduced the BDNF (35). Therefore, the study of the VB

toxicity mechanism would possibly provide us some new ways to eliminate its side effects, which is essential for the clinical application of VB. It seems that different doses of the BVS showed different effects. As we observed, the dose 80 was more effective than the other doses; on the other hand, some side effects such as losing weight were observed.

In this study, a significant increase in the immobility and the climbing time and a decrease in the active time were observed in the AD group compared to the sham group due to depression as the result of injecting $A\beta_{1-42}$ (27) and the BVS injection led to a decrease in the immobility and climbing time and an increase in the active time, which improved symptoms of depression. The injection of $A\beta_{1-42}$ caused a considerable increase in anxiety measured by climbing time in FST, which indicated noradrenergic dysregulation. The FST is a well-known tool for the evaluation of depression-like behaviors in rats. Various studies have represented the active time diagram and the climbing time in FST as indicators for the serotonergic system and the norepinephrine system, respectively (36). An important mechanism for the treatment of AD and depression is through focusing on the alleviation of inflammation. Inflammation in AD is directed by the innate immune system, containing microglia and astrocytes, leading to neurodegeneration and disorders in corticotropin-releasing hormone signaling and decreasing the level of monoamines in the brain (7,37). Various studies have indicated that this system is also active in depression and is accompanied by an increase in microglia (38).

Based on results that *BVS* effectively decreased the volume of senile plaques in the left hippocampus of AD animals, following ip injection of these secretions and their subsequent effects in the brain, it probably passed the blood-brain barrier. Inflammation after AD activates the NFκB pathway which controls the expression of downstream inflammatory mediators such as iNOS, COX-2, IL-8, IL-6, IL-1β, and TNFα. NFκB is a transcription factor that plays a significant role in proliferation, apoptosis, inflammation, and immunity (39). As NFκB is associated with aging and its suppression delays aging, it is possible that focusing on its pathway would be effective in treating AD (40). The use of the hydraulic extract of VB in some skin and liver cancer cells (HepG2, A549) suppressed the NFκB, IκB, and COX-2 and decreased the production of NO. Bufalin and the hydraulic extract of VB reduce the activity of NFκB through bonding with its inhibiting protein (IκB) and inactivate it in the cytosol, thus leading to the reduced release of pro-inflammatory mediators such as iNOS, COX-2, IL-6, IL-1β, and TNFα from macrophages, which is an important strategy for suppressing inflammation (16). The effect of bufalin on reducing the activity of NFκB and decreasing the production of pro-inflammatory mediators has been proven (41). In this study, the dose-dependent side effects such as the reduced weight of the rats were observed. As a strategy, with the use of microbial biotechnology, the therapeutic advantages or half-life of these compounds might be increased in the blood or its side-effects could be decreased (35,42).

CONCLUSION

In general, the results of this study indicated that the injection of $A\beta_{1-42}$ into the brain ventricle induced anxiety and depressive behaviors in the rats. In addition, the use of skin secretions of the *Bufo viridis* toad could improve the anxiety and depression symptoms caused by the induction of AD in a rat model and increase the clearance of senile plaques in the left hippocampus. Generally, BVS can be used as a promising natural option for the treatment of depression and anxiety in the clinical trials in the future. More research is needed to elucidate the mechanism of the action and its optimal treatment.

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CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest in this study.

AUTHORS' CONTRIBUTION

All authors contributed equally to this study.

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