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ORIGINAL ARTICLE

# The Effect of *Ziziphora clinopodioides* Hydroalcoholic Extract on the Neuronal Density and Histopathology of the Hippocampal Area CA2 in Streptozotocin-induced Alzheimer's Model in Male Rats

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**KEYWORDS**

Alzheimer's disease;
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**ABSTRACT:** Alzheimer's disease is a type of dementia and neurodegenerative disorder that affects memory and some cognitive functions. The CA2 area of the hippocampus has pyramidal neurons that plays an important role in cell signaling and neuromodulatory systems. Kakuti (*Ziziphora clinopodioides* Lam.) belongs to the Lamiaceae family and has strong antioxidant compounds. This study was conducted in order to investigate the effect of kakuti hydroalcoholic extract on neuronal density and histopathological lesions of hippocampal CA2 region following Alzheimer's induced by intraventricular injection of streptozotocin in rats. In this research, 72 adult male rats were divided into 9 groups (n = 8) including: control, negative control (Alzheimerized), positive control (Alzheimer + anti-Alzheimer drug Rivastigmine), sham groups (receiving intraventricular administration of artificial cerebrospinal fluid + 200, 400 and 600 mg $kg^{-1}$ kakuti extract) and treatment groups (Alzheimer's + receiving 200, 400, 600 mg $kg^{-1}$ kakuti extract). The neuron density was investigated by the disector method. Congo red and toluidine blue stains were used for histopathology. The data were evaluated with Minitab statistical software and ANOVA test with a significant level of $p \leq 0.001$. The results showed a significant decrease in the neuronal density of the CA2 area in the Alzheimer's group and a significant increase in the treatment group with 400 mg $kg^{-1}$ of Kakuti extract. Histological sections showed the reduction of beta-amyloid plaques in the groups treated with the kakuti extract. These findings show that the hydroalcoholic extract of kakuti is probably effective in improving Alzheimer's conditions and memory and histopathological damage caused by it, and can reduce beta-amyloid plaques and cell death in the CA2 region.

## INTRODUCTION

Alzheimer's disease (AD) is an irreversible and progressive brain disorder that is characterized by the formation of hyperphosphorylated tau protein, neurofibrillary tangles (NFTs), and extracellular senile plaques, and extensive destruction of neurons in different regions of brain [1].

In 2010, the prevalence of this disease in the world was more than 6.35 million people, that is expected to reach 7.65 million people by 2030 and 4.115 million people by 2050 [2].

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Although the exact mechanism of Alzheimer's disease is not known, various mechanisms such as accumulation of amyloid β protein due to incomplete processing of amyloid precursor protein (APP) caused by β secretase enzyme, cholinergic system disorders, neuroinflammation, oxidative stress and toxicity as a result of glutamate excessive stimulation are involved in its pathogenesis [3].

Alzheimer's disease is an important cause of increasing loss of learning and memory and other cognitive functions. The learning process refers to the acquisition of new information that changes in behavior. Memory is a process through which this information is encoded, stored and later read again [4].

In fact, memory is the result of learning and reflects permanent changes in the nervous system. The hippocampus is essential for declarative memory. Memory information is processed in the hippocampus through several distinct sub-pathways [5].

CA2 is a small region between CA3 and CA1 that makes a link of powerful disynaptic circuit linking the input of the entorhinal cortex to the output of CA1. Little data is available on the role of CA2 [6].

Studies have shown that genetically targeted inactivation of CA2 pyramidal neurons causes a severe damage of social memory, inability to remember a conspecific, without changes in sociability or some other hippocampus-dependent behaviors such as spatial and contextual memory [7]. Therefore, these behavioral and anatomical results show CA2 as an important social-cognitive memory processing center. The scientists stated that CA2 pyramidal cell bodies were similar to CA3 cells and were larger than those found in CA1 [8].

CA2 pyramidal cell dendrites lack special spiny dendrites associated with mossy fibers input from the DG, which is characteristic of CA3 pyramidal neurons. CA2 neurons also receive Schaffer collaterals of area CA3 neurons just similar to CA1 area cells. The CA2 region has attracted the attention of researchers due to its relatively high resistance to trauma-induced damage and resistance to synaptic plasticity, compared to other CA regions. CA2 pyramidal neurons also show cell signaling pathways and neuromodulatory effects that are not present in other CA regions [9].

Natural antioxidants in plants can greatly moderate the damage caused to neurons. Kakuti (*Ziziphora clinopodioides* Lam), is a medicinal herb found in Iran and Turky, belongs to the Lamiaceae family, which is rich in thymol and carvacrol. These compounds are known for high antioxidant capacity [10].

Based on this, the purpose of this research is sttudy the effect of the hydroalcoholic Kakuti extract on hippocampal CA2 neuron density and histopathological lesions in sporadic Alzheimer's model in male Wistar rats.

## MATERIALS AND METHODS

### *Preparation of extract*

First, the Kakuti plant was collected from the slopes around Hezar Masjed Mountain and identified by the herbarium of Ferdowsi College and coded as 10313. Then it was dried in the shade and ground, and its hydroalcoholic extract was prepared by Soxhlet extractor.

### *Animal grouping*

Seventy-two adult male Wistar rats (230 to 280 g) were obtained from Razi Institute of Mashhad, and sufficient water and food were available to the animals throughout the study. In order to prevent the effect of environmental conditions on the experiment, the mice were kept in the animal room during the study period with a fixed day and night cycle (12 hours of light/12 hours of darkness) with temperature of 23 ± 2 degrees Celsius. 72 adult male Wistar rats were divided into nine groups of eight including: control, negative control (Alzheimerized), positive control (Alzheimer + anti-Alzheimer drug Rivastigmine), sham groups (receiving intraventricular administration of artificial cerebrospinal fluid + 200, 400 and 600 mg $kg^{-1}$ kakuti extract) and treatment groups (Alzheimer's + receiving 200, 400, 600 mg $kg^{-1}$ kakuti extract) (according to Table 1).

**Table 1.** Grouping of animals.

| Group | Condition |
|---|---|
| **Control** | They did not receive any medication |
| **Negative control** | Cannulated, after a week of recovery, on the first and third day, they received 5 microliters of streptozotocin [11] dissolved in artificial cerebrospinal fluid intraventricularly and bilaterally. |
| **Positive control** | Cannulated, after a week of recovery, on the first and third days, they received 5 microliters of streptozotocin dissolved in artificial cerebrospinal fluid bilaterally. From the first day, they received rivastigmine at a dose of 0.3 mg $kg^{-1}$ dissolved in saline intraperitoneally for 21 days |
| **Sham 200** | Cannulated, after a week of recovery, in the first and third day, they received 5 microliters of artificial cerebrospinal fluid bilaterally. From the first day, they received 200 mg $kg^{-1}$ saline-soluble Kakuti extract intraperitoneally for 21 days |
| **Sham 400** | Cannulated, after a week of recovery, in the first and third day, they received 5 microliters of artificial cerebrospinal fluid bilaterally. From the first day, they received 400 mg $kg^{-1}$ saline-soluble Kakuti extract intraperitoneally for 21 days |
| **Sham 600** | Cannulated, after a week of recoveryon the first and third day, they received 5 microliters of artificial cerebrospinal fluid bilaterally. From the first day, they received 600 mg $kg^{-1}$ saline-soluble Kakuti extract intraperitoneally for 21 days |
| **Treatment 200** | Cannulated, after a week of recovery, on the first and third day, they received 5 microliters of streptozotocin dissolved in artificial cerebrospinal fluid bilaterally. From the first day, they received 200 mg $kg^{-1}$ Kakuti extract dissolved in saline intraperitoneally for 21 days |
| **Treatment 400** | Cannulated, after a week of recovery, on the first and third day, they received 5 microliters of streptozotocin dissolved in artificial cerebrospinal fluid bilaterally. From the first day, they received 400 mg $kg^{-1}$ Kakuti extract dissolved in saline intraperitoneally for 21 days |
| **Treatment 600** | Cannulated, after a week of recovery, on the first and third day, they received 5 microliters of streptozotocin dissolved in artificial cerebrospinal fluid bilaterally. From the first day, they received 600 mg $kg^{-1}$ Kakuti extract dissolved in saline intraperitoneally for 21 days |

### *Intraventricular injection*

First, the animals were anesthetized by intraperitoneal injection of 60 mg $kg^{-1}$ body weight rampon (xylazine) and 6 mg $kg^{-1}$ body weight ketamine and cannulation and injections were performed using the stereotaxic method and Paxinus atlas coordinates [12]. After the experiment, the animals were anesthetized and after the perfusion method, the hippocampus was removed and placed in 10% saline formalin for histological examination. After fixation, the sections entered tissue passage stages, and serial sections were prepared from the hippocampus then the sections were stained and slides were prepared. Photographs were taken of all sections of the CA2 area and the neuronal density of the CA2 area was checked by stereology and the disector method. Congo red and toluidine blue stains were used to examine histopathological lesions.

### *Data analysis*

Data were evaluated with Minitab statistical software and ANOVA test ($p \leq 0.001$).

## RESULTS

Comparison of the average neuronal density of the hippocampus regions in different groups showed that in the CA2 region of the control group, the neuronal density was 3786 ± 55.5. In the negative control group, the neuronal density is 1910 ± 37.3, which has a significant difference compared to the control group ($p \leq 0.001$), which indicates the correct induction of Alzheimer's disease in the negative control group. The amount of neuronal density in the group treated with 200 mg $kg^{-1}$ extract is 2003.9 ± 77.5, which does not show a significant difference with the negative control group. But in the groups treated with the extract dose of 400 mg $kg^{-1}$ and 600 mg $kg^{-1}$ which are 3623.4 ± 96.7 and 3722.3 ± 58.2, respectively, which shows a significant difference with the negative control group; which indicates an increase in neuronal density in these groups compared to the negative control ($p \leq 0.001$). The amount of neuronal density in sham groups with concentrations of 200, 400 and 600 mg $kg^{-1}$ of Kakuti extract is 3801 ± 56.2, 3840 ± 64.1 and 3806 ± 66.8, respectively, and in terms of comparison of the means, there is no significant difference with the control group. The comparison of neuronal density between the positive control group

(3870 ± 70.8) and the groups treated with extract dose of 400 mg $kg^{-1}$ and 600 mg $kg^{-1}$ was not significantly different. While the comparison of the neuronal density of the positive control with the 200 mg $kg^{-1}$ extract treatment shows a significant difference, which indicates group with teloidin blue staining (Figure 3).

no increase in neuronal density in this treatment group. ($p \leq 0.001$) (Figure 1). Figure 2 shows the transverse sections of the hippocampal CA2 region in the experimental

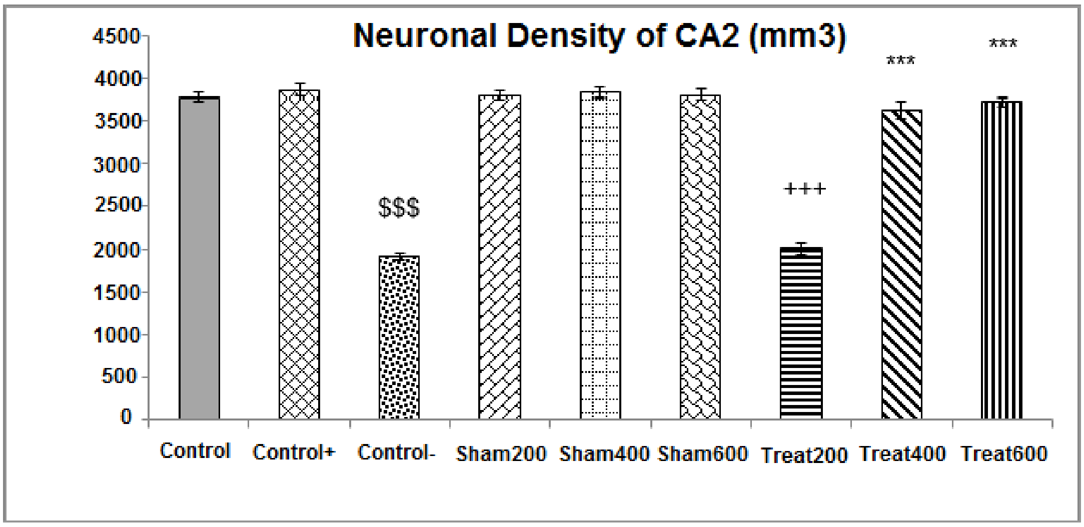

**Figure 1.** Comparison of the effects of hydroalcoholic extract of kakuti for 21 days on neuronal density in the CA2 area of the hippocampus in experimental groups (n=8). Data are shown as mean ± SE. ***: The difference between the treatment groups and the negative control group ($p \leq 0.001$), $$$: The difference between the control group and the negative control group ($p \leq 0.001$); +++: The difference between the positive control group and the treatments ($p \leq 0.001$) Comparison of sham groups with control group showed no significant difference.

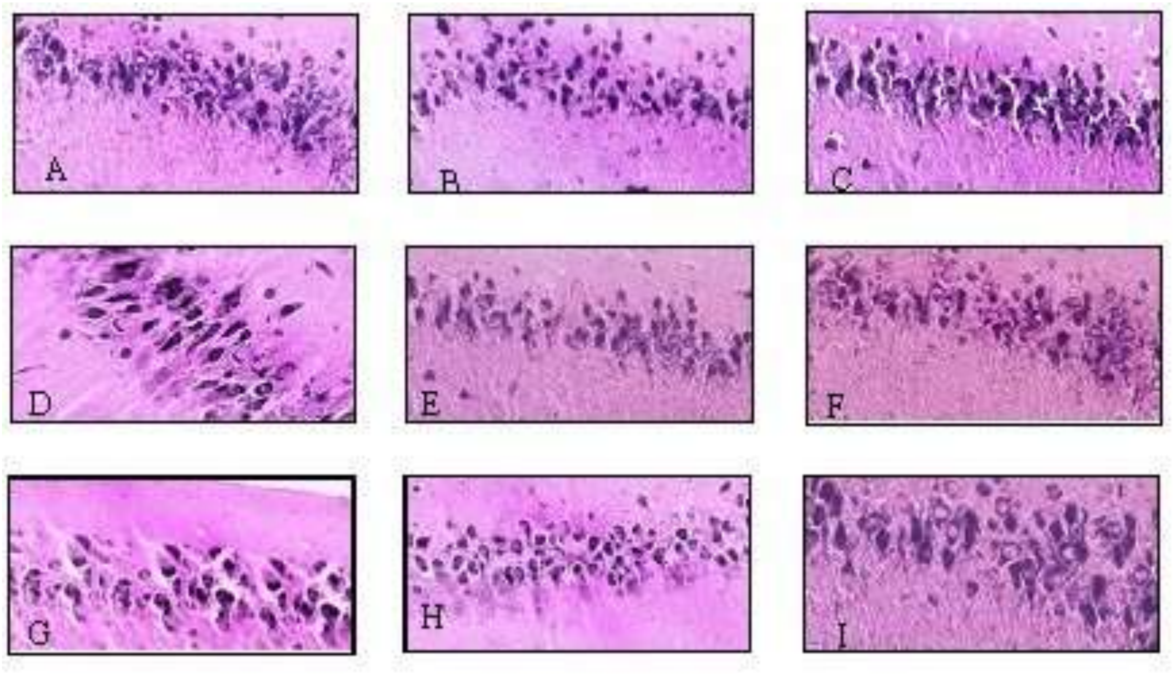

**Figure 2.** Transverse sections of the hippocampal CA2 region in the experimental group with teloidin blue staining (Magnification ×40). Control group (A), positive control group (B), negative control group (C), sham group with 200 mg $kg^{-1}$ kakuti extract (D), sham group with 400 mg $kg^{-1}$ kakuti extract (E), sham group with 600 mg $kg^{-1}$ kakuti extract (F), treatment group with 200 mg $kg^{-1}$ kakuti extract (G), treatment group with 400 mg $kg^{-1}$ kakuti extract (H), treatment group with 600 mg $kg^{-1}$ kakuti extract (I). Degenerate and aggregated neurons are observed scatteredly with increased extracellular space in the negative control group (C) compared to healthy neurons in the control group (A). The amount of damage in the groups treated with kakuti extract has been greatly reduced.

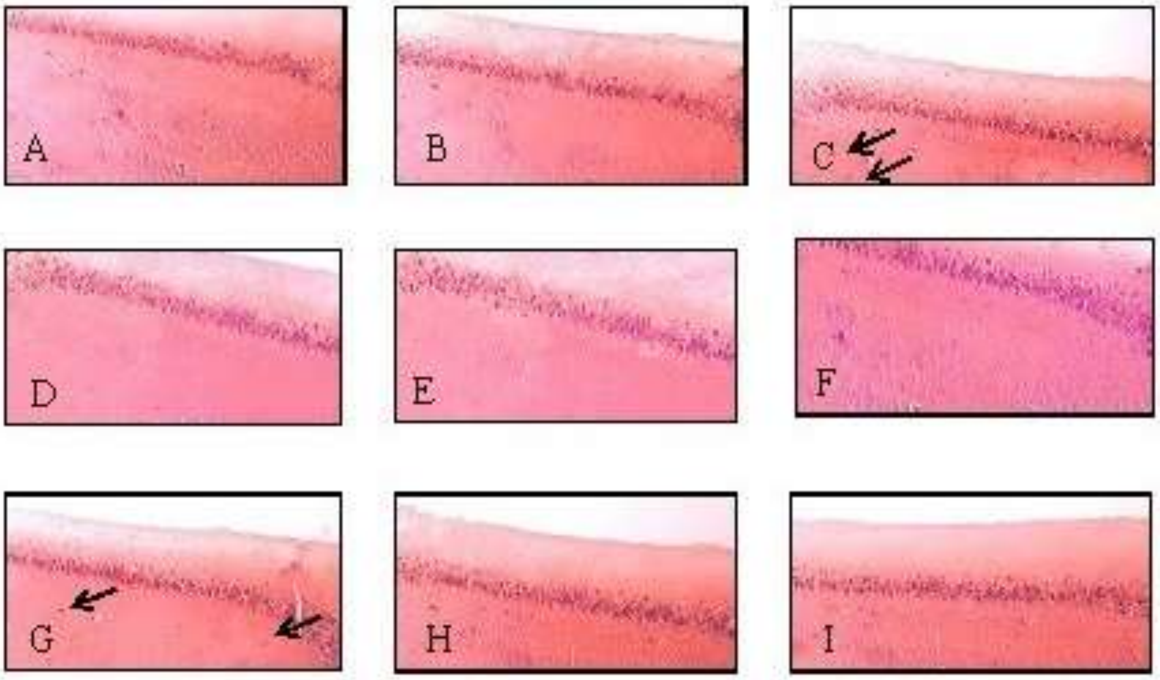

**Figure 3**. CA2 region of the hippocampus in the experimental groups with Congo Red staining (Magnification ×10). Control group (A), positive control group (B), negative control group (C), sham group with 200 mg $kg^{-1}$ kakuti extract (D), sham group with 400 mg $kg^{-1}$ kakuti extract (E), sham group with 600 mg $kg^{-1}$ kakuti extract (F), treatment group with 200 mg $kg^{-1}$ kakuti extract (G), treatment group with 400 mg $kg^{-1}$ kakuti extract (H), treatment group with 600 mg $kg^{-1}$ kakuti extract (I). The control group does not have any amyloid plaques. The negative control group, 21 days after streptozotocin injection, shows the presence of amyloid plaques in the form of red spots. In the treatment group, 21 days after receiving 200 mg $kg^{-1}$ of kakuti extract, amyloid plaques are visible and it is not different from the negative control group. In the treatment group receiving 400 mg $kg^{-1}$ of kakuti extract, amyloid plaques decreased compared to the negative control group. In the treatment group receiving 600 mg $kg^{-1}$ of kakuti extract, the number and density of amyloid plaques has decreased significantly, so that no difference can be seen between this group and the control group.

## DISCUSSION

The effect of hydroalcoholic kakuti extract was compared with the effect of Rivastigmine as a selected drug (positive control group) in the treatment of Alzheimer's disease on sporadic Alzheimer's model rats. The results of the neuronal density of the CA2 region showed that in the negative control group, the neuronal density decreased significantly compared to the control group, and in the treatment groups, the neuronal density increased significantly compared to the negative control. The histopathological findings of this research also showed the accumulation of beta-amyloid plaques in the negative control group compared to the control (Figure 2). Damage to cellular structures by reactive oxygen species, which are generated during normal cell metabolism, plays a credible role in aging-related diseases. Oxidative stress has been identified in Alzheimer's disease and may be a component of amyloid production [13].

The use of antioxidant agents for Alzheimer's treatment is based on the hypothesis of their neuronal protection. An increase in the production of free radicals resulting from oxidative metabolism may increase the neuronal destruction observed in Alzheimer's disease [14].

The hippocampus is naturally susceptible to damage. In general, the production of free radicals is related to normal cellular processes such as cellular metabolism, mitochondrial respiration, lipoxygenase and cyclooxygenase activity [15], which may increase in the brain [16].

Neuronal cell death occurs in response to diverse injuries, such as glucose transport to neurons, reduced oxygen, physical cutting or compression of brain tissue, or abnormal electrical activity like seizures. These injuries are followed by processes including activation of voltage-gated calcium channels, membrane depolarization and cytotoxicity due to excess release of glutamate (with enhancement in intracellular calcium levels) and release of free radicals, can start necrosis and apoptosis signaling pathways [17].

Antioxidants cause a decrease in lipid peroxidation and thereby reduce the destructive effects of free radicals in reducing synaptic plasticity and long-term strengthening of the hippocampus [18].

The protective effect of antioxidants may depend on their ability to hydrogenate, or their ability to scavenge free radicals [19].

Consumption of antioxidants improves spatial learning and increases memory in rats with Alzheimer's disease [20].

The main agent of destroying free radicals are flavonoids, which have hydroxyl [21].

The flavonoid, flavonol and flavonoid compounds present in kakuti plant are considered as a very effective antioxidant. Phenolic elements have strong antioxidant properties. Phenols increase neuronal proliferation and neuronal protection [22].

According to the results of Congo red staining, the comparison of the group receiving Kakuti in doses of 400 and 600 mg $kg^{-1}$ with the negative control group shows that the amount of amyloid plaques has decreased. Beta-amyloid increases the production of free radicals [23].

Considering the vast range of processes that can contribute to cell death, the use of medicinal plants and antioxidant compounds can be considered as a very effective treatment.

## CONCLUSIONS

The result of this research shows that kakuti can have an antioxidant effect depending on the dose and can reduce apoptosis caused by amyloid plaques and be effective in improving memory.

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## ETHICAL CONSIDERATION

The research protocol received approval from the Department of Animal Physiology at the Islamic Azad University, Mashhad Branch, Mashhad, Iran. (Code: IR.IAU.MSHD.REC.1399.108)

### *Conflict of interest*

The authors declare that there is no conflict of interest.

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