Research in Pharmaceutical Sciences, May 2016; 11(3): 227-232
Received: Oct 2015
Accepted: Dec 2015

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Effect of hydroalcoholic extract of *Anethum graveolens* leaves on the dentate gyrus of the hippocampus in the epileptic mice: a histopathological and immunohistochemical study

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Abstract

Anethum graveolens or Dill (local name: Shevid) belongs to the family of Apiaceae (Umbelliferae) and is used traditionally for the treatment of convulsion and diabetes in Iran. This study aimed to investigate the effect of hydroalcoholic extract of *A. graveolens* leaves on the histology of the dentate gyrus of the hippocampus in the epileptic mice kindled by Pentylenetetrazole (PTZ). In this experimental study, the epileptic BALB/c mice kindled by PTZ were randomly divided into four groups of 10 animals each. Three experimental groups received 250, 500 and 750 mg/kg/day of *A. graveolens* extract for 21 days. The control group received phosphate-buffered saline (PBS). After the treatment period, the mice were anesthetized, and their hippocampi were dissected for the histopathological analysis, and immunohistochemical analysis for caspase-3 activity. Histopathological examinations showed that the mean numbers of the healthy neuronal cells in the dentate gyrus of the mice received 500 mg/kg/day of *A. graveolens* extracts were significantly higher than those of the mice received 250 and 750 mg/kg/day of the extracts as well as the control group ($P < 0.05$ and $P < 0.001$, respectively). In addition, the results of immunohistochemical analysis revealed that in mice treated with 500 mg/kg/day of *A. graveolens*; the numbers of caspase-3-positive cells in the dentate gyrus were significantly lower than those of the two other test and the control groups. The findings of this study suggest that 500 mg/kg/day of the *A. graveolens* extract could have protective effect on the dentate gyrus of the hippocampus in the epileptic mice.

Keywords: *Anethum graveolens*; Dentate gyrus; Hippocampus; Epilepsy

INTRODUCTION

Epilepsy is one of the most common neurological disorders in humans and its incidence is 50.4 per 100,000 people (1). Approximately 1% of the general population is affected by epilepsy, and about 10% of them experience a seizure sometime during life (2). The long-term use of chemical drugs such as valproic acid in epileptic patients causes diverse side effects (3). In addition, these drugs treat only about 40% of the seizure cases, and in other cases, they only decrease the frequency of seizure attacks (4). In addition, the alcoholic beverages in the epileptic mice treated with valproic acid, cause damage to the small blood vessels in the central nervous system (5). Therefore, the medicinal plants usage instead of chemical drugs, due to lesser side effects and lesser interference with the biological reactions of the human body, seems to be useful to control the epilepsy. Today, many people in the developing countries use herbal medicines to treat epilepsy (6-11). One of the herbs with antiepileptic properties is *Anethum graveolens*. *A. graveolens* or Dill (local name: Shivid) belongs to the family of Apiaceae (Umbelliferae), and is found in abundance in the East of Iran and Southeastern of Europe. The phytochemical screening of this plant showed that its leaves were rich in tannins, steroids, terpenoids, flavonoids, and cardiac glycosides (12). *A. graveolens* is used

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traditionally for treatment of convulsion and diabetes in Iran (13,14). Our colleagues in Sabzevar University of Medical Sciences reported that *A. graveolens* leaves extract has anticonvulsant activity against seizure induced by pentylenetetrazole (PTZ) in mice (15). Also, Rostampour and coworkers reported that hydroalcoholic extract of this plant, can delay the initiation time of myoclonic and tonic-clonic seizures, and has a protective effect against mortality in PTZ-induced seizure in adult male mice (16). Moreover, *A. graveolens* has anti-hyperlipidemic and anti-fungal effects (17,18). Additionally, it has been shown that the *A. graveolens* leaves essential oil, has anti-inflammatory activity through inhibitory effect on LPS-induced nitric oxide secretion from macrophages (19).

One way to study epilepsy is seizure induction in animal models by injecting PTZ. It has been shown that PTZ induces moderate neurodegeneration in the hippocampus (20). The effects of hydroalcoholic extract of the *A. graveolens* on various organs of the body and seizure have been studied (15,21-26), nevertheless, no study has been yet performed in the case of its effects on the hippocampal dentate gyrus neuronal cells, in PTZ-induced epileptic models. The dentate gyrus of the hippocampus is a major region of the cerebral cortex, which is involved in adult neurons production and short-term memory. Therefore, this study aimed to investigate the effects of the hydroalcoholic extract of the *A. graveolens* leaves on the certain histological parameters of the dentate gyrus of the hippocampus in the PTZ-induced epileptic mice. Expression of caspase-3 as an indicator of apoptosis in the neuronal cells of the dentate was also investigated using immunohistochemistry method.

MATERIALS AND METHODS

Plant materials

A. graveolens leaves were purchased from a local medicinal plant market in Sabzevar (Iran) and authenticated in the Ferdowsi University Herbarium (Mashhad, Iran). A voucher specimen (No. 293-0107-18) was deposited in the Herbarium of the Deparment of Pharmacognosy at School of Pharmacy in Mashhad University of Medical Siences, Mashhad, Iran for future reference.

Hydroalcoholic extraction of A. graveolens leaves

After cleaning and drying, the plants were ground. Then, the resulting powder were soaked with 80% ethanol at a ratio of 1 to 10 (mass/volume) overnight on a shaker. Afterward, the mixture was filtered and concentrated at 40 ºC under reduced pressure using a rotary evaporator. Before injection to animals, the extract was dissolved in sterile PBS at desired concentrations.

Mice and epilepsy induction

In this experimental study, 40 male BALB/c mice (6-8 weeks old, 25–30 g) were provided from the animal house of Sabzevar University of Medical Sciences (Iran). They were handled in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. All animal experiments were approved by the Ethics Committee of Sabzevar University of Medical Science(reference number: IR.MEDSAB.REC.1394.198). Epilepsy was induced using Kindling method by an intraperitoneal injection of 37 mg/kg of PTZ (CAS 54-95-5, Sigma-Aldrich, USA) every other day for 24 days. The seizure behavior was observed after each PTZ injection. The mice showing generalized tonic–colonic seizure (fully kindled mice) were used for the study.

Experimental groups

Fully kindled mice were randomly divided into four groups of 10 animals each. Three experimental groups received intraperitoneally 250, 500 and 750 mg/kg/day of the *A. graveolens* extract for 21 days, while the control group received PBS at the same time. After the treatment period, the mice were deeply anesthetized with ether and transcardially perfused with PBS followed by 4% paraformaldehyde. Following perfusion, the hippocampi were dissected, fixed in 10% fresh-buffered formalin and embedded in paraffin wax for histopathological and immunohistochemical analysis.

Histopathological analysis

Coronal serial sections (5 µm thick) were randomly taken from the paraffin-embedded hippocampi and stained with routine hematoxylin and eosin (H&E) staining method. For each group, the number of healthy neuronal cells in the dentate gyrus was counted separately by two pathologists in 40× microscope field of view (four fields for each specimen, 8 × 8 mm^2 dimension). The number of healthy neuronal cells was calculated as mean ± SD for each group.

Immunohistochemical detection of caspase-3

Immunohistochemical detection of caspase-3 was performed on formalin-fixed, paraffin-embedded hippocampi. First, the sections on slides were deparaffinized in xylene and rehydrated in descending alcohol. After that, for antigen retrieval, they were pretreated in 10 mmol/l citrate buffer (pH, 6.0) in a steamer. Then, the sections were immersed in 3% hydrogen peroxide for 10 minutes to eliminate the endogenous peroxidase activity. Finally, the sections were stained using polyclonal rabbit anti-mouse caspase-3 antibody (Cat #559565, PharMingen, USA), DAKO EnVision systems (K4006, Dako Corporation, USA) and chromogenic substrate, 3,3´ diaminobenzidine tetrahydrochloride (DAB) (27).

Statistical analysis

For data analysis, one way analysis of variance (ANOVA) was performed using SPSS software version 16 (SPSS Inc. USA). Supplementary analyses, including Duncan and Dunnett tests, were performed for pairwise comparison of means between experimental groups, and between the experimental groups and the control group, respectively. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Number of healthy neuronal cells in the dentate gyrus of the epileptic mice

As shown in Table 1, the results of histopathological analysis, revealed that the number of healthy neuronal cells in the dentate gyrus of the epileptic mice treated with different doses of *A. graveolens* extract, were significantly higher than those of control group ($P < 0.05$). Meanwhile, treatment with 500 mg/kg/day of the extract had a remarkable protective effect on the neuronal cells, in comparison with doses 250 and 750 mg/kg/day ($P < 0.001$). In addition, in the mice received 500 mg/kg/day of the extract, the morphological changes of the neuronal cells such as nucleus condensation, nucleus margination and cytoplasmic acidophilia was lower than the other groups (Fig. 1).

Expression of caspase-3

Caspase-3 expression, as an indicator of apoptosis, was assessed by immuno-histochemistry. As shown in Fig. 2, the number of caspase-3-positive neuronal cells in the dentate gyrus of the epileptic groups that received different doses of *A. graveolens* extract, was significantly lower than those of the control group. Meanwhile, more pronounced reduction in caspase-3 expression was observed in the mice treated with 500 mg/kg/day of the extract.

Table 1. The number of healthy neuronal cells in the dentate gyrus of the hippocampus of epileptic mice treated with different doses of *Anethum graveolens* leaves extract.

Group	Number of healthy neuronal cells (mean ± SD)
Test group (250 mg/kg/day *A. graveolens* extract)	5.7 ± 2.1*
Test group (500 mg/kg/day *A. graveolens* extract)	8.6 ± 2.7*'**
Test group (750 mg/kg/day *A. graveolens* extract)	6.8 ± 2.3*
Control group (phosphate-buffered saline)	3.8 ± 1.7

* $P < 0.05$ in comparison with control group, and between test groups, based on ANOVA (Dunnett and Duncan test, respectively)

** $P < 0.001$ in comparison with control group, based on ANOVA (Dunnett test)

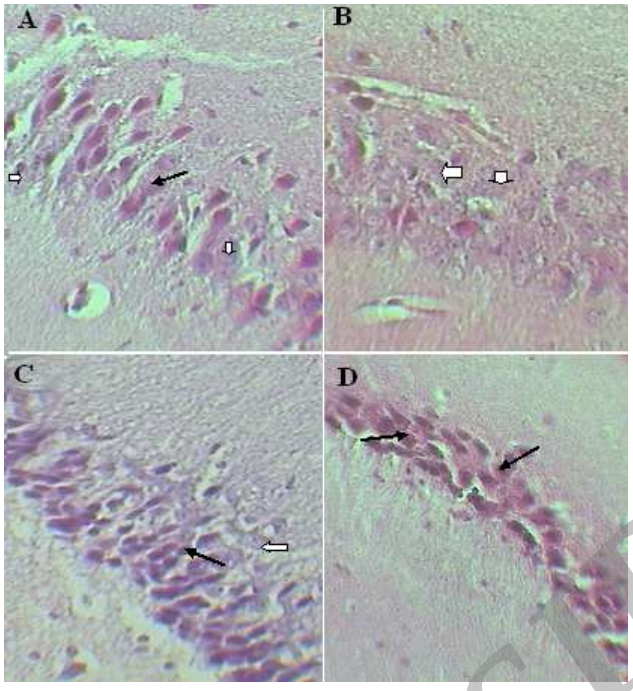

Fig. 1. Histopathological features of the dentate gyrus of the hippocampus in the epileptic mice treated with different doses of the *Anethum graveolens* leaves extract. Lower morphological changes were seen in groups A; receiving 250, B; 500, and C; 750 mg/kg/day of the extract, in comparison with D; control group. White arrows show the healthy neuronal cells, but black arrows show the neuronal cells that underwent morphological changes including nucleus condensation, nucleus margination and cytoplasmic acidophilia. H&E staining. (at 400× magnification). More pronounced effects were observed with 500 mg/kg/day of the extract compared to other two test doses.

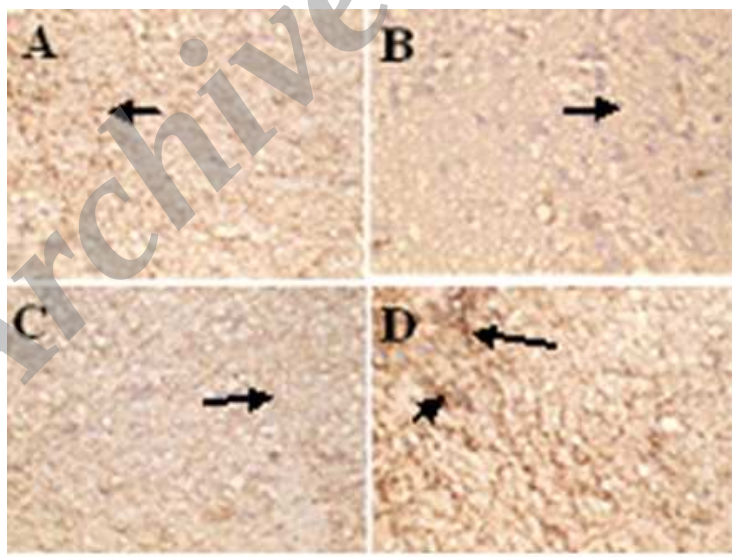

Fig. 2. Immunohistochemical results of caspase-3 expression in the dentate gyrus of the hippocampus in the epileptic mice treated with different doses of *Anethum graveolens* leave extract. Lower immunoreactivity for caspase-3 (brown precipitate) was seen in groups A; receiving 250, B; 500, and C; 750 mg/kg/day of the extract, in comparison with D; control group. Brown precipitates (arrows) showed neuronal cells expressing caspase-3, as an indicator of apoptosis. (at 400× magnification). More pronounced effects were observed with 500 mg/kg/day of the extract compared to other two test doses.

DISCUSSION

This study aimed to investigate the effect of hydroalcoholic extract of the *A. graveolens* leaves on the histology of the dentate gyrus of the hippocampus in the epileptic mice kindled by PTZ. The results of this study showed that the hydroalcoholic extract of *A. graveolens* have protective effect on the dentate gyrus of the hippocampus in the PTZ-induced epileptic mice; hence the intraperitoneal injection of 500 mg/kg/day of *A. graveolens* for 21 days could significantly protect the neuronal cells against the morphological changes and caspase-3 related apoptosis. These effects were also seen with 250 and 750 mg/kg/day of the extract, but to a lesser extent. This may indicate that 500 mg/kg/day, is an optimal dose for the *A. graveolens* extract to exert its protective effect on the dentate gyrus of the hippocampus in epileptic mice.

PTZ kindling induces moderate neurodegeneration in the hippocampus (20). Recently, Nasser and coworkers reported that PTZ exposure significantly increased caspase-3 and Bax (an apoptosis inducer) expression in the rat hippocampal neuronal cell cultures (28). Therefore, increased Bax and caspase-3 expression can be responsible for neuronal cell death in the dentate gyrus of the hippocampus in the PTZ-induced epileptic mice. The results of this study showed that in the epileptic mice received *A. graveolens* extract, the number of caspase-3 positive neuronal cells were lower than those of the control mice. This may indicate the protective effect of the *A. graveolens* extract against the PTZ-induced apoptotic neurodegeneration, probably via inhibition of caspase-3 or by activating the anti-apoptotic proteins such as Bcl-2; however, the latter case needs further research for clarification.

Another mechanism of the epileptic-induced neuronal death, is oxidative stress. It has been shown that the reactive oxygen species may have a putative role in the seizure-induced neuronal death (2). Recently, a study reported that *A. graveolens* essential oil contains antioxidant compounds of limonene and sabinene (19). As pointed out earlier, our results also showed that the mean number of the healthy neuronal cells in the dentate gyrus of the hippocampus of the epileptic mice treated with *A. graveolens* extract, was higher compared to control mice. Therefore, the antioxidant activity of the *A. graveolens* extract may protect the dentate gyrus from deleterious effect of PTZ.

CONCLUSION

In conclusion, this study revealed the protective effect of hydroalcoholic extract of *A. graveolens* on the dentate gyrus of the hippocampus in the PTZ-induced epileptic mice. This finding not only proves the effectiveness of this plant in epileptic disorders, but also support the traditional use of *A. graveolens* for the epilepsy treatment in Iran.

ACKNOWLEDGEMENT

This study was supported by the Vice Chancellor for Research of Sabzevar University of Medical Sciences (Iran). We also thank Dr. Rakhshani for constructive statistical consultants.

REFERENCES

1. Ngugi A, Kariuki S, Bottomley C, Kleinschmidt I, Sander J, Newton C. Incidence of epilepsy: a systematic review and meta-analysis. Neurology. 2011;77(10):1005-1012.
2. Sankaraneni R, Lachhwani D. Antiepileptic drugs, A review. Pediatr Ann. 2015;44(2):36-42.
3. Scorza FA, Arida RM, Cysneiros RM, Priel MR, de Albuquerque M, Cavalheiro EA. The effects of alcohol intake and withdrawal on the seizures frequency and hippocampal morphology in rats with epilepsy. Neurosci Res. 2003;47(3):323-3288.
4. Fischer W, Kittner H. Influence of ethanol on the pentylenetetrazol-induced kindling in rats. J Neural Transm. 1998;105(10-12):1129-1142.
5. Golmohammadi R, Pejhan A, Azhdari-Zarmehri H, Mohammad-Zadeh M. The role of ethanol on the anticonvulsant effect of valproic acid and cortical microvascular changes after epileptogenesis in mice. Neurol Sci. 2013;34(7):1125-1131.
6. Garba K, Yaro A, Ya'u J. Anticonvulsant effects of ethanol stem bark extract of *Lannea barteri* (Anacardiaceae) in mice and chicks. J Ethnopharmacol. 2015;172:227-231.

7. Goyal M, Sasmal D. CNS depressant and anticonvulsant activities of the alcoholic extract of leaves of Ziziyphus nummularia. J Ethnopharmacol. 2014;151(1):536-542.
8. Sharma J, Gairola S, Gaur R, Painuli R, Siddiqi T. Ethnomedicinal plants used for treating epilepsy by indigenous communities of sub-Himalayan region of Uttarakhand, India. J Ethnopharmacol. 2013;150(1):353-370.
9. Snehunsu A, Ghosal C, Kandwal M, Yadav PK, Nayak BS, Rao KR, *et al*. 1-Triacontanol cerotate; isolated from Marsilea quadrifolia Linn. ameliorates reactive oxidative damage in the frontal cortex and hippocampus of chronic epileptic rats. J Ethnopharmacol. 2015;172:80-84.
10. Taghiabadi E, Hosseinzadeh H, Ramezani M, Shafaei H. Anticonvulsant effect of Berberis integerrima L. root extracts in mice. Res Pharm Sci. 2012;7(5):S812.
11. Azanchi T, Pouriaee H, Shafaroodi H, Asgarpanah J. The anticonvulsant effect of Citrus aurantium flowers. Res Pharm Sci. 2012;7(5):S846.
12. Jana S, Shekhawat G. Anethum graveolens: An Indian traditional medicinal herb and spice. Pharmacogn Rev. 2010;4(8):179-184.
13. Nowbandegni AS, Kiumarcy S, Rahmani F, Dokouhaki M, Khademian S, Zarshenas MM, *et al*. Ethnopharmacological knowledge of Shiraz and Fasa in Fars region of Iran for diabetes mellitus. J Ethnopharmacol. 2015;172:281-287.
14. Zargari A. Medicinal plants, 5th Ed. Tehran University Publications, Tehran, Iran, 1991 (pp:528-531).
15. Akaberi A, Mohammad-Zadeh M, Mirmoosavi Seyed J, Tazari MA, Abarashi A. Effects of the aqueous extract of *Anethum graveolens* leaves on seizure induced by pentylenetetrazole in mice. Malays J Med Sci. 2013;20(5)23-30.
16. Rostampour M, Ghaffari A, Salehi P, Saadat F. Effects of hydro-alcoholic extract of *Anethum graveolens* seed on pentylenetetrazol-induced seizure in adult male mice. Basic Clin Neurosci. 2014;5(3):199-204.
17. Chen Y, Zeng H, Tian J, Ban X, Ma B, Wang Y. Antifungal mechanism of essential oil from *Anethum graveolens* seeds against Candida albicans. J Med Microbiol. 2013;62:1175-1183.
18. Hajhashemi V, Abbasi N. Hypolipidemic activity of *Anethum graveolens* in rats. Phytother Res. 2008;22(3):372-375.
19. Kazemi M. Phenolic profile, antioxidant capacity and anti-inflammatory activity of *Anethum graveolens* L. essential oil. Nat Prod Res. 2015;29(6):551-553.
20. Scharfman HE, Gray WP. Relevance of seizure-induced neurogenesis in animal models of epilepsy to the etiology of temporal lobe epilepsy. Epilepsia. 2007;48:33-41.
21. Hosseinzadeh H, Karimi GR, Ameri M. Effects of *Anethum graveolens* L. seed extracts on experimental gastric irritation models in mice. BMC Pharmacol. 2002;2(1):21-what page.
22. Kaur GJ, Arora DS. Antibacterial and phytochemical screening of *Anethum graveolens*, *Foeniculum vulgare* and *Trachyspermum ammi*. BMC Complement Altern Med. 2009;9(1):30-what page.
23. Kojuri J, Vosoughi AR, Akrami M. Effects of *Anethum graveolens* and garlic on lipid profile in hyperlipidemic patients. Lipids Health Dis. 2007;6(5):1476-1512.
24. Monsefi M, Ghasemi M, Bahaoddini A. The effects of *Anethum graveolens L*. on female reproductive system. Phytother Res. 2006;20(10):865-868.
25. Shyu YS, Lin JT, Chang YT, Chiang CJ, Yang DJ. Evaluation of antioxidant ability of ethanolic extract from dill (*Anethum graveolens L.*) flower. Food Chem. 2009;115(2):515-521.
26. Yazdanparast R, Alavi M. Antihyperlipidaemic and antihypercholesterolaemic effects of *Anethum graveolens* leaves after the removal of furocoumarins. Cytobios. 2000;105(410):185-191.
27. Persad R, Liu C, Wu TT, Houlihan PS, Hamilton SR, Diehl AM, *et al*. Overexpression of caspase-3 in hepatocellular carcinomas. Modern Pathol. 2004;17(7):861-867.
28. Naseer MI, Ullah I, Rasool M, Ansari SA, Sheikh IA, Bibi F, *et al*. Downregulation of dopamine D1 receptors and increased neuronal apoptosis upon ethanol and PTZ exposure in prenatal rat cortical and hippocampal neurons. Neurol Sci. 2014;35(11):1681-1688.