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Original Article

Antiapoptotic and antinociceptive effects of *Achillea millefolium L.* aqueous extract in rats with experimental painful diabetic neuropathy

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Abstract

Background and purpose: Neuropathy is one of the common complications of diabetes mellitus. This study aimed to determine the analgesic and antiapoptotic effects of the aqueous extract of *Achillea millefolium* L. (Ach) in rats with experimental painful diabetic neuropathy by behavioral and molecular procedures.
Experimental approach: Thirty male Wistar rats were divided into 5 groups including control, diabetes + saline, and diabetes + Ach extract (doses of 150, 300, and 600 mg/kg/day for 3 weeks, orally). A tail-flick test was performed to assess the pain threshold in different groups. Western blotting test was used to evaluate the apoptotic (Bax, Bcl2, cleaved caspase-3, and cytochrome-c) and inflammatory (TNF-α and NF-κB) protein factors in the lumbar portion of the spinal cord tissue. Also, commercial assay kits were used to evaluate oxidative stress factors (MDA, GPx, and SOD enzyme activity) in the lumbar portion of the spinal cord tissue.
Findings/Results: Results showed that administering Ach extract at the doses of 300 and 600 mg/kg/day significantly increased the nociception threshold in treated diabetic animals compared to untreated diabetic animals. Moreover, the treatment of diabetic animals with Ach extract (300 and 600 mg/kg/day) significantly reduced the oxidative stress, inflammation, and apoptosis biochemical indicators in the lumbar spinal cord tissue compared to the untreated diabetic group.
Conclusion and implications: The findings showed that Ach extract has neuroprotective and anti-nociceptive effects in rats with diabetic neuropathy. The effects can be due to the inhibition of oxidative stress, inflammation, and apoptosis in the spinal cord tissue.

Keywords: Apoptosis; Diabetic neuropathy; Hyperalgesia; Inflammation; Oxidative stress.

INTRODUCTION

Diabetes mellitus is one of the most common chronic metabolic diseases affecting millions of people worldwide (1). Metabolic regulation disorders due to diabetes cause secondary pathophysiological changes in the various organs of the body, which are responsible for the most disability and mortality caused by diabetes (2). Diabetes mellitus is associated with several long-term complications, including neuropathy, retinopathy, and nephropathy (3). Diabetic neuropathy can present as polyneuropathy, mononeuropathy, or autonomic neuropathy (4). Neuropathic pain, which usually occurs in the lower extremities, is also a feature of diabetic polyneuropathy (5). Hyperglycemia is a major component of the onset and progression of diabetic neuropathy in patients with the disease (6). A recent study has focused on the biochemical and molecular processes leading to diabetic neuropathy complications (1). *In vivo* and *in vitro* models of diabetic neuropathy have been studied to identify the major biochemical mechanisms causing the complications, which can be an important step in their treatment (7,8).

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Most of the mechanisms are related to the cellular metabolic and oxidation-reduction systems (9-11). Increased cellular oxidative stress activates the polymerase pathway (ADP-ribose). It alters the expression of genes involved in regulating inflammation and neuronal function, ultimately leading to cell destruction and death through apoptosis mechanisms (2,12,13). As mentioned above, neurodegeneration caused by high blood glucose in diabetic conditions causes neuropathic pain through various neuroanatomical, neurophysiological, and neurochemical mechanisms (5). Neuropathic pain occurs either spontaneously or as a result of a mildly painful stimulus and causes painful symptoms (14). *Achillea millefolium L.* (Ach), commonly named yarrow, is one of the well-known plants belonging to the *Asteraceae* family. The plant is one of the most famous medicinal plants widely used in ancient medicine in different cultures, European, and Asian countries to treat many diseases. The cultivation and consumption history of the plant dates back to 3000 years ago. The flowering branches of plants are used in traditional medicine which are collected in summer (15). The aromatic plant has a variety of medicinal properties, such as anti-inflammatory, antioxidant, and antiapoptotic properties (16-18). Studies have shown that luteolin, apigenin, and kaempferol as the main polyphenolic compounds of Ach are responsible for the anti-inflammatory, antioxidant, and neuroprotective properties of this herb (19-23). Also, it has been shown that Ach extract exhibited an antinociceptive effect in animal models (24). According to the mentioned pharmacological effect of Ach and its main phenolic compounds, this study aimed to determine the antiapoptotic and antinociception effects of aqueous extract of Ach in an animal model with experimental diabetic neuropathy.

MATERIALS AND METHODS

Drugs and chemicals

Streptozotocin (STZ) was purchased from Sigma-Aldrich Company (St. Louis, Missouri, USA). Malondialdehyde (MDA), glutathione peroxidase (GPx), and superoxide dismutase (SOD) commercial biochemical assay kits were procured from Zellbio Company (Lonsee, Germany). Bcl-2-associated protein x (Bax), B-cell lymphoma 2 (Bcl2), cleaved caspase-3, cytochrome-c, tumor necrosis factor-alpha (TNF-α), and nuclear factor kappa B (NF-κB) antibodies were obtained from Abcam Company (Cambridge, UK). Polyvinylidene fluoride (PVDF) paper and an enhanced chemiluminescence (ECL) kit were procured from Roshe Company (Roche Diagnostics GmbH, Mannheim, Germany).

Animals

In this study, 30 male Wistar rats weighing 200-250 g were provided by the animal house of the Rafsanjan University of Medical Sciences. The animals were housed under standard conditions including a temperature of 23 ± 1 °C, free access to sufficient water and food, and a 12-h dark/12-h light cycle. Minimizing animal suffering was considered throughout the experimental period. All procedures were approved by the Ethics Committee of Rafsanjan University of Medical Sciences (ethics code: IR.RUMS.REC.1399.111) and performed by Guidelines for the Care and Use of Laboratory Animals (NIH, Publication No. 85-23, revised 1985; European Communities Directive 2010/63/EU).

Experimental groups

The animals were randomly divided into 5 experimental groups (6 rats in each group), as follows: group 1 (control) included non-diabetic rats receiving normal saline orally for 3 weeks; group 2 (diabetes + saline) included diabetic rats receiving normal saline orally for 3 weeks; groups 3, 4, and 5 included diabetic rats receiving Ach extract orally at the doses of 150 (diabetes + Ach 150), 300 (diabetes + Ach 300), and 600 mg/kg/day (diabetes + Ach 600) for 3 weeks, respectively (18).

Plant material extraction and high-performance liquid chromatography analysis

The Ach plant was bought from the Isfahan Botany Herbarium in July 2021 (specimen NO. 4007). The flowering branches of the plant were cut off and ground after being cleaned and

dried. Two hundred mL of distilled water was percolated with 2 g of powdered plant for 24 h. The extract was dried and evaporated after being filtered using filter paper (8-10 µ). According to Ayoobi's study, the recommended human dose is 2-4 g of dried flowers and stem (25) equivalent to 250-500 mg of the dried aqueous extract, and we utilized it in the current investigation. To quantify the flavonoids in the extracted sample high-performance liquid chromatography (HPLC) was used.

Experimental protocol

Induction of diabetes and treatment with Ach extract

The induction of diabetes was performed by a single subcutaneous injection of STZ (55 mg/kg) (7). STZ was freshly dissolved in 0.1 mol/L citrate buffer (CB). Non-diabetic rats receiving an injection of CB were used as the control group. One week after the STZ injection, the blood sample was collected *via* the lateral tail vein. Then, blood glucose level was measured enzymatically by a glucose oxidase peroxidase kit (Pars Azmon Co., Iran). Blood glucose above 250 mg/dL was considered a measure of diabetes (7). One week after STZ injection and the induction of diabetes, the aqueous extract of Ach was dissolved in saline and given to the respective experimental groups by a gastric tube daily for 3 weeks.

Tail-flick test to measure nociception threshold

A tail-flick test was used to measure the nociception threshold in the experimental groups. In the test, the beam intensity of the device was adjusted between 4 and 6 s in the middle third of the tail of the control animals. A cut-off time of 15 s was also considered to prevent any tissue damage in the animal's tail. The test was performed 3 times with a 3-min interval by a tail-flick device (Ugo Basile, Italy). Then, the mean of tail retraction time was considered the nociception threshold (tail-flick latency) for each animal. The test was performed once a week for 4 weeks (8).

Tissue extraction and preparation for molecular assessments

As described previously (8), at the end of the behavioral study period, the rats were anesthetized by CO_2 inhalation and decapitated. The vertebrae had been severed through the pelvic girdle. Hydraulic extrusion was accomplished by inserting a 16-gauge needle into the sacral spinal canal and injecting ice-cold saline. The spinal cord tissue thus obtained was immediately placed on ice in a glass petri dish, and the dorsal half of the lumbar spinal cord was dissected. Tissue samples were weighed, immediately frozen, and stored in a liquid nitrogen tank. After that, the isolated tissue was homogenized in ice-cooled radioimmunoprecipitation assay buffer containing Tris-HCl (10 mM, pH 7.4), 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 0.1% Na deoxycholate, 1% NP-40, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2.5 µg/mL of leupeptin, and 10 µg/mL of aprotinin), and 1 mM sodium orthovanadate (8). The homogenated tissues were centrifuged at 4 °C for 20 min (14000 rpm), and the supernatant was collected. Protein concentration in the supernatant was measured by the Bradford method (7,8,26,27).

Western blot analysis

Western blot test was used to evaluate the expression of some apoptotic (Bax, Bcl2, cleaved caspase 3, and cytochrome-c) and inflammatory (TNF-α and NF-κB) proteins in the dorsal portion of lumbar spinal cord tissue. Forty µg of each protein sample was used for electrophoresis on 12.5% SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were electrically transferred from the SDS-PAGE to the PVDF paper. PVDF paper was then immersed in a blocking buffer (non-fat milk with a concentration of 5% in Tris-buffered saline containing 0.1% Tween 20 detergent (TBS-T)) (26) for 2 h at room temperature. TBS-T solution was used for 5 min (3 times) to remove the excess blocking buffer on PVDF paper. PVDF paper was then immersed in a blocking buffer (TBS-T, 150 mM NaCl, 20 mM Tris-HCl with pH 7.5, and 0.1% Tween 20) containing the primary antibodies (1:1000 (µL)) overnight at 4 °C. To remove the excess solution of primary antibodies, the PVDF paper was washed with TBS-T solution. Then, a secondary antibody conjugated to horseradish peroxidase was used. The antibody-antigen

complexes were detected *via* the ECL system and exposed to lumi-film chemiluminescent detection film (Roche Diagnostics GmbH, Mannheim, Germany). β-actin immunoblotting was used as a loading/internal control. Band densities were evaluated using the respective band densitometry. The ImageJ image-processing program (1.52r, National Institutes of Health, USA) was used to analyze the intensity of expression. The values were expressed as the tested protein/β-actin ratio for each sample.

Oxidative stress measurement

The oxidative stress markers including MDA and the enzyme activities of GPx and SOD in the lumbar spinal cord tissue were evaluated using the manufacturer's instructions for commercial biochemical assay kits.

Statistical analysis

Shapiro-Wilk test was used to check the normality of data distribution. The equality of variances was evaluated using Levene's test. The obtained data from the behavioral and molecular analysis were expressed as mean ± SEM. Two-way repeated measure ANOVA followed by Sidak post-test was used for comparing glucose serum level and body weight between the start and end of the study. One-way ANOVA followed by the post hoc tests of Bonferroni and Tukey was used for comparing glucose level and body weight among groups in each time point (start or the end of the study), respectively. Two-way ordinary ANOVA followed by the Tukey post hoc test was used to compare the tail-flick data in the similar time point among groups. Moreover, one-wayANOVA followed by Tukey post hoc test was used to compare the biochemical and molecular data. The significance level was considered $P < 0.05$.

RESULTS

Phytochemical analysis of Ach extract

The HPLC results for quantifying the flavonoids in the extracted sample found 1.6 mg/g of apigenin and 0.3 mg/g of luteolin (Table 1).

The effect of aqueous extract of Ach on serum glucose and body weight

One week after the STZ injection, the glucose serum level of the diabetic groups increased significantly compared to the control group, and the increment was maintained until the end of the experimental period (Table 2). Also, the body weight in the diabetes + saline group was significantly reduced at the end of the experimental period compared to the control group. Table 2 reveals that the treatment of diabetic rats with the 300 and 600 mg/kg doses of aqueous extract of Ach significantly reduced the serum level of glucose and increased body weight at the end of the experiment compared to the diabetes + saline group. Treatment with the 150 mg/kg dose of aqueous extract of Ach did not show significant improving effects on the serum level of glucose and body weight in diabetic animals at the end of the study compared to the diabetes + saline group (Table 2).

Table 1. Phenolic compounds in Ach extract in HPLC test.

Polyphenolic compounds	Content (mg/g)
Luteolin	0.3 ± 0.01
Apigenin	1.6 ± 0.12

The data were expressed as mean ± SEM. Ach, *Achillea millefolium*; HPLC, high-performance liquid chromatography.

Table 2. The effect of Ach aqueous extract (150, 300, and 600 mg/kg/day) on the serum level of glucose and body weight.

Experimental groups	Serum level of glucose (mg/dL)		Body weight (g)	
	Start of the study	End of the study	Start of the study	End of the study
Control	94.00 ± 1.653	95.00 ± 2.160	220.0 ± 6.202	247.0 ± 5.837$^{\Delta\Delta\Delta}$
Diabetes + saline	304.8 ± 10.85***	303.3 ± 15.57***	226.5 ± 6.265	197.5 ± 7.854$^{\Delta\Delta\Delta***}$
Diabetes + Ach 150	298.8 ± 15.67***	296.0 ± 16.91***	225.5 ± 6.874	207.8 ± 7.613$^{\Delta\Delta\Delta}$
Diabetes + Ach 300	301.0 ± 21.57***	285.3 ± 23.25$^{***\#}$	226.0 ± 5.158	229.2 ± 5.712$^{\#\#}$
Diabetes + Ach 600	305.0 ± 17.27***	284.0 ± 19.98$^{\Delta***\#}$	225.7 ± 6.386	237.2 ± 4.956$^{\Delta\Delta\#\#\#}$

The data were expressed as mean ± SEM. $^{\Delta}P < 0.5$, $^{\Delta\Delta}P < 0.01$, and $^{\Delta\Delta\Delta}P < 0.001$ indicate the significant differences compared with the start of the study in the respective group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus diabetes + saline group; $^{***}P < 0.001$ versus the respective control group. Ach, *Achillea millefolium.*

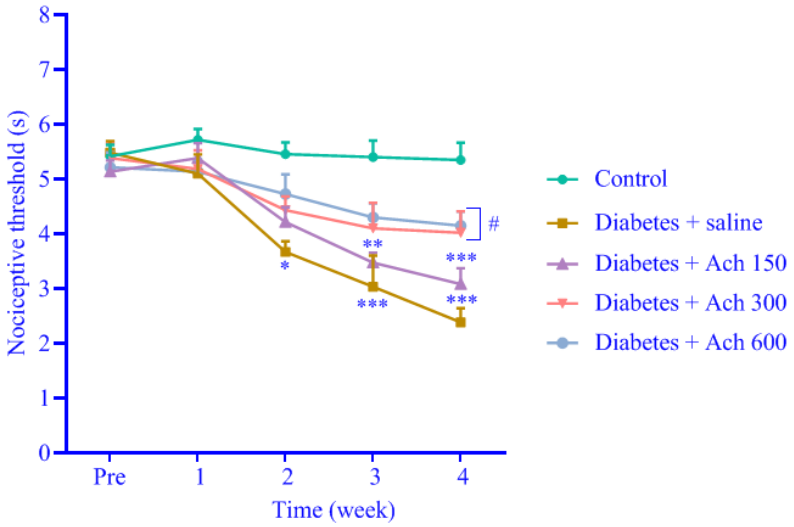

Fig. 1. The effect of Ach aqueous extract (150, 300, and 600 mg/kg/day) on the progress of diabetes-induced thermal hyperalgesia. Each line indicates the nociceptive threshold in each experimental group. The time point "pre" was referred to before STZ injection. The data were presented as mean ± SEM and analyzed using two-way ANOVA followed by Tukey's post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate the significant differences compared with the control group at a similar time point; $^{\#}P < 0.05$ versus the diabetes + saline group at a similar time point. Ach, *Achillea millefolium.*

The effect of Ach extract on the nociception threshold

Two weeks after STZ injection, the diabetes + saline group showed a significant decrease in nociception threshold compared to the control group and continued until the end of the study. In addition, the treatment of diabetic animals with the 300 and 600 mg/kg doses of Ach significantly increased the nociception threshold compared to the diabetes + saline group. It is worth mentioning that treatment with the 150 mg/kg dose of Ach had no significant effects on the nociception threshold compared to the untreated diabetic animals (Fig. 1).

The effect of Ach extract on oxidative stress level in the lumbar spinal cord tissue

The data showed a significant increase in MDA (as an indicator of lipid peroxidation) levels in lumbar spinal cord tissue in the untreated diabetic group compared to the control group (Fig. 2A). The results also showed a significant decrease in the MDA levels in diabetic groups treated with Ach at the doses of 300 and 600 mg/kg compared to the untreated diabetic group, indicating a decrease in oxidative stress in the spinal cord tissue (Fig. 2A). In addition, the levels of GPx and SOD enzyme activities (as the cellular antioxidant defense system) reduced in the untreated diabetic group compared to the non-diabetic group, significantly (Fig. 2B and 2C). The activity levels of GPx enzyme in the diabetic groups treated with the 300 and 600 mg/kg doses of Ach showed a significant increase compared to the untreated diabetic group (Fig. 2B). In addition, the treatment of diabetic animals with the 300 and 600 mg/kg doses of Ach significantly elevated the SOD enzyme activity in the lumbar spinal cord tissue compared to diabetes + saline group (Fig. 2C).

The effect of Ach extract on apoptotic biomarkers in the lumbar spinal cord tissue

According to Fig. 3, the expression levels of Bax, cytochrome-c, and cleaved caspase-3 pro-apoptotic proteins as well as the Bax/Bcl2 ratio in the lumbar spinal cord increased in the untreated diabetic group compared with the control group. However, the treatment of diabetic groups with 300 and 600 mg/kg doses of Ach decreased the expression of cytochrome-c, cleaved caspase-3, and Bax/Bcl2 ratio compared to the untreated diabetic group.

The expression of Bax showed a significant reduction in the only diabetes + Ach 600 group when compared with the diabetes + saline group. The findings can be considered a decrease in the level of apoptosis in the lumbar spinal cord tissue. Furthermore, the expression level of Bcl2 (as an antiapoptotic protein) showed a significant decrease in the untreated diabetic group compared to the non-diabetic rats. On the other hand, the diabetic group treated with Ach at the dose of 600 mg/kg showed a significant increase in Bcl2 protein expression compared to the untreated diabetic group.

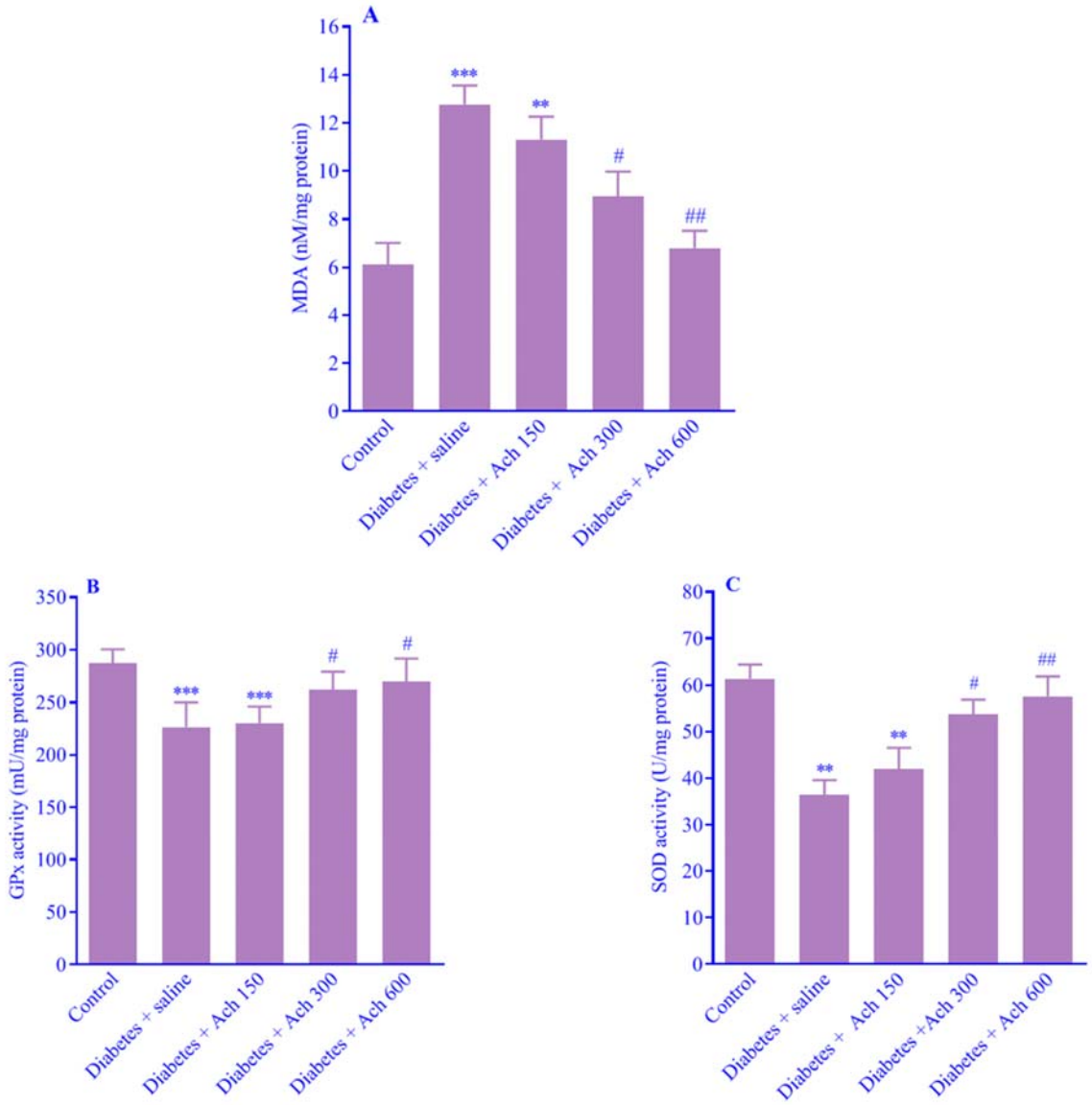

Fig. 2. The effect of Ach aqueous extract (150, 300, and 600 mg/kg/day) on (A) MDA; (B) GPx activity; and (C) SOD activity in the dorsal portion of the lumbar spinal cord in the experimental groups. The data were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Tukey's post hoc test. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate the significant differences compared with control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus diabetes + saline group. MDA, Malondialdehyde; GPx, glutathione peroxidase; SOD, superoxide dismutase; Ach, *Achillea millefolium*.

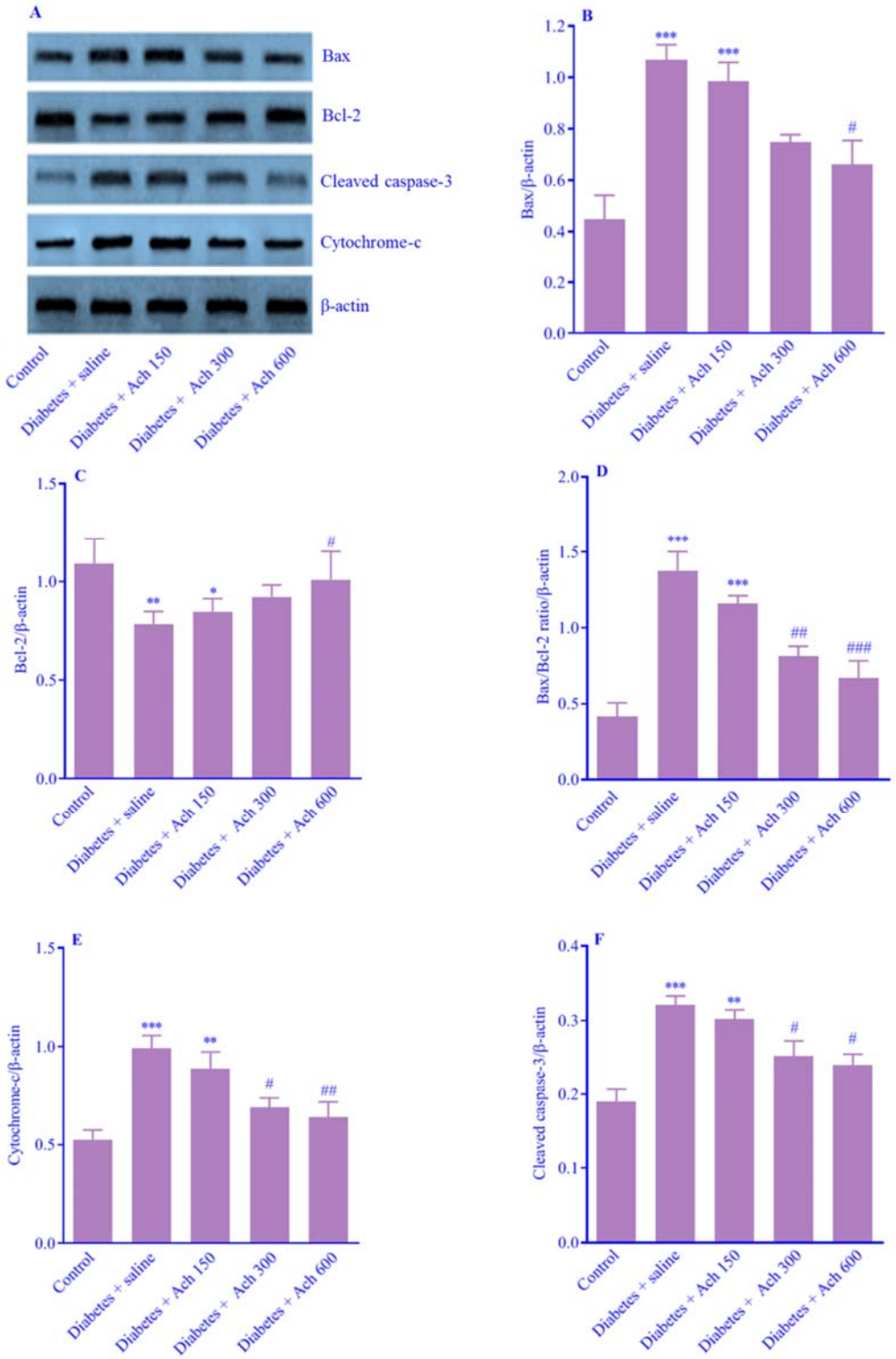

Fig. 3. The effect of Ach aqueous extract (150, 300, and 600 mg/kg/day) on apoptosis biomarkers (A) SDS-PAGE; (B) Bax; (C) Bcl-2; (D) Bax/Bcl-2 ratio; (E) cytochrome-c; and (F) cleaved caspase-3 in the dorsal portion of the lumbar spinal cord in the experimental groups. The data were shown as mean ± SEM and analyzed using one-way ANOVA followed by Tukey's post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate the significant differences compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus diabetes + saline group. Ach, *Achillea millefolium;* SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

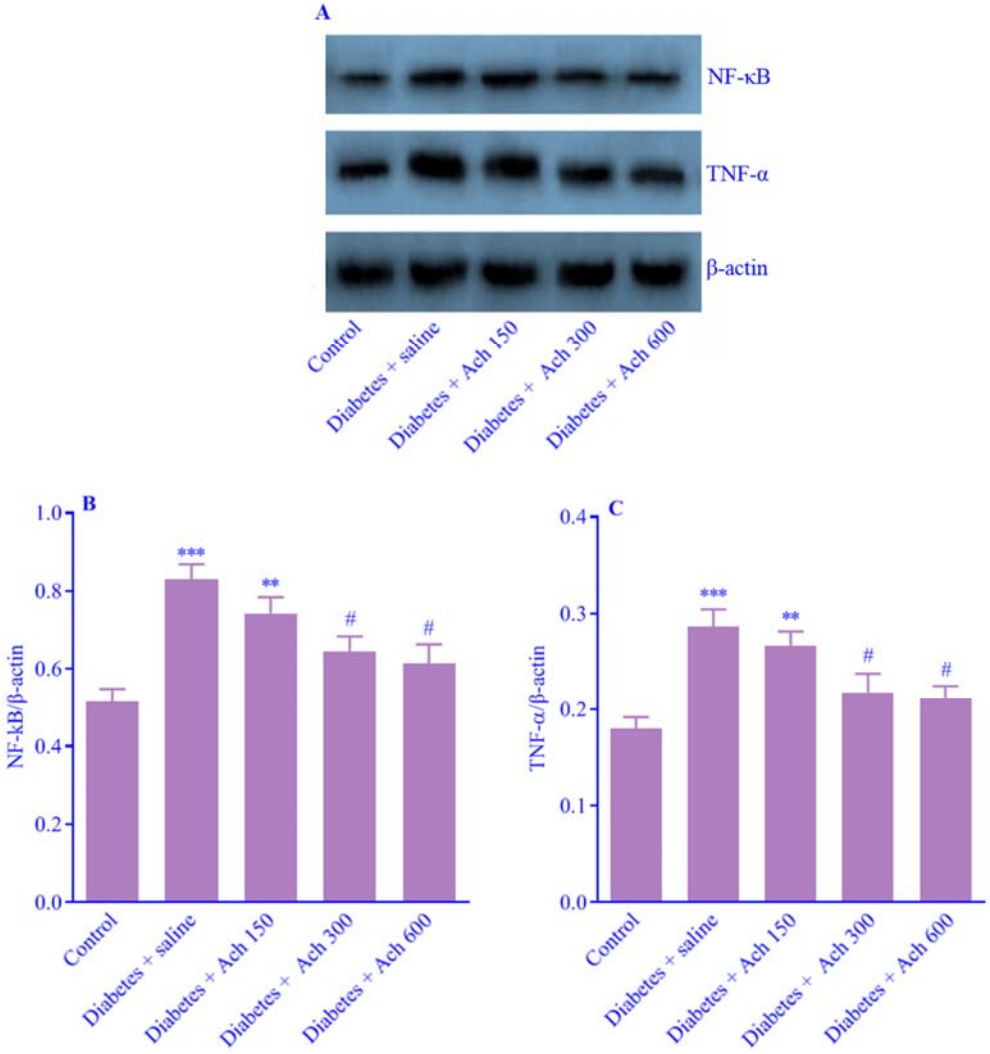

Fig. 4. The effect of Ach aqueous extract (150, 300, and 600 mg/kg/day) on the inflammatory biomarkers of (A) SDS-PAGE; (B) NF-κB; and (C) TNF-α in the dorsal portion of lumbar spinal cord. The data were shown as mean ± SEM and analyzed using one-way ANOVA followed by Tukey's post hoc test. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences compared to the control group; $^{\#}P < 0.05$ versus diabetes + saline group. NF-κB, Nuclear factor kappa B; TNF-α, tumor necrosis factor alpha; Ach, *Achillea millefolium*; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The effect of Ach extract on the inflammatory biomarkers in lumbar spinal cord tissue

The results showed that the expression levels of NF-κB and TNF-α as inflammatory biomarkers in the lumbar spinal cord increased in the untreated diabetic group compared to the control group, significantly (Fig. 4). On the other hand, the diabetic groups treated with Ach at the doses of 300 and 600 mg/kg exhibited the decreased expression levels of NF-κB and TNF-α proteins compared to the untreated diabetic group, significantly (Fig. 4).

DISCUSSION

Diabetic neuropathy is one of the most common complications of diabetes mellitus. However, the main causes of this complication have not yet been well known (4). In addition, the direct treatment of diabetic neuropathy and the reduction of pain caused by complications using available drug therapies is not very successful (6). Therefore, it is important to study the mechanisms of diabetic neuropathy and identifying the compounds and drugs that

treat it. In the present study, the tail-flick test was used to show the incidence of hyperalgesia, as a prominent feature of diabetic neuropathy. The findings showed that 2 weeks after the induction of diabetes, the animals withdraw their tails 2 to 3 s less than non-diabetic animals in response to the thermal noxious stimulus. As mentioned earlier, the incidence of hyperalgesia is due to the development of diabetic neuropathy (4). The present study showed that Ach extract could improve the nociception threshold in diabetic rats. The findings are in line with previous studies regarding the incidence of hyperalgesia as one of the main signs of the development of diabetic neuropathy (5,7,8,27). Many neuroanatomical, neurophysiological, and neurochemical mechanisms are involved in developing neuropathic pain (6,14). Neuropathic pain occurs either spontaneously or as the result of a mildly painful stimulus and causes painful symptoms (5). The results also showed that diabetes increases MDA levels and decreases the activities of GPx and SOD antioxidant enzymes in spinal cord nerve tissue. The findings indicated diabetes-induced oxidative stress in spinal cord nerve tissue. It was shown that reactive oxygen species (ROS) production and oxidative stress increase in hyperglycemia and diabetic conditions (9,10,28). In this regard, it has been found that oxidative stress is one of the major causes of neuronal death and the development of pathological conditions which can lead to the incidence of pain in the peripheral and central nerve fibers (29,30). The present study indicated that Ach extract was able to reduce oxidative stress by reducing MDA levels and increasing the activity of GPx and SOD enzymes. In line with the present study, Chou *et al*. showed that Ach oil increases the activity of the antioxidant enzymes of SOD, GPx, and catalase in cultured macrophage cells (31). As mentioned above, apigenin and luteolin are the main phenolic compounds found in Ach extract. It has been demonstrated that the administration of apigenin reduces oxidative stress in various tissues or cells in oxidative stress situations (32,33). It has also been shown that the administration of luteolin decreases the levels of oxidative stress by decreasing the MDA levels and enhancing SOD activity in the kidney tissue of diabetic rats (34). Many studies have shown that the occurrence of apoptosis in nerve tissues can cause hyperalgesia in diabetic neuropathy conditions (7,8,35). The results of the current study showed that the expression of anti-apoptotic proteins including Bax, Bcl2, cleaved caspase 3, and cytochrome-c elevated in the lumbar spinal cord tissue of diabetic rats. On the other hand, Bcl2 anti-apoptosis protein expression decreased in the lumbar spinal cord tissue of diabetic animals. The present findings also showed that the treatment of diabetic animals with Ach extract could improve the apoptotic parameters closer to normal and thus prevent the cytotoxic effects of hyperglycemia in the lumbar spinal cord tissue. Our previous studies also showed the overexpression of cleaved caspase-3, Bax/Bcl2 ratio, and cytochrome-c as pro-apoptotic proteins (7,26,27). In this regard, it has been observed that increased Bax expression causes a rapid increase in mitochondrial calcium, which can lead to cellular apoptosis (36). On the other hand, when Bax overexpression is inhibited, apoptosis due to excessive calcium uptake by mitochondria is also prevented (37). Although calcium signaling can stimulate ATP production through activating calcium-sensitive dehydrogenases in mitochondria, the accumulation of intra-mitochondrial calcium ions may increase ROS production (38). The excessive production of ROS in mitochondria can release stimulants inducing apoptosis (such as Bax, cytochrome-c, and caspases proteins) from the intra-membrane space of mitochondria to the cytoplasm and thus cause programmed cell death (36). Another complication of diabetes involved in developing hyperalgesia is inflammation in nervous tissues (4,13). Previous findings have indicated that an increase in blood glucose through elevating some inflammatory mediator proteins, such as TNF-α and NF-κB can lead to diabetic neuropathy (39,40). The current results revealed that the expression level of NF-κB and TNF-α inflammatory proteins decreased in the diabetic neuropathy situation. Therefore, it can be suggested that Ach extract can reduce the inflammatory parameters in the lumbar spinal cord tissue. It has been shown that the

administration of apigenin to diabetic mice reduces the inflammatory (TNF-α, interleukin 6 (IL-6), and NF-κB) and apoptotic (Bcl-2, Bax, and caspase-3) factors in the kidney tissue (41). A study demonstrated that luteolin can improve diabetic cardiomyopathy *via* decreasing NF-κB (as pro-inflammatory cytokine) expression in diabetic mice (23). Moreover, Chou's study showed that Ach oil reduces the production of inflammatory factors such as IL-6 and TNF-α in cultured macrophage cells (31). Zolghadri *et al*. also demonstrated that the administration of Ach extract could reduce the gene expression of IL-1β and inducible nitric oxide synthase, as inflammatory and oxidative stress factors, in the pancreatic tissue as well as increase insulin concentration and decrease serum glucose in diabetic rats (42).

CONCLUSION

The present study indicated that Ach extract could exert analgesic and neuroprotective effects against diabetic neuropathy in rats. Molecular mechanisms underlying the protective effects of Ach extract, at least partly, can be related to inhibiting apoptosis, oxidative stress, and inflammation in the lumbar spinal cord nerve tissue of diabetic rats.

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Conflicts of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

A. Kaeidi and A. Shamsizadeh conceived and designed the study; A. Kaeidi and J. Hassanshahi analyzed the data; A. Kaeidi, M.R. Rahmani, and J. Hassanshahi conducted the experiments and drafted the study; M. Moradi, A. Kaeidi, M.R Rahmani, and J. Hassanshahi performed additional experiments and contributed to providing the revised paper. M. Moradi and M.R Rahmani contributed equally to the project. The final version of the manuscript was approved by all authors. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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