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ORIGINAL PAPER

Neuroprotective Effects of Thymoquinone in Acrylamide-Induced Peripheral Nervous System Toxicity Through MAPKinase and Apoptosis Pathways in Rat

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Abstract

Acrylamide (ACR) is extensively used in industrial areas and has been demonstrated to induce neurotoxicity via oxidative stress and apoptosis. In this study, we assessed the probable protective effects of thymoquinone (TQ), an active constituent of *Nigella sativa*, against ACR-induced neurotoxicity. ACR (50 mg/kg, i.p., for 11 days) and TQ (2.5, 5 and 10 mg/kg, i.p., for 11 days) were administered to rats. On 12th day, gait score was examined and rats were sacrificed. Malondialdehyde (MDA) and reduced glutathione (GSH) contents were determined in sciatic nerve. Furthermore, western blotting was conducted. The exposure of rats to ACR caused severe gait disabilities. The MDA and GSH contents were increased and decreased, respectively. ACR decreased P-ERK/ERK ratio and myelin basic protein (MBP) content, but significantly increased P-JNK/JNK, P-P38/P38, Bax/Bcl-2 ratios and caspase 3 and 9 levels. Concurrently administration of TQ (5 and 10 mg/kg) with ACR, prevented gait abnormalities and meaningfully reduced MDA and elevated the GSH contents. Furthermore, TQ (5 mg/kg) elevated the P-ERK/ERK ratio and MBP content while reduced the P-JNK/JNK, P-P38/P38 ratios and apoptotic markers. MAP kinase and apoptosis signaling pathways were involved in ACR-induced neurotoxicity in rat sciatic nerve and TQ significantly reduced ACR neurotoxicity. TQ afforded neuroprotection, in part, due to its anti-oxidative stress and anti-apoptotic mechanisms.

Keywords Acrylamide · Thymoquinone · MAP kinase signaling pathway · Sciatic nerve · Neuroprotective · Apoptosis

Introduction

Acrylamide (ACR) with the molecular formula of CH2=CH–CO–NH2, IUPAC name of 2-propenamid, and also chemical names such as prop-2-enamide, 2-propenamide, acrylic amide or ethylenecarboxamide; is easily dissolved in water and organic solvents such as methanol or ethanol [1, 2]. There are concerns that ACR is formed in high-carbohydrate foods and especially in food rich in amino acid-asparagine—during frying, deep frying and baking (at the temperature exceeding 120 °C and via Millard's reaction). Mean daily intake of ACR is between 0.14 and 1.31 µg/kg body weight/day for Europeans while for Americans it is 0.43 to 1.1 µg/kg body weight/day [3]. But acceptable daily intake limit of ACR is 1 µg/kg of body weight/day [4–6].

Neurotoxicity is the most well-documented effect of ACR in human and experimental animals [7]. The symptoms are sensory and motor dysfunction, skeletal muscle weakness, weight loss, and ataxia [8]. A variety of mechanisms have been attributed to ACR causing neurotoxicity. Oxidative stress is one of the most important mechanisms leading to ACR-induced neurotoxicity [9]. The enhancement in lipid peroxidation, decline in antioxidant capacity in nervous tissue and sciatic nerve of Wistar rats were observed after administration of ACR [10]. Peripheral neuropathy and altered electrophysiology of the sciatic nerve induced by ACR, accompanied by the symptoms of redox imbalance, were also indicated [10]. It has also been shown that

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exposure to ACR may lead to central and/or peripheral distal axonopathy [11]. A myelin sheath has encompassed a normal peripheral myelinated axon [12], which can be affected by ACR. This monomer, in turn, is able to damage the axonal membrane resulting in demyelination [13].

It has been proven that decreased levels of reduced glutathione (GSH) may cause neurodegenerative diseases [14]. Exposure to ACR leads to a decrease in cellular GSH content and increase in reactive oxygen species (ROS) generation [10]. On the other hand, malondialdehyde (MDA), as a biomarker of lipid peroxidation in several diseases including neurodegenerative diseases [15], elevated in mice brain which were exposed to ACR [16].

Several lines of experimental evidence have demonstrated that ACR is able to induce apoptosis via different mechanisms such as Mitogen-Activated Protein Kinases (MAPKs) including the extracellular signal–regulated kinases (ERK1/2), the c-Jun NH 2-terminal kinases (JNKs) and P38 [17]. In a study, ACR activated phosphorylation of P38 and JNK kinases via increase in ROS production in macrophages exposed to ACR [18]. Moreover, in an in vitro model, it was shown that phosphorylation of JNK and p38 was meaningfully augmented after treatment of PC12 cells with ACR, whereas ERK1/2 phosphorylation was meaningfully decreased [19]. Lakshmi et al. in 2012 showed that ACR is able to induce apoptosis in the cerebral cortex of Wistar rats [20]. Also, treatment of PC12 cells with ACR escalated ROS generation and caused apoptosis [21]. Sumizuwa in 2007 obtained the same results in which ACR induced apoptosis in neuronal cells (SH-SY5Y cell line) via ERK pathway and caspase-3 activation [22]. In addition, ACR, by dint of increasing the expression of Bax, pro-caspase 3 and decreasing Bcl-2 led to apoptosis [23]. Due to the importance of ACR neurotoxicity, many scientists have been trying to find medicinal plants which are able to protect against neurotoxic effects of ACR [9, 24, 25]. In our previous works, we have studied and reported the effects of some medicinal plants or their constituents such as saffron [26], rutin [27], chrysin [28], linalool [29] on neurotoxicity induced by ACR.

In recent years considerable attention has been paid to *Nigella sativa*, known as black seed, which has been used to cure different types of diseases for several centuries [30, 31]. *N. sativa* and its main constituent, thymoquinone (TQ), have various pharmacological activities including anti-oxidative [32, 33], anti-apoptotic [34], anti-inflammatory [35], neuroprotective [36], hepatoprotective [37, 38], anti-asthmatic [39], gastroprotective [40], renoprotective [41] and anti-cancer [42, 43]. Their use in several clinical trials has also been promising. *N. sativa* and TQ have had beneficial effects to cure patients with inflammatory and auto-immune disorders, as well as metabolic syndrome [44].

Neuroprotection, as one of the most important effects of *N. sativa* and TQ, has been investigated in different experiments and a variety of mechanisms has been suggested [45–48]. In a study, it was demonstrated that pretreatment of rats with *N. sativa* extracts decreased thiobarbituric acid-reactive substance (TBARS) and increased GSH, superoxide dismutase and catalase levels. Antioxidant, free radical quenching and anti-inflammatory properties could be the main mechanisms of its neuroprotective effects [49]. TQ increases GSH via directly reacting with free radicals [50]. In another study, Radad in 2014 showed that TQ could ameliorate neuronal degeneration, axonal demyelination and degeneration of hippocampal and cerebellar neurons induced by lead acetate in rats [51]. TQ has anti-apoptotic properties via different mechanisms [34, 52, 53]. TQ prevented amyloid β_{1-40}-induced apoptosis and neurotoxicity via inactivation of caspases 3, 8 and 9 [54]. Treatment of prenatal rat cortical neurons with metformin and TQ prevented apoptosis via elevating Bcl-2 expression, inhibiting the activation of caspase 3 and caspase 9 and decreasing the cleavage of Poly [ADP-ribose] polymerase 1 [55].

In a model of hydrogen peroxide-mediated oxidative stress in human neuronal cells, it was shown that TQ decreased P53, AKT1, ERK1/2, P38, JNK, and NF-κB and could be used as a therapeutic agent against neurodegenerative diseases [56]. TQ was also able to lessen D-galactosamine- and lipopolysaccharide-evoked P-ERK, P-JNK and P-P38, notifying that TQ could prevent acute hepatic failure via inhibiting MAP kinase signaling pathway [53]. Being the downstream effectors in antioxidant responses, MAP kinase signaling exhibits redox modulatory effects. In fact, GSH depletion leads to the activation of MAPK pathway [57].

To our knowledge, no information is available on the effect of TQ on ACR-induced neurotoxicity of the sciatic nerve in which to investigate the MAP kinase and apoptotic pathways. Hence, we decided to conduct the present research to assess the neuroprotective, antioxidant, and anti-apoptotic effects of TQ in ACR-induced neurotoxicity in Wistar rats.

Materials and Methods

Chemicals and Reagents

ACR (C3H5NO, > 99% purity), *n*-butanol, phosphoric acid, potassium chloride (KCl) and thiobarbituric acid (TBA) were purchased from Merck Company, Germany; trichloro acetic acid (TCA), Tetrabutylammonium and TQ from Sigma Company, Germany; Polyvinylidene fluoride membrane from Millipore (Billerica, MA, USA) and DTNB (5, 5′-Dithiobis 2-nitrobenzoic acid) from Sigma Company, USA were prepared.

Experimental Design

Forty-eight adult male Wistar rats (weighing 230–250 g) were obtained from the animal house of school of Pharmacy, Mashhad University of Medical Sciences, Mashhad, IR. Iran. Animals were caged in eight groups and freely given food and water. 12 h light–dark cycles with 21–24 °C temperature and 40–60% humidity were kept at animal house. Rats were used in agreement with the ethical committee acts and guidelines of Mashhad University of Medical Sciences (ethical number: 931569). After 7 days of acclimation, 8 groups of rats were assigned randomly to one of the groups as shown in Table 1. For i.p. (intraperitoneal) injections, TQ was dissolved in Tween 80 (0.8% v/v) and saline (NaCl 0.9%) solution. The choice of ACR dose was based on previous studies and preliminary experimental results and showed the levels of toxic intake of ACR in experimental animals [28]. The choice of TQ dose was based on previous studies which indicated TQ had protective effects on tissue damage [33].

Behavioral Tests

Gait Score

The gait scores were examined in each of the animals of all the groups. Following observation, a gait score was assigned from 1 to 4 (Table 2). A blinded, trained investigator carried out the behavioral assessment. Each rat was observed 3 times, each time 3 min [8].

Sample Collection

Twenty-four hours after the end of the treatment period, rats (n = 6) were decapitated and the sciatic nerve was extracted.

Table 1 Study design

Groups	Treatment	Dosage	Route of exposure	Duration (days)
1	Control	–	i.p.	11
2	ACR	50 mg/kg	i.p.	11
3	ACR + TQ	50 mg/kg + 2.5 mg/kg	i.p.	11
4	ACR + TQ	50 mg/kg + 5 mg/kg	i.p.	11
5	ACR + TQ	50 mg/kg + 10 mg/kg	i.p.	11
6	TQ	5 mg/kg	i.p.	11
7	TQ	10 mg/kg	i.p.	11
8	Vit E + ACR	200 mg/kg	i.p.	6*

ACR acrylamide, *TQ* thymoquinone, *Vit E* Vitamin E, *i.p.* intraperitoneal

*Vit E was administered every other day

Table 2 Criteria for rat gait score

Score	Behavioral index
1	The rat is unaffected or normal
2	The rat is a little bit affected and described by weakness, slight ataxia, active, foot splay
3	The rat is moderately affected and described by less active, foot splay with limb spread during ambulation
4	The rat is intensely affected with above-mentioned symptoms accompanying with inability to support body weight, dragging hind-limbs and inability to rear

To extract the sciatic nerve, rats were anaesthetized with ketamine (10 µl) and xylazine (20 µl) [58], and surgical exposure of the left and right sciatic nerve via a dorsolateral muscle splitting incision was accomplished [59]. Then the samples were snap-frozen in liquid nitrogen and stored at − 80 °C.

Lipid Peroxidation Assay

MDA is the main aldehyde by-product and marker of lipid peroxidation in biological systems [60]. The hemogenate from sciatic nerve in 10% KCl was mixed with 3 ml phosphoric acid (1%) and 1.0 ml TBA (0.6%). TBARS formed when test tubes were heated in 95 °C boiling water for 45 min. The content inside the test tubes was cooled by placing them in cold water and 4 ml of *n*-butanol was added and vortexed for 1.0 min. Centrifuge was accomplished for 10 min at 3000×*g* and the organic layers were eliminated and transferred to a fresh test tube. The absorbance was recorded at 532 nm (maximum absorbance for the TBARS) using a spectrophotometer (Jenway 6105 UV/Vis, UK). MDA levels were expressed as nmol/g tissue [29, 61].

Determination of GSH Content

The determination of GSH content was based on the reductive cleavage of DTNB by sulfhydryl groups to form a yellow color [62]. The sciatic nerve tissues were mixed in phosphate buffer saline (0.1 M, pH 7.4) to prepare a tissue homogenate of 10% PBS. Then, 0.5 ml of tissue homogenate was mixed with 0.5 ml of 10% TCA. The homogenate was vortexed and then centrifuged for 10 min at 3000 g. Then the supernatant was collected and 2.5 ml phosphate buffer (pH 8) and 0.5 ml DTNB were added to the supernatant. The absorbance was read at 412 nm by using a spectrophotometer (Jenway 6105 UV/Vis, UK). Finally, GSH standard curve was plotted and the results were expressed as nmol/g tissue [63].

Western Blotting Test

At specific time-points, sciatic nerve samples were placed in a lysis buffer containing 10 mM sodium azide, 1.0 mM sodium orthovanadate (Na_3VO_4), 1.0 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 50 mM Tris–HCl (pH 7.4), 2 mM EGTA, 2 mM EDTA, 0.2% W/V sodium deoxycholate, and complete protease inhibitor cocktail (Roche, Mannheim, Germany). The resulting homogenate was sonicated on ice with three 10 s bursts at high intensity with a 10 s cooling period among each burst and then centrifuged at 10,000×*g* revolutions per minute for 10 min at 4 °C. Bradford assay kit (BioRad, USA) was used to determine the protein concentration [64]. Each sample was mixed 1:1 V/V with 2× SDS blue buffer, boiled, aliquoted and kept in the − 80 °C freezer. Samples were loaded and electrophoresed on a 12% (for ERK, P-ERK, JNK, P-JNK, P38, P-P38, Bax, Bcl-2, caspase 3 and caspase 9) or 15% (for MBP) SDS polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane (BioRad, USA). The membranes were blocked with 5% non-fat milk powder (skimmed milk) for 2 h or 5% bovine serum albumin for 1 h at 37 °C and washed with Tris-Buffered Saline and Tween 20 (TBST) for three times. Then, they were incubated overnight (16–18 h) at 4 °C on a rocker with mouse anti-Phospho-JNK (Cell Signaling #9255, 1:1000), mouse anti-Phospho-P38 (Cell Signaling #9216, 1:1000), mouse anti-Phospho-ERK1/2 (Cell Signaling #9106, 1:1000) and mouse anti-MBP (Cell Signaling #13344, 1:1000) antibody. Other membranes were incubated (2 h) on a rocker with rabbit anti-JNK (Cell Signaling #9252, 1:1000), rabbit anti-P38 (Cell Signaling #9212, 1:1000), rabbit anti-ERK1/2 (Cell Signaling #4695, 1:1000), rabbit Bax (Cell Signaling #2772, 1:1000), rabbit Bcl-2 (Cell Signaling #2870, 1:1000), rabbit caspase 3 (Cell Signaling #9665, 1:1000) and rabbit caspase 9 (Cell Signaling #7237, 1:1000) antibody. Membranes were washed with TBST for three times. Then, the membranes were incubated with rabbit or mouse horseradish peroxidase-conjugate anti-IgG (Cell Signaling #7074, 1:3000; Cell Signaling #7076, 1:3000, respectively) for 1.5 h in room temperature. Enhanced chemiluminescence was used to visualize the peroxidase-coated bands. The integrated optical densities of bands were measured using Alliance 4.7 Gel doc (UK). Densitometric analysis for protein bands was performed using UV Tec software (UK). The protein levels were normalized relative to the corresponding bands of β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control protein.

Statistical Analysis

Results were expressed as mean ± SD. GraphPad Prism 6.0 (GraphPad Prism Software Inc., San Diego, CA, USA) was used for statistical analysis. Statistical comparisons in western blot were made by using one-way ANOVA followed by Turkey's. P values less than 0.05 were considered to be statistically significant. For the gait abnormalities data were expressed as median with interquartile range for each group and statistical analysis were performed with nonparametric test Kruskal–Wallis.

Results

Effect of TQ on ACR-Induced Gait Abnormalities

Rats receiving only ACR showed advanced gait abnormalities comparing to control group at the end of the experimental period (11 days). These rats exhibited foot splay, twisting of hind-limbs and difficulty in movement ($P < 0.001$ vs. control). Interestingly, rats receiving 5 mg/kg TQ and 50 mg/kg ACR, revealed a considerably lower intensity of neuropathic symptoms comparing to ACR group ($P < 0.01$). At the end of 11 days, treatment of rats with 200 mg/kg Vitamin E and 50 mg/kg ACR caused a significant decrease in gait scores comparing to ACR-administered rats ($P < 0.01$) (Fig. 1).

Effect of TQ on ACR-Induced Lipid Peroxidation in Sciatic Nerve

A severe lipid peroxidation in sciatic nerve was evidenced by a massive increase in the MDA levels in rats receiving 50 mg/kg ACR compared to control group ($P < 0.001$). After co-treatment of 5 and 10 mg/kg TQ with 50 mg/kg ACR, the MDA levels were significantly decreased ($P < 0.001$ and $P < 0.01$, respectively) comparing with the ACR group. Also, the MDA levels decreased when 200 mg/kg Vitamin E was co-administered with ACR to rats comparing to ACR group ($P < 0.01$) (Fig. 2).

Effect of TQ and ACR on GSH Content

GSH levels were measured to evaluate the state of oxidative stress. GSH levels were considerably lower in ACR-administered rats ($P < 0.001$) compared to control group. TQ at the doses of 5 and 10 mg/kg, co-administered with ACR, meaningfully increased GSH levels in comparison with ACR group ($P < 0.001$ and $P < 0.05$, respectively). Furthermore, the GSH levels increased when 200 mg/kg Vitamin E was co-administered with ACR ($P < 0.001$) compared with ACR group (Fig. 3).

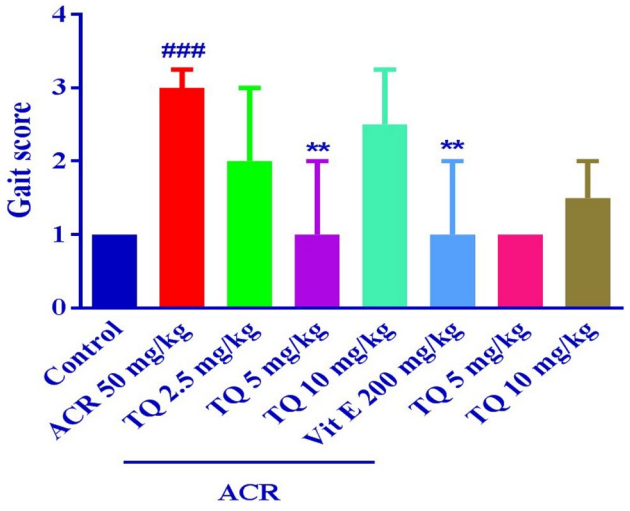

Fig. 1 Effect of TQ on behavioral index (gait score) in rats, exposed to ACR. Data are expressed as median with interquartile range, (n = 6). ###P < 0.001 vs. Control and **P < 0.01 vs. ACR-administered rats. TQ: Thymoquinone, ACR: Acrylamide, Vit E: Vitamin E

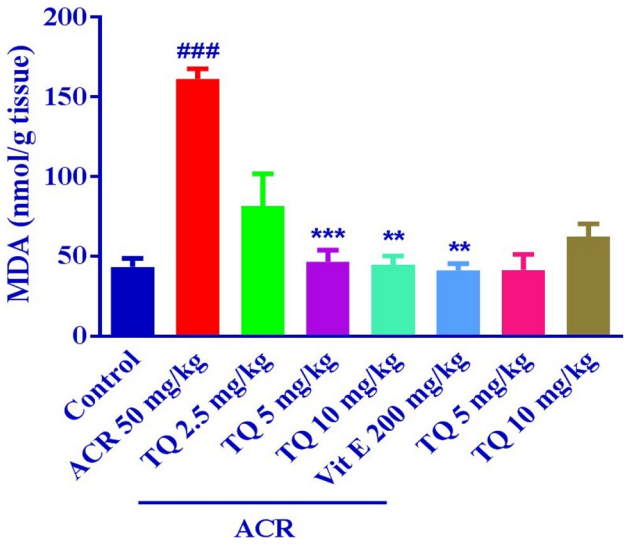

Fig. 2 Effect of TQ on sciatic nerve lipid peroxidation in rats, exposed to ACR. Data are expressed as mean ± SD, (n = 6). ###P < 0.001 vs. control, **P < 0.01 and ***P < 0.001 vs. ACR-administered rats. TQ: Thymoquinone, ACR: Acrylamide, Vit E: Vitamin E

Effect of TQ and ACR on the Activities of MAP Kinase Pathway Proteins (ERK, JNK and P38)

As shown in Fig. 4, ACR at the dose of 50 mg/kg, significantly decreased the P-ERK/ERK ratio (P < 0.01) comparing to control group. TQ 5 mg/kg co-administered with

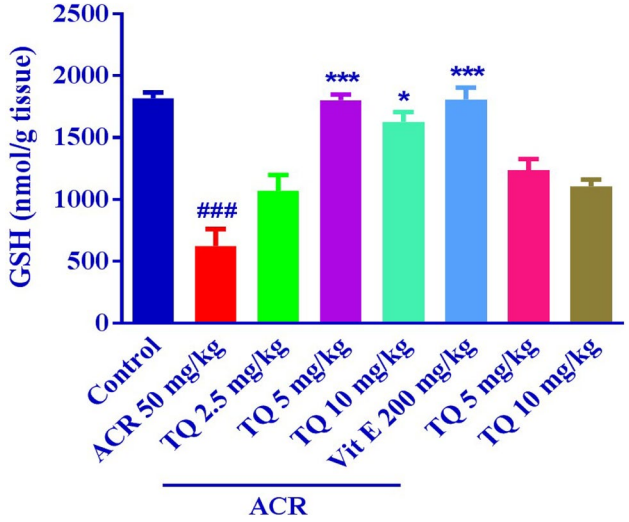

Fig. 3 Effect of TQ on the GSH content following ACR treatment in rats. Data are represented as mean ± SD, (n = 6). ###P < 0.001 vs. NS, ***P < 0.001 and *P < 0.05 vs. ACR-administered rats. TQ: Thymoquinone, ACR: Acrylamide, Vit E: Vitamin E

ACR 50 mg/kg, was able to increase the P-ERK/ERK ratio (P < 0.001) comparing to the ACR-administered rats. Furthermore, Vitamin E 200 mg/kg significantly increased the P-ERK/ERK ratio (P < 0.01) compared to the ACR-administered rats.

ACR at the dose of 50 mg/kg, as shown in Fig. 5, caused significant changes in the P-JNK/JNK ratio comparing to control group (P < 0.001). TQ 5 mg/kg co-administered with ACR 50 mg/kg, significantly decreased the P-JNK/JNK ratio (P < 0.001) comparing to the ACR-administered rats. Furthermore, Administration of vitamin E 200 mg/kg with ACR 50 mg/kg decreased the P-JNK/JNK ratio (P < 0.01) compared to the ACR-administered rats.

ACR at the dose of 50 mg/kg was able to significantly elevate the ratio of P-P38/P38 (P < 0.001) comparing to control group. Interestingly, co-administration of TQ 5 mg/kg with ACR 50 mg/kg could reduce the P-P38/P38 ratio (P < 0.001) comparing to the ACR-administered rats. In addition, Vitamin E 200 remarkably elevated the P-P38/P38 ratio (P < 0.001) compared to the ACR group (Fig. 6).

Effect of TQ and ACR on MBP Level

Administration of ACR at the dose of 50 mg/kg significantly diminished the MBP content (P < 0.001 vs. control group). TQ 5 mg/kg co-administered with ACR 50 mg/kg, produced increase in the MBP content (P < 0.001) as compared to the rats receiving ACR. Additionally, Vitamin E 200 mg/kg significantly increased the MBP content (P < 0.001) compared to the ACR-administered rats (Fig. 7).

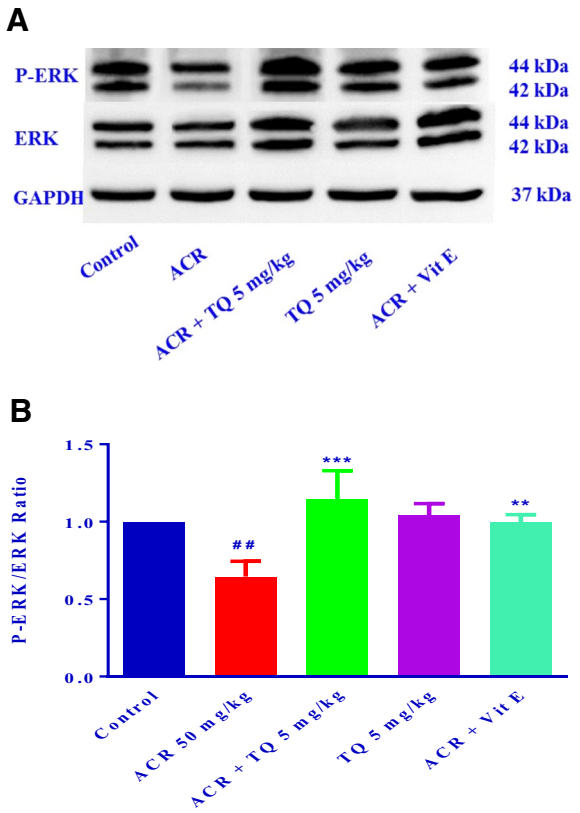

Fig. 4 Effect of TQ (5 mg/kg) and ACR (50 mg/kg, i.p.) given alone or concurrently, on the ratio of P-ERK/ERK in the sciatic nerve of rats through western blotting analysis. **a** The blots and **b** the bars exhibiting the densitometry analysis of western blots for P-ERK and ERK proteins. Data were analyzed by one-way ANOVA following Tukey's for multiple comparisons. ##P < 0.01 vs. control, ***P < 0.001 and **P < 0.01 vs. ACR-administered rats. TQ: thymoquinone, ACR: acrylamide, Vit E: Vitamin E

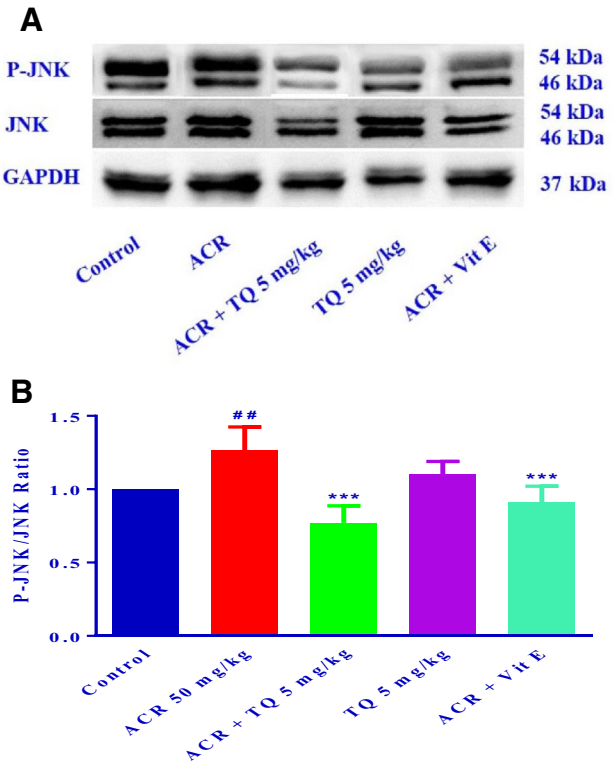

Fig. 5 Effect of TQ (5 mg/kg) and ACR (50 mg/kg) given alone or concurrently, on the ratio of P-JNK/JNK in the sciatic nerve of rats through western blotting analysis. **a** The blots and **b** The bars exhibiting the densitometry analysis of western blots for P-JNK and JNK proteins. Data were analyzed by one-way ANOVA following Tukey's for multiple comparisons. ##P < 0.01 vs. control, ***P < 0.001 vs. ACR-administered rats. TQ: Thymoquinone, ACR: Acrylamide, Vit E: Vitamin E

Effect of TQ and ACR on Apoptotic Factors (Bax/Bcl-2, Caspase 3 and Caspase 9)

To measure the expression of Bax, Bcl-2, caspase 3 and caspase 9 in the rats' sciatic nerve, western blotting test was done. According to Fig. 8b, the levels of Bax protein increased in the ACR-treated rats whereas no meaningful alteration in the levels of Bcl-2 protein was detected; consequently, the Bax/Bcl-2 ratio significantly increased (P < 0.01) comparing to control group. On the other hand, co-administration of TQ 5 mg/kg and ACR 50 mg/kg meaningfully declined the Bax/Bcl-2 ratio compared to ACR-treated rats (P < 0.001). Moreover, Vitamin E 200 mg/kg considerably decreased the Bax/Bcl-2 ratio (P < 0.01) compared to the ACR-administered rats.

Figure 8c shows that the levels of cleaved caspase 3 protein elevated in the ACR-treated rats (P < 0.05) comparing to control group. In contrast, co-administration of TQ 5 mg/kg and ACR 50 mg/kg profoundly reduced the cleaved caspase 3 protein levels compared to ACR-treated rats (P < 0.001). Besides, Vitamin E 200 mg/kg and ACR 50 mg/kg considerably reduced the cleaved caspase 3 protein levels (P < 0.01) compared to the ACR-administered rats. According to Fig. 8d, the levels of cleaved caspase 9 protein increased in the ACR-treated rats (P < 0.01) in comparison with control group (Fig. 8). While, co-administration of TQ 5 mg/kg with ACR 50 mg/kg significantly decreased the cleaved caspase 9 protein levels compared to ACR-treated rats (P < 0.001). In addition, Vitamin E 200 mg/kg and ACR 50 mg/kg substantially decreased the cleaved caspase 9 protein levels (P < 0.001) compared to the ACR-administered rats.

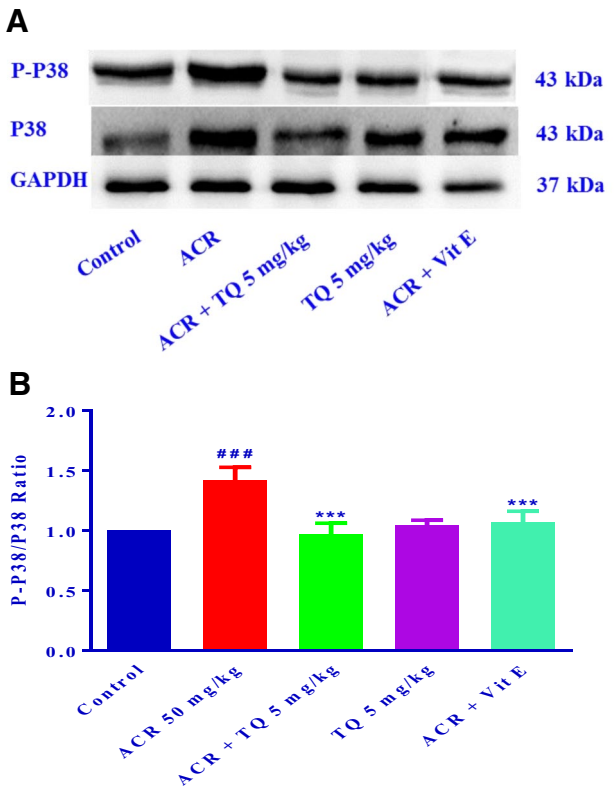

Fig. 6 Effects of TQ (5 mg/kg) and ACR (50 mg/kg) given alone or concurrently, on the ratio of P-P38/P38 in the sciatic nerve of rats through western blotting analysis. **a** The blots and **b** The bars exhibiting the densitometry analysis of western blots for P-P38 and P38 proteins. Data were analyzed by one-way ANOVA following Tukey's for multiple comparisons. ###$P<0.001$ vs. control, ***$P<0.001$ vs. ACR-administered rats. TQ: Thymoquinone, ACR: Acrylamide, Vit E: Vitamin E

Discussion

The current study investigated the effects of TQ in ACR-induced peripheral neurotoxicity via MAP kinase and apoptosis pathways.

ACR, as a well-known toxicant for the nervous system in humans and animals, leads to a neurotoxic syndrome with specific symptoms including ataxia, skeletal muscle weakness and weight loss [23]. Accordingly, in this study, rats treated with ACR (50 mg/kg, i.p., for 11 days), showed paralysis of forelimbs and hind limbs. Severely-intoxicated rats dragged their feet along the open field floor. These effects of ACR were in accordance with our previous and other studies [26, 29, 65]. Wistar rats exposed to ACR (50 mg/kg, i.p., for 11 days) exhibited behavioral index changes and body weight loss [26]. On the other hand, rats receiving TQ (5 and 10 mg/kg, i.p., for 11 days) concurrently with ACR, showed less or no signs of paralysis in their limbs. The protective effect of TQ could be due to its antioxidant properties [66].

It has been demonstrated that exposure to ACR can decrease GSH content and increase MDA levels which results in elevation of ROS [29, 67]. It has also been proven that ACR-induced oxidative stress can lead to tissue damage including sciatic nerve [10]. We showed before that MDA levels were increased in cerebral cortex after ACR administration to rats while TQ treatment remarkably decreased lipid peroxidation [45]. *N. sativa* and TQ have been shown to possess anti-oxidative properties and prevent lipid peroxidation in several studies [33, 68]. In a rat model of ischemia–reperfusion injury, *N. sativa* seeds oil and TQ, showed an overall protective effect against lipid peroxidation [33]. In the present study, rise in MDA levels and decline in GSH content caused by ACR were observed in rat sciatic nerve. Increase in ROS production can lead to apoptosis [69]. In this regard, Prasad et al. demonstrated that ACR was able to increase markers of oxidative stress such as ROS, MDA and nitric oxide in sciatic nerve, cortex and cerebellum. These alterations were counteracted by eugenol and isoeugenol [70]. TQ at the dose of 5 and 10 mg/kg was effectively able to reduce the elevated levels of MDA and increase the reduced levels of GSH induced by ACR. However, TQ at the dose of 2.5 mg/kg was not effective in protecting against ACR neurotoxicity. TQ showed its anti-apoptotic properties via preventing oxidative stress, because this phytochemical compound was able to reverse all these alterations.

In addition, according to the gait score, MDA and GSH results, we chose the most effective dose of TQ (5 mg/kg) among different doses (2.5, 5 and 10 mg/kg) for western blotting test.

In general, MAP kinase signaling pathway includes ERK, JNK, and P38 signaling proteins which contribute to the pathology of several neurodegenerative conditions [71]. Increase in ROS can result in activation of the P38 and JNK MAP kinase signaling pathway [72]. ACR has been shown to inactivate ERK via increasing caspase 3 levels [22]. JNK activation, in turn, increases the activity of caspase 3 which eventually leads to apoptosis [19]. In the current study we showed that ACR could decrease the P-ERK/ERK ratio and significantly increase the P-P38/P38 and P-JNK/JNK ratios in rat sciatic nerve, as compared to control group. These results were in consistent with the results of Pan et al. in which they demonstrated that phosphorylation of JNK and P38 were considerably increased after ACR exposure to PC12 cell line, signifying that JNK and P38 proteins were activated by this monomer. While phosphorylation of ERK substantially decreased after ACR exposure [19]. On the other hand, TQ (5 mg/kg) was able to increase the P-ERK/ERK ratio and significantly decrease the P-P38/P38 and P-JNK/JNK ratios comparing to ACR-treated rats, suggesting that this phytochemical compound could prevent apoptosis. In this regard, TQ has been demonstrated to be

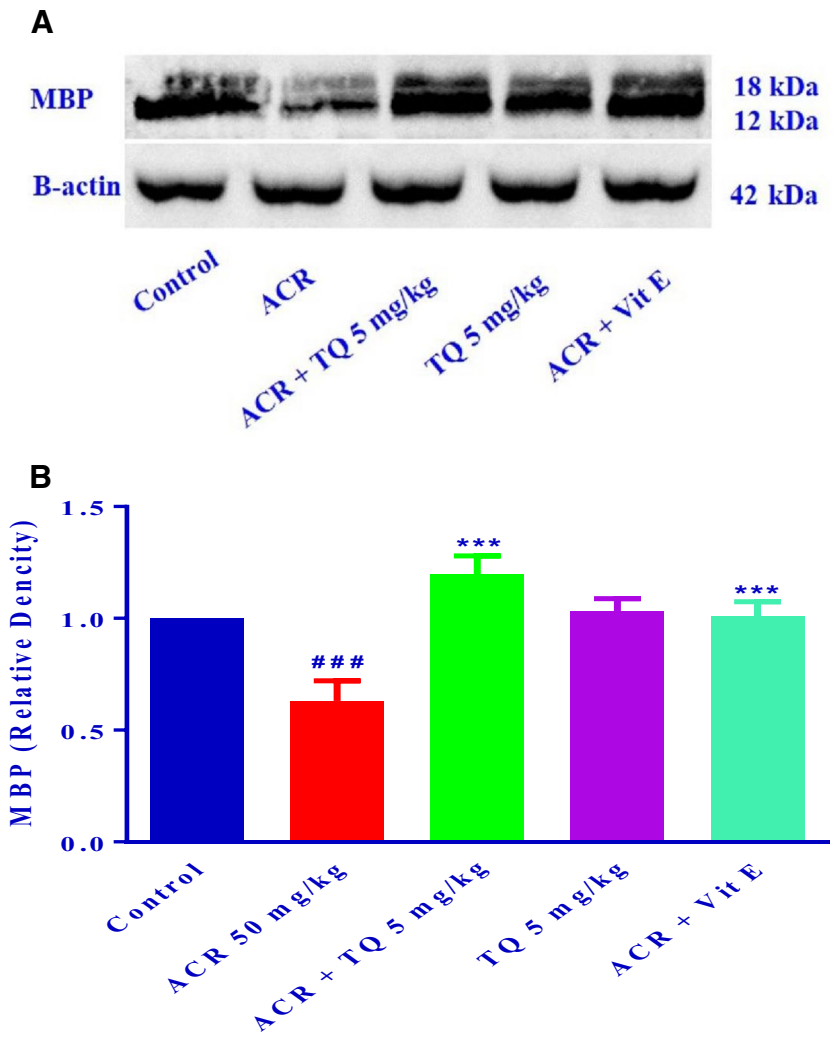

Fig. 7 Effects of TQ (5 mg/kg) and ACR (50 mg/kg) given alone or concurrently, on the MBP content in the sciatic nerve of rats through western blotting analysis. **a** The blots and **b** The bars exhibiting the densitometry analysis of western blots for MBP protein. Data were analyzed by one-way ANOVA following Tukey's for multiple comparisons. ###P < 0.001 vs. control, ***P < 0.001 vs. ACR-administered rats. TQ: Thymoquinone, ACR: Acrylamide, Vit E: Vitamin E

efficacious against neurodegenerative diseases. It protected SH-SY5Y cells against H_2O_2 by modulating the expression of antioxidant-related genes including superoxide dismutase 1, superoxide dismutase 2 and catalase, and signaling genes including P53, AKT1, ERK1/2, P38 MAPK, JNK, and NF-κB [56].

Bax and Bcl-2 proteins are the members of the Bcl-2 family which contradict each other. The elevation in the pro-apoptotic protein, Bax, shows an increase in the tissue apoptosis. Also the elevation in the anti-apoptosis protein, Bcl-2 protein, shows anti-apoptotic activity. Thus, alterations in Bax and Bcl-2 proteins indicate the apoptosis level of tissue cells [73]. Activation of caspases is also an important incident of cell apoptosis [74]. At the initiation and execution phases of apoptosis, different caspases might be activated. Caspase 9 is the upstream initiator while caspase 3 is one of the downstream effectors which play the principal role in the initiation of apoptosis [75]. Previous studies proposed that ACR caused elevation in Bax/Bcl-2 protein expression ratio, and caspases 3 and 9 levels [76, 77]. The results of the current study indicated that Bax/Bcl-2 protein expression ratio, and caspases 3 and 9 levels in the sciatic nerve of ACR-treated rats increased as compared to the control group. TQ at the dose of 5 mg/kg was able to reverse these alterations. Hence, TQ (5 mg/kg) could prevent apoptosis induced by ACR via reducing the Bax/Bcl-2 ratio, and caspases 3 and 9 levels. These findings were in consistent with our other study in which we proved that TQ was able to decrease the increased levels of caspases 3 and 9 and Bax/Bcl-2 protein expression ratio [47]. Therefore, TQ can afford neuroprotection, at least in part, due to its anti-apoptotic properties.

MBPs help the myelin sheath be stabilized via forming complexes with negatively-charged lipids. They are hydrophilic proteins located on myelin membranes [78]. The data obtained from different experiments have demonstrated that ACR led to reduction in MBP content [79, 80]. In this matter, our results indicated that ACR at the dose of 50 mg/kg significantly decreased the content of MBP in sciatic nerve while TQ (5 mg/kg) elevated MBP content. These results

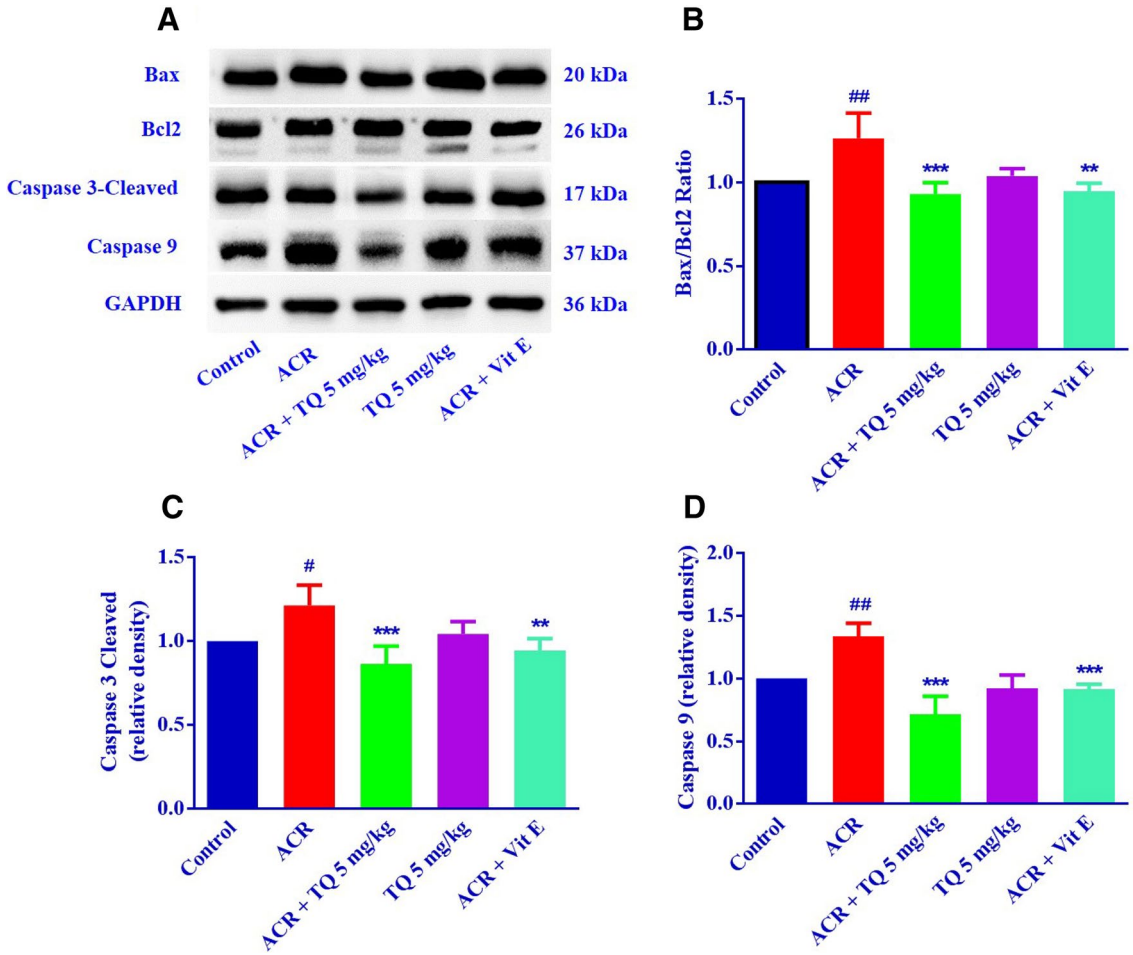

Fig. 8 Effects of TQ (5 mg/kg) and ACR (50 mg/kg) given alone or concurrently, on apoptotic factors in the sciatic nerve of rats through western blotting analysis. **a** The blots and **b, c** and **d** the bars exhibiting the densitometry analysis of western blots for Bax/Bcl-2 ratio, caspase 3 and caspase 9 proteins, consecutively. Data were analyzed by one-way ANOVA following Tukey's for multiple comparisons. #P < 0.05 and ##P < 0.01 vs. control, **P < 0.01 and ***P < 0.001 vs. ACR-administered rats. TQ: Thymoquinone, ACR: Acrylamide, Vit E: Vitamin E

were in agreement with the results of Elgholam et al. in which ACR treatment led to decreased content of MBP and rosemary aqueous extract increased the level of this protein [80].

In the present study, as positive control, we used vitamin E since it has been demonstrated to serve as a neuroprotective by exerting antioxidant activity against ACR-induced neurotoxicity [28, 29]. The data obtained from the present study showed no difference between TQ 5 mg/kg and vitamin E in preventing oxidative stress in sciatic nerve.

Conclusion

In conclusion, we showed that MAP kinase and apoptosis signaling pathways were involved in ACR-induced neurotoxicity in rat sciatic nerve. ACR induced apoptosis, characterized by increase in MDA levels, Bax/Bcl-2 ratio, caspase 3 and caspase 9 levels, and P-P38/P38 and P-JNK/JNK ratios, and decrease in GSH content and P-ERK/ERK ratio (Fig. 9). Consequently, TQ showed its protective effects against ACR-induced neurotoxicity in the sciatic nerve of rats by reversing ACR-induced alterations in these molecules and proteins.

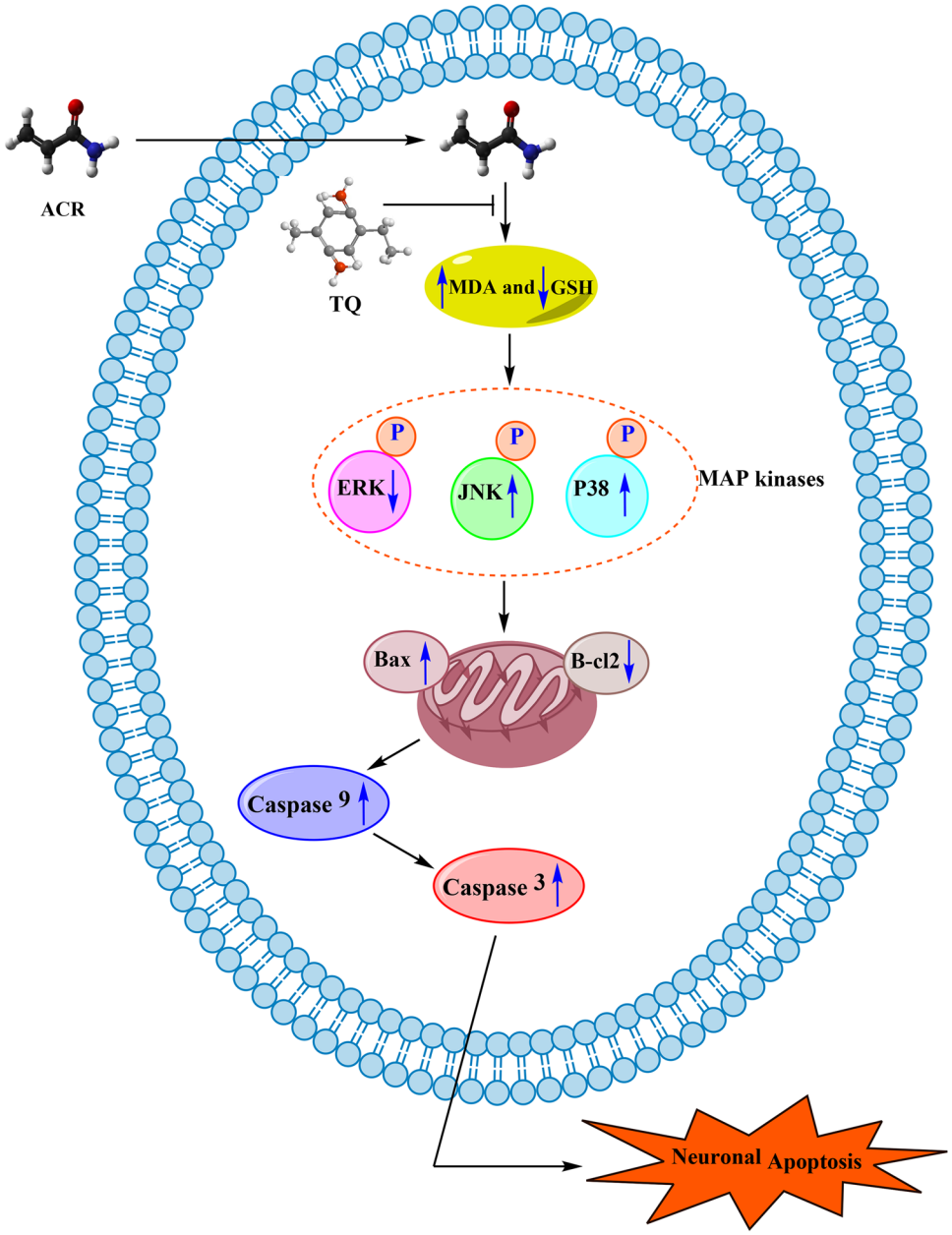

Fig. 9 Acrylamide mechanism in inducing neuronal apoptosis and the role of thymoquinone in preventing neuronal apoptosis

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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