

Original Research Article

Ameliorative Potential of Morin in Streptozotocin-Induced Neuropathic Pain in Rats

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Abstract

Purpose: To investigate the protective effect of morin, a naturally occurring bioflavonoid of Moraceae family, in experimentally-induced diabetic neuropathy (DN) in rats.

Methods: Diabetes was induced by a single injection (65 mg/kg, ip) of streptozotocin (STZ). Morin (15 and 30 mg/kg/day) oral treatment was started 3 weeks after diabetes induction and continued for 5 consecutive weeks. Pain threshold behavior tests were performed at the end of the treatment. In sciatic nerve, inflammatory cytokines (TNF- α , IL-1 β , IL-6), nerve growth factor (NGF) and insulin growth factor (IGF-1) were determined using ELISA kits, while thiobarbituric acid reactive substances (TBARS), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) levels were assessed.

Results: Diabetic animals showed apparent decreased paw-withdrawal (39 %, $p < 0.05$) and tail-flick (31 %, $p < 0.05$) latency as compared with control group. All the measured biomarkers were altered ($p < 0.05$ to 0.001) in diabetic rats compared with control non-diabetic animals. Morin treatment attenuated hyperalgesia and analgesia ($p < 0.05$) respectively. Morin treatment of diabetic rats at both doses significantly decreased the levels of cytokines ($p < 0.01$), glucose ($p < 0.01$) and TBARS ($p < 0.001$), but increased NGF ($p < 0.01$), IGF-1 ($p < 0.01$) and GSH ($p < 0.01$) levels in sciatic nerves compared to untreated diabetic animals. Inhibited activities (U/mg protein) of SOD (1.08 ± 0.16) and CAT (2.77 ± 0.36) in sciatic nerve of diabetic rats also found corrections (2.09 ± 0.11 , $p < 0.01$) and (4.53 ± 0.57 , $p < 0.01$) after morin (30 mg/kg/day) treatment, compared with untreated diabetic animals.

Conclusion: These findings demonstrate the protective effect of morin mediated through reduction of oxidative stress and inflammatory process, and suggest the therapeutic potential of morin in the attenuation of diabetic neuropathy.

Keywords: Morin, Diabetes, Neuropathy pain, Oxidative stress, Anti-inflammatory

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INTRODUCTION

Neuropathic pain is a form of chronic pain induced by damage or abnormal function of central or peripheral nervous system [1] and it also associated with diabetes metabolic syndromes. It usually results in sensory abnormalities such as burning sensations, hyperalgesia, allodynia and dysesthesia, leading

to alteration in patient's quality of life as well patient's emotional well-being [2]. DN is estimated to affect about 15% - 25% in type-1 and 30% - 40% in type-2 diabetic patients, causing disabilities and a high mortality rate [3]. The pathophysiological mechanisms of DN include a complex network of unified vascular [4]; metabolic [5] and neurotropic [6] defects, which end with electrophysiological discrepancies,

abnormal sensory perception and progressive damage and loss of unmyelinated and myelinated nerve fibers [7]. Oxidative stress can be also one of the caustic mechanisms associated with DN and evidence about its possible role in the development of diabetic complications are now well-documented [8]. Hyperglycemia was reported to induce lipid peroxidation and generation of reactive oxygen species (ROS) in sciatic nerves [9]. Moreover, oxidative stress was suggested to provoke sciatic nerve dysfunction and reduced endoneurial blood flow in diabetic rats [10,11].

Morin is well-known naturally occurring bioflavonoids. It can be isolated from herbs and fruits belonging to Moraceae family such as onion, seed weeds, mill (*Prunusdulcis*), fig (*Chlorophoratorinctoria*), almond (*P. guajava* L), red wine, and Osage orange [12]. Experimentally morin has showed different pharmacological potentials including antioxidant [13,14] and anti-inflammatory [15]. Recently, morin was found to have protective effects against diabetic-induced hepatotoxicity, nephropathy [16] and osteopenia [17] in rats. Limited data are available on the possible beneficial effects of morin on diabetic-induced neuropathy. Using morin as a natural antioxidant might show effective, economical and safe option for DN patients. Thus, the present study was designed to evaluate the potential preventive role of morin on experimental DN model in male Wistar albino rats.

EXPERIMENTAL

Animals and experimental models

Male Wistar albino rats, approximately 3 months old, weighing 260 – 285 g were received from Experimental Animal Care Center (King Saud University, Riyadh, Saudi Arabia). They were maintained under controlled conditions of temperature (22 ± 1 °C), humidity (50-55 %), and light (12 h light/dark cycles) and were provided with Purina chow (Manufactured by Grain Silos & Flour Mills Organization, Riyadh, Saudi Arabia) and drinking water *ad libitum*. A single intraperitoneal injection of STZ (Sigma Chemicals, USA) was used at a dose of 65 mg/kg body weight to induce diabetes in overnight fasted rats. STZ was freshly dissolved in 0.1 M citrate buffer, pH 4.5 [18]. Control rats received an equal volume of citrate buffer. Diabetes was confirmed 72 h later by considering animals with fasting blood glucose values more than 250 mg/dL as diabetic [18]. Animals were divided into five groups (n = 6) as following; 1) Control (C), 2) Morin (30 mg/kg/day, gavage), 3) Diabetic (D) 4) Morin (15 mg/kg/day, gavage)

treated to diabetic rats (D + M15) and 5) Morin (30 mg/kg/day, gavage) treated to diabetic rats (D + M30). Morin (SIGMA Chemicals, USA) was suspended in 0.5 % CMC (vehicle) and treatment started (0.5 ml/100 g body weight) orally by gavage after three weeks of diabetic induction and continued for five consecutive weeks. The animals in control and diabetic groups received same volume of 0.5 % CMC as vehicle. Behavioral assessments were under taken after 24 h of last treatment. All procedures including euthanasia procedure were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996) and the Ethical Guidelines and approval EGA-223-14 of the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia).

Evaluation of mechanical hyperalgesia (Randall & Selitto method)

Mean right and left paw pressure thresholds were determined by using the paw pressure analgesia meter (MK-20D Analgesia meter, Muromachi Kikai Co Ltd, Japan) [19]. Pressure linear increase rate fixed as 10 mmHg/sec and the cut-off pressure was at 500 mmHg to avoid tissue injury. The pressure was applied to the center of the hind paw. When the animals displayed pain by withdrawal of the paw, the applied paw pressure was recorded by an analgesia meter and expressed as mmHg. Three tests separated by at least 10 min were performed for each animal, and the mean value was recorded.

Tail flick test

Using a tail flick apparatus (Tail Flick model DS 20 Sorrel Apelex, France), acute nociception was induced according to the method described by Sugimoto *et al* [20]. In Brief, the tail flick latency of the restrained animal was measured by focusing the intensity controlled beam of light on the distal last 2 cm of the animal's tail. Thereafter, the time taken to remove the tail from the noxious thermal stimulus was recorded. For each animal, 2 to 3 recordings were made at an interval of 15 min and the mean value was used for statistical analysis.

Sample collection and tissue preparation

At end of the treatment and behavioral assessments, animals were fasted overnight and blood samples were collected through cardiac puncture under deep anesthesia and then

sacrificed. Sciatic nerves were rapidly removed and dipped in liquid nitrogen for a minute and kept in deep freezer at - 80 °C till analysis [18]. Blood samples were centrifuged at 3,000 rpm for 10 min and serum samples were stored at - 20 °C till analysis. Sciatic nerves were homogenized in a cold 50 mM phosphate buffered saline (pH 7.4) by a glass homogenizer (Omni International, Kennesaw, GA, USA). The homogenate was centrifuged at 1000 rpm for 10 min at 4 °C to isolate nuclei and unbroken cells. After discarding the pellets, a portion of supernatant was centrifuged again at 12000 rpm for 20 min to obtain post-mitochondrial supernatant.

Determination of glucose and insulin

Serum glucose levels were measured by using the commercially available kit (RANDOX Laboratories Ltd., UK) while insulin serum level was assayed using ELISA kit (Bio-Source, Europe S.A., Belgium).

Determination of NGF and IGF-1 and inflammatory cytokines

Sciatic nerve levels of NGF, IGF-1, TNF- α , IL-1 β and IL-6 were assessed and quantified by using ELISA technique (R & D systems, USA) according to the manufacturer's instructions.

Evaluation of thiobarbituric acid reactive substances (TBARS)

The TBARS levels were estimated in homogenate of sciatic nerve by using TBARS assay kit (ZeptoMetrix, USA). Briefly, 2.5 ml of the kit provided reaction buffer was mixed with 100 μ l of the homogenate. The mixture was then heated at 95 °C for 60 min. After cooling and centrifugation, the absorbance of the supernatant was measured at 532 nm using a spectrophotometer.

Evaluation of reduced glutathione (GSH)

The method described by Sedlak and Lindsay [21] was used to estimate GSH levels. Briefly, homogenate was mixed with 0.2 M Tris buffer, pH 8.2 and 0.1 mL of 0.01 M Ellman's reagent, [5,5'-dithiobis-(2-nitro-benzoic acid)] (DTNB). Samples were then centrifuged at 3000 rpm at room temperature for 15 min. The absorbance of the clear supernatants was recorded to measure the concentration of GSH using spectrophotometer at 412 nm.

Determination of superoxide dismutase (SOD) and catalase (CAT) activities

With the aid of nitroblue tetrazolium as the indicator, SOD activity in sciatic nerve was estimated in the post-mitochondrial supernatant according to the method described by Kono [22]. Nitroblue tetrazolium reduction to blue for mazon mediated by superoxide anions, generated by the oxidation of hydroxylamine hydrochloride, was measured at 560 nm under aerobic conditions. After SOD addition, this reduction was inhibited and the extent of inhibition is taken as a measure of the activity. On the other hand, CAT activity was estimated in the post-mitochondrial supernatant by the method of Aebi [23] using H₂O₂ as substrate. In brief, H₂O₂ decomposition by CAT was monitored following the decrease in absorbance at 240 nm. Both SOD and CAT activities were expressed as units/mg protein.

Statistical analysis

Data are expressed as means \pm SEM. Statistical analysis was carried out using one-way ANOVA followed by Newman-Keuls as post hoc test. $p < 0.05$ was considered statistically significant. All statistics tests were conducted using Graph Pad Prism (version 5) software.

RESULTS

Fasting glucose levels markedly ($p < 0.001$) increased while the insulin values decreased in STZ-induced diabetic rats compared to controls. Morin treatment with low dose (15 mg/kg) and high dose (30 mg/kg) to diabetic rats significantly decreased the glucose levels $p < 0.05$ and $p < 0.01$ respectively while compared to untreated diabetic animals. Insulin levels were significantly ($p < 0.05$) increased in diabetic rats after morin (30 mg/kg/day) treatment for five weeks (Table 1).

Levels of NGF and IGF-1 in sciatic nerves of the diabetic animals were significantly ($p < 0.05$) reduced compared to control rats. In morin higher dose (30 mg/kg/day) treated diabetic animals, the NGF and IGF-1 values were significantly ($p < 0.05$) elevated compared to untreated diabetic rats. Experimental diabetes induction resulted in a significant ($p < 0.001$) increase in sciatic nerve TBARS and a decrease ($p < 0.01$) in the GSH levels. The enzymatic activities of SOD and CAT were significantly ($p < 0.01$) inhibited in diabetic rats when compared to control animals. Morin treatment with higher dose to diabetic rats markedly decreased the elevated

TBARS levels in sciatic nerve compared to untreated diabetic animals. The inhibited GSH levels in diabetic rats was significantly attenuated with the morin treatments (15 and 30 mg/kg/day) $p < 0.05$ and $p < 0.01$ respectively. The inhibited enzymatic activities of SOD and CAT in sciatic nerve of diabetic rats also revealed significant ($p < 0.05$) enhancement by the five weeks morin (30 mg/kg/day) treatment (Table 1) in comparison with diabetic untreated group.

As indicated by the paw pressure analgesia and tail flick tests, the pain threshold of the diabetic rats was significantly ($p < 0.05$) decreased as compared to control animals. Administration of morin to diabetic animals for 5 consecutive weeks with the higher dose (30 mg/kg/day) significantly ($p < 0.05$) improved the pain thresholds (Fig 1).

The concentrations of pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6 in sciatic nerve were significantly ($p < 0.01$) increased in diabetic rats compared to control animals. All these biomarkers were markedly ($p < 0.05$) inhibited in sciatic nerve of morin (30 mg/kg/day) treated

diabetic rats when compared to untreated diabetic animals. However, morin treatment with higher dose to normal rats for five consecutive weeks could not alter the normal values of these cytokines (Fig 2).

DISCUSSION

Experimentally-induced diabetes by STZ in rodents is a well-known animal model to investigate metabolic and pharmacological changes associated with diabetes [24,25]. Hyperglycemia is suggested to be implicated in the development of diverse diabetic complications, such as retinopathy, nephropathy, neuropathy, foot ulcers, diabetic osteopenia and micro- and macro-vascular complications [26]. DN and the associated neuropathic pain is one of the most common diabetic complications that approximately occur in 50 % of the diabetic patients. In the current investigation, male rats was employed as an experimental model due to male rodents' lack of hormonal fluctuation, which makes developing metabolic disease less difficult than in female rats. Moreover, the STZ model of

Table 1: Effect of morin on serum glucose and insulin levels as well as on NGF, IGF-1, TBARS, GSH, SOD and CAT activities in sciatic nerve of normal and diabetic rats

Parameter	Control rats (vehicle)	Morin (30 mg/kg) treated to normal rats	Diabetic rats (vehicle)	Morin (15 mg/kg) treated to diabetic rats	Morin (30 mg/kg) treated to diabetic rats
Glucose (mg/dl)	90.75 \pm 7.55	87.56 \pm 3.47	415.59 \pm 19.62 ^{***a}	376.12 \pm 17.19 ^b	306.29 \pm 29.18 ^b
Insulin (ng/ml)	23.43 \pm 1.74	24.34 \pm 1.21	9.21 \pm 1.57 ^{***a}	11.96 \pm 0.78	14.16 \pm 0.85 ^b
NGF (pg/mg protein)	98.71 \pm 13.18	98.89 \pm 7.36	54.57 \pm 10.93 ^a	76.94 \pm 6.76	90.56 \pm 7.87 ^a
IGF-1 (pg/mg protein)	440.80 \pm 73.89	439.80 \pm 35.04	204.50 \pm 20.55 ^a	326.30 \pm 47.51	410.50 \pm 44.57 ^b
TBARS (nM/mg protein)	12.61 \pm 2.74	13.14 \pm 1.56	37.00 \pm 6.18 ^{***a}	28.19 \pm 2.21	22.78 \pm 1.54 ^b
GSH (nM/mg protein)	12.55 \pm 2.13	12.07 \pm 1.39	4.69 \pm 0.56 ^{***a}	9.47 \pm 0.86 ^b	10.47 \pm 1.78 ^b
SOD (U/mg protein)	2.38 \pm 0.32	2.34 \pm 0.35	1.08 \pm 0.16 ^{**a}	1.76 \pm 0.25	2.09 \pm 0.11 ^b
CAT (U/mg protein)	5.67 \pm 0.61	5.65 \pm 0.41	2.77 \pm 0.36 ^{**a}	3.61 \pm 0.37	4.53 \pm 0.57 ^b

Values are expressed as Mean \pm SEM, n = 6. The statistical significance was considered as ^{*} $p < 0.05$, ^{**} $p < 0.01$ and ^{***} $p < 0.001$ in comparison with ^(a) control group (C) and ^(b) diabetic morin treated, compared with diabetic untreated group

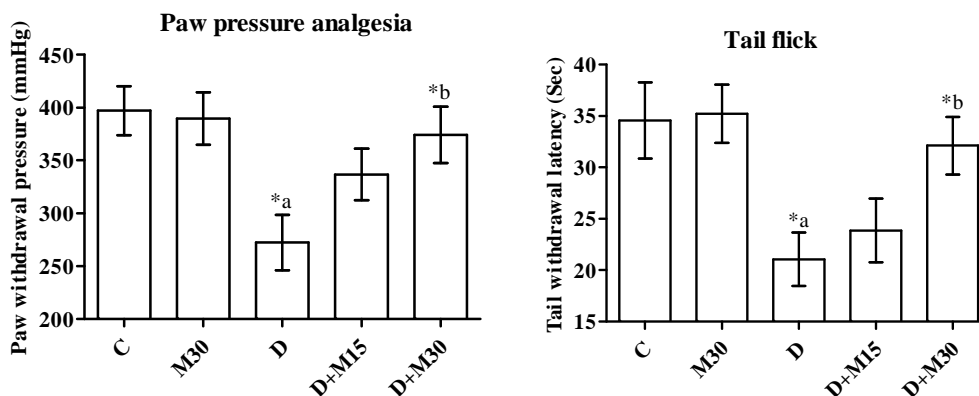


Fig 1: Effects of morin on mechanical and thermal analgesia of diabetic and non-diabetic animals; values are expressed as mean \pm SEM, n = 6; ^{*} $p < 0.05$; ^{**} $p < 0.01$ as compared with ^(a) control group (C) and ^(b) diabetic morin treated, compared with diabetic untreated group

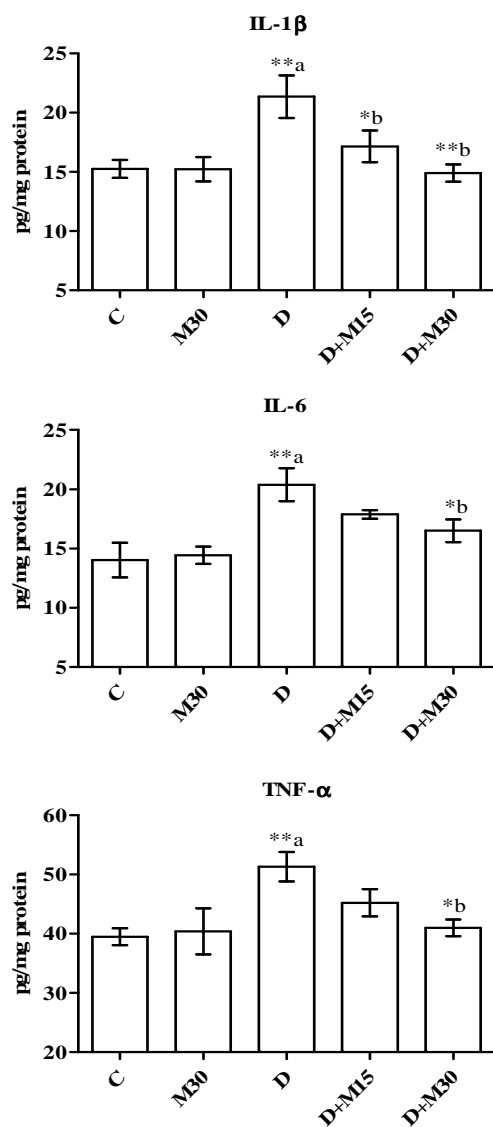


Fig. 2: Effects of morin on TNF- α , IL-1 β and IL-6 levels in sciatic nerve of diabetic and non-diabetic animals; values are expressed as Mean \pm SEM, n = 6. One-way ANOVA followed by Student-Newman-Keuls multiple comparisons test were applied. * $p < 0.05$; ** $p < 0.01$ as compared with ^(a) control group (C) and ^(b) diabetic morin treated, compared with diabetic untreated group

DN used in the current and several other experimental investigations, is standardized against numerous reference anti-diabetic drugs such as glibenclamide and sitagliptin [27,28] as well as antineuropathic pain medications such as pregabalin [29].

In the present study, diabetic animals showed a significant delay in both paw withdrawal and tail withdrawal latencies than that observed in control animals. These findings indicated the development of mechanical and thermal

hyperalgesia which are in agreement with earlier investigators [25,30]. It has well-documented that NGF plays a vital role in both survival and maintenance of sympathetic and sensory nerves. It shows an important neuro-protective function and causes axonal growth. Pathological condition that alters levels of NGF can lead neurons to loss of their function and death [31]. IGF-1, a structurally similar hormone to insulin with anabolic effects, plays also an important function in cellular growth and proliferation. It is also a potent inhibitor of programmed cell death. The growth-promoting properties of IGF-1 are reported in almost all biological tissues, especially liver, cartilage, bone, skeletal muscle, lungs, nerves, skin, and kidney. IGF-1 can also control the growth and development of nerve cells as well as synthesis of DNA [32]. In the present study, levels of both NGF and IGF-1 were markedly decreased in the sciatic nerves of the diabetic animals indicating loss of neuro-integrity and increased rate of nerve cells apoptosis.

Oxidative stress is a major contributor in the development of DN [10,33-34] and its association is mainly due to autoxidation of mono-saccharides and proteins [35]. Findings of the present study showed a remarkable elevated level of lipid peroxidation biomarker TBARS in sciatic nerve of diabetic animals. The potent endogenous antioxidant, GSH, is considered an early defense against free radicals. In agreement with earlier studies, GSH levels in the sciatic nerve of diabetic rats was significantly inhibited in the present study [10]. Moreover endogenous defense mechanisms against free radicals which include the antioxidant enzymes (SOD and CAT) are considered as major antioxidant enzymes that are involved mainly in antioxidative process. However, SOD protect biological tissues from highly reactive superoxide anions (O_2^-) by converting them to H_2O_2 and hyperglycemia is known to reduce the activity of SOD in sciatic nerve of animals, which might involve non-enzymatic glycosylation [36].

These reported data are in agreement with results from the present study wherein decreased SOD activity was found in diabetic rats isolated nerves [37]. Whereas CAT, plays a vital role in the catalytic decomposition of harmful H_2O_2 to O_2 and H_2O . Decreasing the activity of CAT in diabetes reduces the cellular protection and makes tissues more subjected to free radicals attack. Over results also showed association between DN and oxidative stress by inhibiting the enzymatic activities in diabetic rats

compared to normal. Thus, the concurrent decrease in endogenous antioxidant defenses system makes sciatic nerves more vulnerable to hyperglycemia-induced oxidative stress. Current findings clearly showing the link between pro-inflammatory biomarkers such as TNF- α , IL-1 β and IL-6 and the development of DN by increasing their levels compared to controls. It has been documented that, these inflammatory cytokines are involved in systemic inflammation and in stimulation of acute phase reaction and the elevated levels of those inflammatory mediators are found to be a consequence of hyperglycemia and insulin resistance in diabetes [38,39].

Several *in vivo* and *in vitro* studies have reported the potent antioxidant and free-radical scavenging activities of flavonoids. Such properties are the main reasons for their well-recognized health benefits. Morin is considered as an effective antioxidant that has been used as a herbal medicine and food preservative and it has been reported to have several beneficial activities in different biological tissues including antioxidant and/or free radical scavenging actions [40-42]. Our present results further strengthened the beneficial effects of morin as it showed protection against STZ-induced DN in rats. However, these effects may be attributed to its antioxidative, anti-inflammatory and anti-diabetic properties. Indicators for nerve conduction properties are improved by the treatment of morin to diabetic rats for 5 consecutive weeks. Further justification can be presented as it improved the mechanical and thermal analgesic conditions by delaying the paw and tail withdrawal latencies in diabetic rats. Furthermore, factors that regulate nerve cells growth and development such as NGF and IGF-1 were also enhanced by morin in the present study.

It is well-known that morin has potent ameliorative properties against ROS development and also act as a free radical scavenger [43]. Nandhakumar *et al* study showed that morin can markedly inhibit mammary carcinoma associated oxidative stress by enhancing the reduced activities of the antioxidant enzymes SOD, CAT, and GPx and the increased levels of lipid peroxidation products TBARS and hydroperoxides in rats [12]. The antioxidant properties of morin are suggested to be through the hydroxyl groups present at the C-3 and C-5, besides at C-4. These hydroxyl groups are very effective in quenching free radicals developed during oxidative stress [44].

In present study, morin in both administered doses significantly inhibited GSH reduced levels, while only the higher dose restored TBARS elevated level in sciatic nerves of diabetic rats and also the activities of antioxidant enzymes SOD and CAT were bring back to normal in higher taken dose of morin.

The reduction of oxidative stress biomarkers may be also due to inhibition of hyperglycemia as it has been recognized to trigger oxidative stress and to be involved in the pathogenesis of DN. Another reason for the beneficial effects of morin is the reported anti-inflammatory properties, which were demonstrated in earlier *in vitro* and *in vivo* studies [45,47]. Recently Chen *et al* reported that the morin treatments suppress the production of several pro-inflammatory biomarkers expressions such as NO, PGE-2, iNOS and COX-2 [48]. Our findings showed similar results where the higher dose of morin (30 mg/kg) produced significant inhibition against the elevated pro-inflammatory cytokines such as TNF- α , IL-6 and IL-1 β in experimentally-induced diabetic rats.

CONCLUSION

The results obtained from the present study reveal that morin ameliorates hyperglycemia-induced mechanical or thermal hyperalgesia and lowers neuropathic pain by reducing oxidative stress in the nerve of diabetic rats by virtue of its antioxidative and anti-inflammatory properties. Morphological assessments also show that the damage caused by STZ to the sciatic nerve is also markedly reduced by the administration of morin. Finally, these findings suggest that morin treatment might be beneficial in chronic diabetics exhibiting neuropathy.

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