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Original Article

Anti-amnesic and analgesic effects of Moringa oleifera in diabetic rats: possible hypoglycemic and antioxidant mechanisms

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Abstract

This study aimed to evaluate the effects of ethanolic extract of dried Moringa oleifera leaves (MO) administered intragastrically at different doses for 30 days on diabetes-induced amnesia and hyperalgesia in rats, as well as to investigate the possible underlying mechanisms. Rats received MO extract (100, 200, and 400 mg/kg) or vehicle starting at the onset of hyperglycemia and continuing for 30 days. Passive avoidance learning (PAL) and memory tasks were used to assess memory, while formalin test was applied to analyze chemical hyperalgesia. Diabetes led to impairments in both phases of the PAL and memory test. MO (200 and 400 mg/kg) protected diabetic rats from learning and memory impairment and enhanced cognition of control animals. In the formalin test, MO at 200 and 400 ng/kg reversed chemical hyperalgesia of diabetic rats, while showing analgesic effects in healthy rats. The most significant hypoglycemic and antioxidant effects were shown with MO 400 mg/kg in diabetic animals. MO (200 and 400mg/kg) enhanced cognitive function and induced analgesia in diabetic rats, possibly by hypoglycemic and antioxidant mechanisms. Therefore, it may be a promising source for management of diabetic neurological complications that deserves notice and further studies.

Keywords: Antioxidant, Acquisition, Hyperalgesia, Memory, *Moringa oleifera*, Rats.

1. Introduction

Moringa oleifera Lam, commonly referred to as the 'wonder tree' or 'natural nutrition of the tropics,' thrives in tropical and subtropical regions [1]. *M. oleifera* (MO), being abundant in nutrients such as minerals, proteins, and antioxidants, serves as an excellent choice for human and animal nutrition [3], particularly in both impoverished and developed nations. This herb has been widely utilized for both prophylactic and therapeutic reasons in traditional medicine [4,5]. Examples of medical capabilities encompass antibacterial, anticancer, antiulcer, hypotensive, anti-inflammatory, antispasmodic, hypolipidemic, and antidiabetic characteristics [6-9].

There is significant interest in herbal remedies for the treatment of diabetes and its associated neurological problems [9-11]. Cognitive impairments and hyperalgesia, an increased sensitivity to painful stimuli, are regarded as primary neurological consequences of diabetes [12]. Streptozotocin (STZ)-induced diabetes is broadly and commonly used for investigating diabetes in animal studies. Diabetic mice have deficiencies in passive avoidance learning (PAL) and memory, along with chemical hyperalgesia, evidenced by heightened flinching behaviors following formalin injection into the paw [13-15].

Given the aforementioned findings, MO may be a good alternative for the management of diabetes complications. This research aimed to assess the effects of orally administered MO at varying doses on cognitive impairment in diabetic mice through the use of PAL and memory tasks. We additionally examined the preventive impacts of MO on chemical hyperalgesia in diabetic animals by the formalin test.

2. Materials and methods

2.1. Animals

One week before the initiation of the experiments, sixty-four mature male Wistar rats (200- 250) were acclimatized to the laboratory setting, which included a 12-hour light-dark cycle and a temperature range of 20 °C. Subsequent to the acclimation phase, the rats were randomly allocated into six groups. The Guide for the Care and Use of Laboratory Animals (NIH publication 86–23; modified 1985; http://www.oacu.od.nih.gov/regs/guide/guidex.htm) was adhered to during the handling of the ani-

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mals. The University of Zabol ethical review committee granted ethical approval under license number IR.UOZ.REC.1404.001. All experiments were performed between 10:00 and 14:00, with each animal being employed just once.

2.2. Drugs

Streptozotocin (STZ) was procured from Pharmacia & Upjohn (USA), while Ketamine HCl was acquired from Rotexmedica (Trittau, Germany). Both drugs were supplied through intraperitoneal injection.

2.3. Experimental design

In this research, we used four diabetic groups and four control groups (N = 8). Administration of STZ (50 mg/kg, *i.p.*) induced diabetes in animals. If glucose levels in plasma exceeded 250 mg/dL three days after STZ injection, the rat was considered diabetic. After diabetes diagnosis, the rats were administered either a vehicle or 100, 200, and 400 mg/kg of MO extract (*i.g*), daily for four weeks. The dosages of MO employed in this investigation were established based on preliminary and previously published studies [15-17]. Cognitive and nociceptive assessments were performed at the end of the treatment period.

2.4. Plant materials and preparation

The desiccated leaves of MO were obtained and taxonomically authenticated by Dr. Dehghani from the Department of Biology at the University of Zabol. The extraction procedure adhered to the approach defined in prior studies [18]. The leaves were ground into a coarse powder utilizing an automatic mixer. The resultant powder (2.96 kg) was further macerated in 500 ml of a 50% V/V ethanol-water solution at ambient temperature (26 ± 1°C) for 48 hours, with intermittent agitation. We concentrated the filtrate at 40 °C under reduced pressure to completely evaporate the extraction solvent. The final extract produced a black soluble crude residue weighing approximately 201.45 g, equivalent to 6.61% w/w.

2.5. Passive avoidance learning (PAL) test (step through test)

The apparatus and methodology were explained in previous papers [14, 19, 20]. In summary, the device comprised two identically sized chambers, one light and one dark, each measuring 20×40×20 cm, constructed from opaque plastic walls. The floor of each room was fitted with parallel stainless-steel bars (3mm in diameter, placed 1cm apart), linked to an electric shock generator. A rectangular aperture was situated between the two rooms, which could be sealed by an opaque guillotine door.

2.5.1. Training

All animals underwent two trials to acclimate to the device. Five seconds after that, the animals were placed in the illuminated section, and the door was elevated. Upon the animal entrance into the other section, the door was secured, and it was relocated to its home cage. Thirty minutes later, the habituation process was conducted again, followed by the initial trial for acquisition. During the initial adaptation session, the latency to entrance into the dark section was defined as step-through latency, STLa. During the learning phase, the rat was subjected to a continuous current foot shock of 50Hz square wave at 1mA for 1 second when entering the dark section. The training technique was reiterated and concluded if the animal stayed in the light section for 120 uninterrupted seconds. Upon completion of the learning phase, the rat was extracted and situated in its home cage.

2.5.2. Retention test

Twenty-four hours after the training session, the retention test was conducted. Following 5 seconds after placing the animals in the lit chamber, the door was raised, and both the step-through latency (STLr) and the time spent in the dark compartment (TDC) were recorded for a duration of 300 seconds. Terminated if the animal did not enter into the dark section (getting 300 s).

2.6. Nociceptive testing

The apparatus was the same as described previously [21-23]. Thirty minutes before administering vehicle or extract, each rat received a single formalin injection (2.5%, 50 mL, *s.c.*) in hind paw. The evaluation of pain responses commenced immediately upon the formalin injection and continued for 60 minutes. The pattern of nociceptive response was biphasic, consisting of an initial acute pain phase (phase 1: 0–5 minutes), a short interval of quiescence, and a following extended tonic pain phase (phase 2: 10–60 minutes). A weighted scoring system was utilized to examine pain behaviors in this test. Nociceptive responses were subsequently quantified according to the weighted behaviors based on prior investigations [21-23].

2.7. Plasma glucose levels measurement

Upon completion of the experiment, all animals were first weighed and then euthanized under anesthesia produced by Ketamine HCl (50 mg/kg, *i.p.*). Blood samples were obtained to evaluate plasma glucose levels by a kit (Zistshimi, Tehran, Iran) and a spectrophotometer (UV3100, Shimadzu, Tokyo, Japan), as well as to analyze antioxidant properties.

2.8. Evaluation of antioxidant parameters

The level of superoxide dismutase (SOD) enzyme was analyzed based on an earlier study [24]. The rate of H_2O_2 consumption was examined spectrophotometrically at 240 nm to assess the activity of catalase (CAT) [25].

2.9. Statistical analysis

We used SPSS statistical software (version 21.5) to analyze all obtained data (expressed as mean ± S.E.M). A one-way analysis of variance (ANOVA) was employed for the data analysis. To conduct multiple comparisons among the various experimental groups, Tukey's test was utilized. We considered p-values less than 0.05 as statistically significant.

3. Results

3.1. Effects of MO administration on the PAL and memory in non-diabetic rats

Prior to the electrical shock, the STLa across various groups did not exhibit a significant difference in the initial acquisition trial ($P > 0.05$, data not shown). The trials required for acquisition exhibited differences between the 200 mg/g and 400 mg/g treated control groups (1.37 ± 0.18 and 1.25 ± 0.16, respectively) and the untreated controls (2.75 ± 0.25) ($P < 0.01$, $P < 0.01$) (Fig. 1A). In the memory

test, MO (100 mg/kg) did not significantly affect STLr ($P > 0.05$). MO at dosages of 200 mg/g and 400 mg/g resulted in increased STLr values of 148.5 ± 3.59 and 265.6 ± 12.3, respectively, compared to controls, which had a value of 119.52 ± 5.2, as shown in Figure 1B ($P < 0.05$, $P < 0.001$, respectively). Additionally, the controls treated with MO (200 and 400 mg/kg) exhibited a reduction in TDC (96.87 ± 7.9 and 98.8 ± 3.2, respectively) as shown in Figure 1C ($P < 0.05$, $P < 0.01$, respectively).

3.2. Effects of MO administration on PAL and memory in diabetic rats

The acquisition trial numbers were significantly elevated in diabetic rats ($P < 0.001$), as illustrated in Fig. 1A. In the retention test, diabetic subjects demonstrated a lower STLr (41.7 ± 4.62) and a higher TDC (208.7 ± 3.98) relative to the control group (115.62 ± 5.41, 136.25 ± 3.32, respectively) (P's < 0.001, Figs. 1B and 1C). Administration of MO (100 mg/kg) did not change the acquisition trial numbers of diabetic animals. In contrast, dosages of 200 and 400 mg/kg of MO resulted in a decrease in the trial numbers when compared to untreated diabetic animals (P's < 0.001) (Fig. 1A). Higher doses of MO in diabetic rats significantly enhanced STLr in the retention test (P's < 0.001) (Fig. 1B). The increased TDC noted in diabetic rats was reduced following MO administration (200 and 400 mg/kg) ($P < 0.01$, $P < 0.001$, respectively) (Fig. 1C). In contrast, MO at 100 mg/kg did not yield any differences in STLr and TDC in diabetic rats (P's > 0.05) (Figs. 1B and 1C).

3.3. Effects of MO administration on chemical hyperalgesia in non-diabetic animals

Figures 2A and 2B illustrate the pain scores in phase 1 and the area under the curve (AUC) of scores in phase 2 of formalin test. The administration of MO (100 mg/kg) did not affect the pain scores of control groups in either phase (all P-values > 0.05) (Figures 2A and 2B). MO (200 and 400 mg/day) decreased pain scores of phase 1 as well as the AUC of scores in phase 2 (Figs. 2A and 2B) in comparison to the untreated control rats.

3.4. Effects of MO administration on the chemical hyperalgesia in diabetic rats

In untreated diabetic animals, the scores and AU scores were significantly elevated (3.82 ± 0.23 and 146.1 ± 2.6, respectively) in comparison to control animals (3.49 ± 0.23 and 95.8 ± 2.71, respectively) (P's < 0.001, Figs. 2A and 2B). This suggests that diabetic rats exhibit significant chemical hyperalgesia. Diabetic rats administered MO at doses of 200 and 400 mg/kg exhibited reduction in nociceptive scores ($P < 0.01$, $P < 0.001$, respectively, Fig. 2A) as well as a decrease in the AUC of scores (P's < 0.001, Fig. 2B) in comparison to untreated diabetic rats. MO administration at dose of 100 mg/kg did not produce significant alterations in nociceptive scores in each phase of the test (P's > 0.05, Figs. 2A and 2B).

3.5. Effects of MO administration on plasma glucose levels and body weight

Before starting the experiment, plasma glucose levels and body weight among the animals showed no significant differences (see Table 1). No significant disparities were observed among the various groups prior to the intervention. The administration of MO (100 mg/day) to the control rats did not produce any significant changes in plasma glucose levels ($P > 0.05$). MO treatment (200 and 400 mg/kg) in control animals caused a small decrease in the levels of plasma glucose (Table 1). Both un-treated and MO 100 mg/kg –treated diabetic rats exhibited significantly elevated plasma glucose levels compared to control rats (P's < 0.001). The administration of MO at doses of 200 and 400 mg/kg to diabetic subjects led to a significant reduction in glucose levels by the end of the experiments (P's < 0.001). Additionally, plasma glucose levels did not differ significantly between diabetic animals receiving MO (200 and 400 mg/day) and untreated control animals

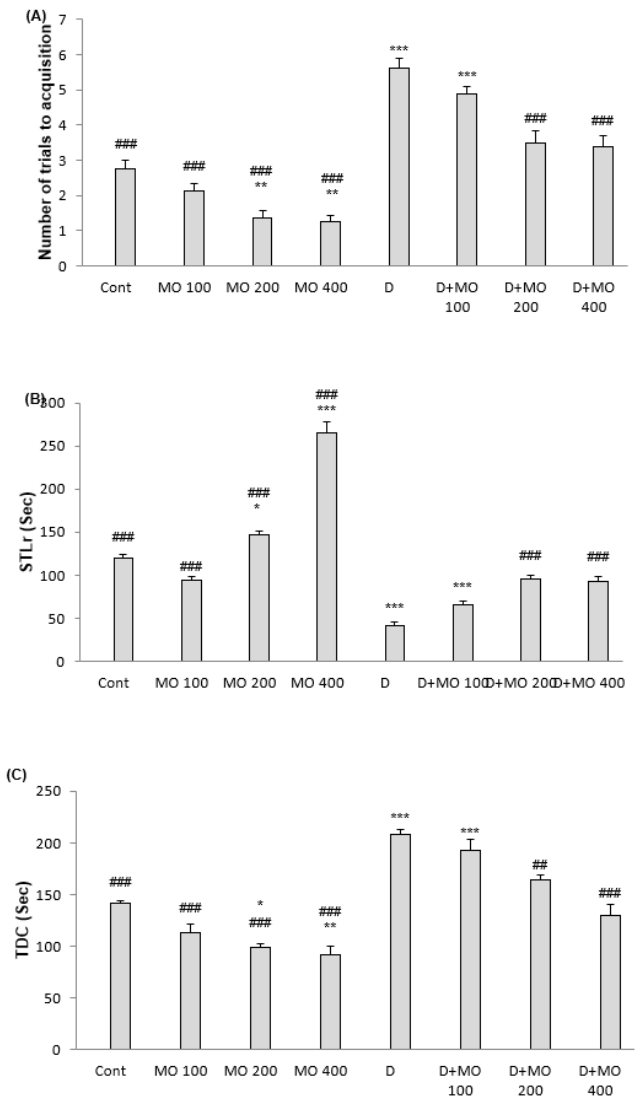

Fig. 1. The number of trials required for acquisition (A), the step-through latency (STLr) in the retention test (B), and the time spent in the dark compartment during the retention test (TDC) (C) across various groups: control, control treated with MO (100 mg/kg) (MO 100), control treated with MO (200 mg/kg) (MO 200), control treated with MO (400 mg/kg) (MO 400), diabetic (D), diabetic treated with MO (100 mg/kg) (D+MO 100), diabetic treated with MO (200 mg/kg) (D+MO 200), and diabetic treated with MO (400 mg/kg) (D+MO 400) (N = 8). The data are expressed as mean ± standard error of the mean (S.E.M.). The asterisk denotes a significant difference relative to the control group (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). The hashmark denotes a significant difference when compared to the diabetic (D) group (## $P < 0.01$ and ### $P < 0.001$).

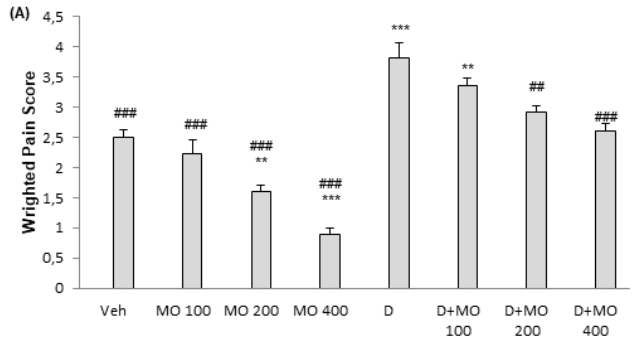

Fig. 2. 2A. The weighted pain scores during the initial phase of the formalin test across various groups: control, control with MO (100 mg/kg) (MO 100), control with MO (200 mg/kg) (MO 200), control with MO (400 mg/kg) (MO 400), diabetic (D), diabetic with MO (100 mg/kg) (D+MO 100), diabetic with MO (200 mg/kg) (D+MO 200), and diabetic with MO (400 mg/kg) (D+MO 400) (N = 8). The data are expressed as mean ± S.E.M. The asterisk denotes a significant difference relative to the control group (** P < 0.01 and *** P < 0.001). The hashmark denotes a statistically significant difference when compared to the diabetic (D) **2B**. The area under the curves (AUCs) of pain scores was analyzed for the following groups: control, control treated with MO (100 mg/kg), control treated with MO (200 mg/kg), control treated with MO (400 mg/kg), diabetic, diabetic treated with MO (100 mg/kg), diabetic treated with MO (200 mg/kg), and diabetic treated with MO (400 mg/kg) (N = 8). The data are expressed as mean ± S.E.M. The asterisk denotes a significant difference relative to the control group (*** P < 0.001). The hashmark denotes a statistically significant difference when compared to the diabetic (D) group (### P < 0.001).

(P's > 0.05) (Table 1).

The body weight of both untreated and MO 100 mg/kg–treated diabetic rats decreased significantly at the end of experiments (P's < 0.001). MO treatment (200 and 400 mg/kg) to diabetic animals decreased the weight loss seen in treated animals compared to untreated diabetics at the end of the experiments (P's < 0.01) (Table 1).

3.6. Effects of MO administration on SOD and CAT antioxidant activity

Diabetic rats exhibited reduced SOD and CAT activity levels in comparison to healthy animals at the end (Table 2).

Levels of superoxide dismutase (SOD) and catalase (CAT) activities of different experimental groups (control treated with MO (100 mg/kg) (MO 100), control treated with MO (200 mg/kg) (MO 200), control treated with MO (400 mg/kg) (MO 400), Diabetic (D), diabetic treated with MO (100 mg/kg) (D+MO 100), diabetic treated with MO (200 mg/kg) (D+MO 200), and diabetic treated with MO (400 mg/kg) (D+MO 400) at the end of experiments.

Groups	SOD	CAT
Cont	12.10 ± 0.1	6.38 ± 0.34
MO 100	12.92± 0.24	7.4 ± 0.19
MO 200	15.28± 0.47**	11.31 ± 0.29**
MO 400	17.30±0.22***	14.45 ± 0.14***
D	5.25± 0.31	2.44 ± 0.21
D+MO 100	5.92± 0.14	3.67 ± 0.12
D+MO 200	9.3± 0.27	6 ± 0.13
D+MO 400	11.4± 0.4	7.13 ± 0.09

The asterisk shows significant difference than control group (** P < 0.01 and *** P < 0.001).

The administration of MO at a dose of 100 mg/kg did not alter the activities of these two antioxidant enzymes (P's > 0.05). Administration of MO (200 and 400 mg/kg) resulted in a significant increase in SOD and CAT activities in both diabetic and non-diabetic animals (Table 2).

4. Discussion

The antidiabetic impacts of medicinal plants are significant for diabetes treatment due to their accessibility, low incidence of adverse effects, and cost-effectiveness. Research findings suggest that administering MO at doses of 200 and 400 mg/kg to diabetic rats can reverse cognitive impairments, shown in the PAL test, and chemical hyperalgesia, shown in the formalin test. Higher doses of MO improved cognitive function and exhibited antinociceptive effects in non-diabetic animals. The reduction in the number of trials necessary for successful acquisition in the PAL task suggests an improvement in the acquisition process. The increase in STLr and the decrease in TDC observed during the retention test further indicate enhanced memory retention. In the present study, diabetic rats displayed challenges in learning, as evidenced by the greater number

Table 1. Plasma glucose and body weight of different experimental groups (control treated with MO (100 mg/kg) (MO 100), control treated with MO (200 mg/kg) (MO 200), control treated with MO (400 mg/kg) (MO 400), Diabetic (D), diabetic treated with MO (100 mg/kg) (D+MO 100), diabetic treated with MO (200 mg/kg) (D+MO 200), and diabetic treated with MO (400 mg/kg) (D+MO 400)) in the beginning and end of experiments.

Groups	Initial plasma glucose (mg/dL)	Final plasma glucose (mg/dL)	Initial body weight (g)	Final body weight (g)
Cont	79.8 ± 4.1	81.57 ± 7.1###	264.16 ± 3.4	315.37 ± 4.6###
MO 100	89. 2 ± 5.1	83. 8 ± 6.32###	270.2 ± 4.8	320 ± 5.2###
MO 200	83 ± 2.2	64.25 ± 5.6###	273.1 ± 2.9	336 ± 4.95###
MO 400	78.5 ± 6.32	50.87 ± 1.8**###	269.4 ± 3.2	358.5 ± 5.4**###
D	81.7 ± 2.83	394.1 ± 7.42***	262.9± 4.1	220.6 ± 3.2***
D+MO 100	78.1 ± 5.46	357.1 ± 6.54***###	272.2 ± 2.3	224 ± 5.56***
D+MO 200	90.8 ± 2.9	110.83 ± 3.9###	268.5 ± 3.3	286.7 ± 7.21###
D+MO 400	89.25 ± 4.1	95.21 ± 2.9###	272.1± 2.9	293.8 ± 3.4###

The asterisk shows significant difference than control group (** P < 0.01 and *** P < 0.001). The hashmark deicts significant difference in comparison with diabetic (D) group (### P < 0.001).

of trials required for acquisition in both the PAL and memory tasks. During the retention assessment, decreases in STLr and increases in TDC were indicated, underscoring the memory retention deficits associated with diabetes. Administration of MO (200 and 400 mg/kg) to diabetic rats reduced the number of trials required for acquisition, indicating the treatment's protective effect against acquisition deficits. This treatment restored the reduced STLr and alleviated the elevated TDC observed in diabetic rats during the retention trial. The cognitive-enhancing effects of MO in this study align with previously reported outcomes of MO leaf extract (400 mg/kg, i.g.) in improving spatial memory and providing neuroprotection in a rat model of age-related dementia [8]. Additionally, chronic oral administration of ethanolic extract from MO leaves restores brain monoamines (norepinephrine, dopamine, and serotonin) in an experimental model of Alzheimer's disease [6, 11]. The other effects of MO in inhibiting acetylcholinesterase (AChE) activity, inducing vasodilation and monoamine transmitter regulatory activity [10,12] could also be involved in the memory-enhancing effects reported in this study.

The formalin test is a significant behavioral assessment for indicating hyperalgesia in diabetic rats due to its extended response duration, making it particularly useful for experimental research and intervention. The administration of formalin into the paw induces a biphasic nocifensive flinching response, significantly more intense in diabetic rats, as evidenced by previous studies [26, 27]. Diabetic rats exhibit notable chemical hyperalgesia in the formalin test. MO (200 and 400 mg/kg) mitigated the chemical hyperalgesia in both phases 1 and 2 of the formalin test, showing that MO may affect both central and peripheral mechanisms involved in these phases [28]. Moreover, MO (200 and 400 mg/kg) induced anti-nociception in non-diabetic animals. Research demonstrates that MO extract markedly decreases nociceptive behaviors and inflammation in experimental models of inflammatory pain, including subcutaneous carrageenan and collagen, as well as in the acetic writhing test, thereby supporting our current findings.

The pathophysiology of neurological disorders in diabetes is complex and not fully understood; however, hyperglycemia and oxidative stress are significant contributing factors [15, 28, 29]. The recent in vivo study demonstrated that oral administration of MO at doses of 200 and 400 mg/kg in diabetic rats over 30 days led to a reduction in glucose levels. The observed improvement in abnormal behaviors in the diabetic animals in this study may be partially ascribed to the hypoglycemic effects of MO. The hypoglycemic effect of MO observed in this study is consistent with previous research that has documented the antidiabetic properties of MO extracts in experimental diabetes [30-33]. The release of insulin, which enhances glucose uptake and glycogen synthesis in diabetic animals, could account for the hypoglycemic effects of MO extract [34].

Two robust antioxidant biomarkers were measured to assess the effect of MO in this study. Diabetes induction blocked the antioxidant defense system, as evidenced by a reduction in the activities of these two primary enzymatic antioxidants. The findings align with prior studies indicating diminished efficiency of antioxidant enzymes, including SOD, CAT, and glutathione peroxidase, in diabetic animals [35,36]. The enhancing activity of these two robust antioxidants in diabetic rats treated with MO at 200 and 400 mg/kg may contribute to the observed protective effects of these treatments.

Our findings demonstrated that MO (200 and 400 mg/kg) decreased blood glucose of healthy animals. This finding is compatible with the hypoglycemic property of 200 mg/kg MO aqueous extract in normal rats [17]. Furthermore, MO (400 mg/kg) induced a significant weight gain in the healthy rats and prevented the decreased body weight of diabetic animals in this study. These findings show that MO may be beneficial for the dietary control of diabetes after further confirmation.

No effects of MO on motor function have been documented at dosages analogous to those used in this research [3, 8, 37]. Moreover, although STLa during the initial acquisition trial showed no variation among the animal groups, the nootropic effects of MO are unrelated to any influence on locomotion.

The results demonstrate the memory-enhancing and antinociceptive properties of MO in STZ-induced diabetic rats and non-diabetic animals for the first time, perhaps through hypoglycemic and antioxidant processes. This information indicates that MO is a promising and abundant source for developing novel and therapeutic strategies in diabetes care.

Conflict of interest

There is no conflict of interest.

Ethics approval

The animal experiment was approved by the University of Zabol ethical review committee (IR.UOZ.REC.1404.001).

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