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ORIGINAL RESEARCH

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Umbelliferone isolated from *Zosima absinthifolia* roots partially restored erectile dysfunction in streptozotocin-induced diabetic rats

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Abstract

Erectile dysfunction (ED) is a common problem in diabetic men, which affects the quality of life. *Zosima absinthifolia* (*Vent.*) Link (*syn. Zosima orientalis Hoffm.*) is the only member of Zosima genus growing in Turkey. Its fruits and aerial parts have been used in folk medicine as a sedative and digestive problems. This study aimed to investigate the possible beneficial effect of intracavernosal injection of umbelliferone isolated from *Z. absinthifolia* Link roots on ED of streptozotocin-induced diabetic rats. Adult Sprague–Dawley ($n = 20$) rats were divided into control and streptozotocin-induced diabetic groups. In vivo erectile responses were also repeated after intracavernosal injection of umbelliferone (1 μM). Umbelliferone-induced relaxant responses in the presence of inhibitors of nitric oxide synthase (NOS) and cyclic guanosine monophosphate (cGMP) were assessed in corpus cavernosum (CC) strips from control rats. The relaxant responses of CC strips from both groups were evaluated in the presence or absence of umbelliferone (100 μM). In vivo erectile responses in diabetic rats were significantly lower than in controls, which were partially prevented by umbelliferone. Umbelliferone resulted in a relaxation of CC in a concentration-dependent manner. NOS and cGMP inhibitors antagonized the umbelliferone-induced relaxations in CC. Acetylcholine, sodium nitroprusside, a phosphodiesterase type 5 inhibitor, sildenafil and electrical field stimulation-induced relaxation responses in diabetic CC strips were potentialized after pre-incubation with umbelliferone. In conclusion, intracavernosal administration of umbelliferone may prevent ED in diabetic rats by recovering of NO/cGMP pathway. These results may be supported by further studies using combinations of umbelliferone and phosphodiesterase type 5 inhibitors for the treatment of diabetic ED.

Keywords Erectile dysfunction · Umbelliferone · Diabetes · Corpus cavernosum

Introduction

Diabetes is commonly associated with erectile dysfunction (ED), which greatly affects the quality of life (Mazzilli et al. 2015; Rastrelli et al. 2015). The risk of ED in diabetic men is threefold higher than in healthy men (Mazzilli et al. 2015; Rastrelli et al. 2015). Several etiologies including vasculogenic and neurogenic factors are mostly involved in diabetes-induced ED. Furthermore, various treatments for ED have less efficacy in the diabetic condition compared to the non-diabetic condition. For instance, diabetic men often exhibit a poor response to first-line oral phosphodiesterase type 5 (PDE-5) inhibitors (Ruan et al. 2016). Thus, several alternative treatment options appear to be required for the management of diabetes-induced ED. For a long time, herbal medicine has been an alternative treatment option for the management of ED, and over 15% of men use natural-based therapies (Evans et al. 2011). Yohimbe, Korean ginseng, and Ginkgo biloba are popular examples in ED

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treatment (MacKay 2004; Jang et al. 2008). Recently, we showed that treatment with pomegranate juice improved ED in a diabetic rat model (Onal et al. 2016).

Apiaceae is one of the richest families including coumarins (Iwase et al. 2017). *Zosima absinthifolia* (Vent.) Link (syn. *Zosima orientalis* Hoffm.) is the only member of *Zosima* genus grows in Turkey. The aerial parts of the plant are used as a vegetable (Bahadir et al. 2011). It is commonly known as "ayı eli" or "peynir otu" in Turkey (Bahadir et al. 2011). It has been reported that *Z. absinthifolia* has biological activities such as antioxidant, antibacterial, and anti-inflammatory effects (Al-Shamma and Mitscher 1979; Razavi et al. 2013). Previous phytochemical studies have shown that *Z. absinthifolia* includes alkaloids and coumarins such as deltoin, imperatorin, pimpinellin, bergapten, isobergapten, sphondin isopimpinellin, and umbelliferone (Bahadir et al. 2011; Baser et al. 2000). In addition, our current study has demonstrated anticholinesterase and antioxidant activities of extracts and essential oils from the aerial part, root, flower, fruit as well as bergapten, imperatorin, pimpinellin, and umbelliferone-isolated of the roots from *Z. absinthifolia* (Karakaya et al. 2019). Umbelliferone is a derivative of coumarin also known as 7-hydroxycoumarin. Umbelliferone has several pharmacological activities such as antioxidant (Ramesh and Pugalendi 2006) and antidiabetic effects (Ramesh and Pugalendi 2005). Furthermore, a previous study showed a decrease in diabetic nephropathy after umbelliferone treatment (Garud and Kulkarni 2017). Umbelliferone can be an acceptable candidate due to all these biological activities for the treatment of diabetic ED. In the present study, we firstly evaluated the possible restorative effect of umbelliferone on ED in streptozotocin (STZ)-induced diabetic rats.

Materials and methods

Plant material

The flowering plants of *Z. absinthifolia* (Vent.) Link were collected in 2014 from Erzurum (East of Turkey) and kept in the Herbarium of Atatürk University, Faculty of Pharmacy (No. 23847).

Extraction and purification

The roots of *Z. absinthifolia* were grounded and macerated for 8 h/3 days with methanol in a water bath not exceeding 45 °C using a mechanical mixer at 300 rpm (Heidolph, Schwabach, Germany). The extracts were filtered and concentrated till dryness using a rotary evaporator (Heidolph, Schwabach, Germany). Then, they were dispersed in methanol–water and fractionated with 400 ml of n-hexane dichloromethane, ethyl acetate, and butanol in a separatory funnel. Each fraction was concentrated for dryness. Dichloromethane was mixed with silica gel and further subjected to column chromatography (52.5 × 6.9 cm) with a gradient of hexane/ethyl acetate, as eluent to give a total of 720 fractions. Eluting with n-hexane was started and continued by increasing polarity by the addition of ethyl acetate in a ratio of 5%. Fractions (76–181) eluted with 25% ethyl acetate in hexane were dried and rechromatographed with hexane-ethyl acetate in increasing gradient elution. The fraction eluted with hexane-ethyl acetate (5%) yielded a white solid residue (130 mg, 0.18%) was named umbelliferone.

Identification of umbelliferone using nuclear magnetic resonance (NMR) and mass spectroscopy

Experiments using the NMR (^{1}H, ^{13}C, HMBC, HMQC, COSY, and TOCSY) were done on a Varian Mercury at 400 MHz. The chemical shifts were evaluated in deuterochloroform and expressed (ppm). IR spectra were registered on a Horiba FT-720 IR spectrometer utilizing a KBr disk.

To provide NMR data for umbelliferone

It was obtained as a white solid powder, 130 mg, yield: 0.18%; mp 223–225 °C; Rf 0.39; UV (MeOH) λ_{max} (log ε) 217 (2.72), 244 (3.54), 259 (4.11), 278 (4.16), 323 (3.79); nm; IR (KBr) ν_{max} 3412, 1679, 1609 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 6.14 (1H, *d*, *J* = 9.3 Hz, H-3), 7.78 (1H, *d*, *J* = 9.3 Hz, H-4), 7.37 (1H, *d*, *J* = 8.7 Hz, H-5), 6.72 (1H, *d*, *J* = 8.4 Hz, H-6), 6.63 (1H, *s*, H-8); ^{13}C-NMR ($CDCl_3$, 400 MHz): δ = 162.34 (C-2), 110.87 (C-3), 144.54 (C-4), 111.71 (C-4′), 129.37 (C-5), 113.11 (C-6), 161.74 (C-7), 102.02 (C-8), 155.82 (C-8′). ESIMS *m/z* 163.24 $[M + Na]^+$. HR-ESI-MS: *m/z* (neg): 160.9869 $[M–H]^-$.

Experimental animals

Adult male Sprague–Dawley rats (350–400 g) were divided into two groups: (1) control and (2) diabetic rats received a dose of STZ (40 mg/kg, i.p.) within a citrate buffer (pH = 5.5) on the day of use (Cengiz et al. 2017). Measurement of blood glucose levels was performed using an Accu-Chek glucometer (Roche Diagnostics, Indianapolis, IN) after the induction of diabetes. Rats were housed in separate cages on a 12-h light–dark cycle and were fed standard chow and water ad libitum. This study was approved by the Institutional Animal Care and Use Committee of Ankara University (2014-15-86).

In vivo evaluation of erectile function

To measure in vivo evaluation of erectile function, intracavernosal pressure (ICP, mmHg) was monitored in rats

which were anesthetized with ketamine (50 mg/kg, i.p.). The trachea was cannulated (polyethylene, [PE]-240 tubing) to sustain the airway, and the carotid artery was cannulated (PE-50 tubing) to measure the main arterial pressure (MAP, mmHg), with a transducer (Statham, Oxnard, CA) attached to a data acquisition system (Biopac MP 100 System, Santa Barbara, CA). A 25-gauge needle filled with 250 U/ml of heparin and connected to polyethylene-50 tubing was inserted into the penis right crura connected to a pressure transducer to measure constantly ICP. The right major pelvic ganglion and cavernosal nerve (CN) were defined. A stainless-steel bipolar hook electrode for stimulation was situated around the CN posterolateral to the prostate on one side, and the MAP and ICP were constantly measured with pressure transducers. The CN was stimulated (2.5, 5, and 7.5 V, 15 Hz, 30 s train duration) with a square pulse stimulator (Grass Instruments, Quincy, MA). The measurements were repeated after intracavernosal administration of umbelliferone (1 μM) in groups (Yilmaz-Oral et al. 2017).

Isometric tension measurements

Cavernosal tissue strips were placed in organ bath chambers, maintained in Krebs-bicarbonate solution (containing, mM: NaCl; 118.1, KCl; 4.7, KH_2PO_4; 1.0, $MgSO_4$; 1.0, $NaHCO_3$; 25.0, CaCl; 22.5, and glucose; 11.1, pH 7.4). The strips (1 × 1 × 9 mm) were dissected and mounted under 1 g of resting tension in a 20-ml organ bath. The organ chamber temperature was maintained at 37 °C by a circulating water bath and continuous bubbling with a mixture of 95% O_2 and 5% CO_2. The tissues were allowed to equilibrate for a minimum of 60 min, and the bath solution was replaced every 15 min. Electrical field stimulation (EFS) of the autonomic nerves (duration: 15 s; amplitude: 50–90 V; frequency: pulse width: 5 ms) was accomplished by the use of platinum electrodes, positioned on either side of the tissue strip (Grass Instruments, Quincy, MA).

In the first series of experiments, corpus cavernosum (CC) strips were precontracted with phenylephrine (Phe, 10 μM) and allowed to relax after administration of umbelliferone. The relaxation response curves to umbelliferone were also repeated in the presence of the nonspecific nitric oxide synthase (NOS) inhibitor, L-NAME (L-N(G)-Nitroarginine Methyl Ester, 100 μM) and soluble guanylate cyclase inhibitor (cGMP), ODQ (1H-[1,2,4]-oxadiazolo [4,3-a] quinoxaline-1-one, 30 μM).

In the second series of experiments, acetylcholine (ACh), EFS, sodium nitroprusside (SNP), and a PDE-5 inhibitor sildenafil-induced relaxation responses were evoked after precontraction of CC strips with Phe (10 μM) in the presence or absence of umbelliferone (100 μM).

Statistical analysis

All data values were expressed as mean ± SEM. Statistical differences were determined by variance analysis followed by the complementary analysis of Bonferroni. A P value < 0.05 was considered to be significant. At the end of the experiment, each CC strip was weighed. All contractile responses were expressed as mg of tension developed per mg of CC tissue and relaxant responses were measured as a percentage of Phe-contraction.

Drugs

All drugs were purchased from Sigma Chemical Co. (St. Louis, MO).

Results and discussion

The present study provides importantly basic mechanistic information regarding of dose-dependent relaxation to umbelliferone, a bioactive component obtained from *Z. absinthifolia* in rat CC. The major findings of the study showed that (i) umbelliferone partially restored in vivo erectile function in diabetic rats; (ii) umbelliferone itself relaxed rat CC in a concentration-dependent manner; (iii) the NO-cGMP pathway played a significant role in mediating umbelliferone-induced relaxation; and (iv) umbelliferone contributed to normalization of the reduced relaxation responses to ACh, EFS, and sildenafil in diabetic CC in vitro.

The methanol extract of the roots of *Z. absinthifolia* was fractionated using solvents with different polarities (*n*-hexane, dichloromethane, ethyl acetate, and *n*-butanol). For the bioguided fractionation study the effective dichloromethane extract was first submitted to a silica gel column and eluted with a gradient of *n*-hexane:ethyl acetate (100:0 → 0:100, v/v) and ethyl acetate:methanol (100:0 → 0:100, v/v), and eight fractions (Fr. A–H) were obtained. Fr. H was submitted on a silica gel column using *n*-hexane: ethyl acetate (65:35, v/v) and the resulting fraction was chromatographed on silica gel column using *n*-hexane:ethyl acetate (90:10, v/v) to give compound umbelliferone. Then, umbelliferone (7-hydroxycoumarin) was isolated and identified (Fig. 1). A previous study has shown that four known coumarins namely, bergapten, imperatorin, pimpinellin as well as umbelliferone were purified and identified (Karakaya et al. 2019). Similarly, previous phytochemical studies showed the presence of umbelliferone in *Z. absinthifolia* (Bahadir et al. 2011; Baser et al. 2000). In addition, umbelliferone was found in the root of plants (Apiaceae) (Kim et al. 2006; Kong et al. 1996).

Body weight in diabetic rats was significantly lower than in control rats ($p < 0.001$, Fig. 2a). Blood glucose levels in the diabetic group were significantly higher than in the control group ($p < 0.001$, Fig. 2b).

Penile erection in response to cavernous nerve stimulation was determined in vivo in the STZ-induced diabetic animal model. Our data demonstrated that diabetes decreased the in vivo erectile response and the in vitro relaxant response of CC to EFS. ICP/MAP values in diabetic rats were lower than in control rats ($p < 0.01$; Fig. 3a), which was partially returned by intracavernosal administration of umbelliferone (1 µM, $p < 0.05$). In addition, total ICP values were decreased in the diabetic group when compared with the control group ($p < 0.01$; Fig. 3b). After intracavernosal administration of umbelliferone (1 µM), total ICP values were completely restored in the diabetic group at 5 and 2.5 voltage levels. However, ICP at 7.5 voltage in treated rats were partially improved ($p < 0.05$, Fig. 3b). Similarly, in in vitro studies, the nitrergic relaxation response to EFS in diabetic rats at 20 Hz was decreased in the diabetic group compared with the control group, which was restored by the incubation with umbelliferone (100 µM). There was no difference in EFS-induced relaxation response in control rats between the absence and presence of umbelliferone (Fig. 5b). There is no previous data to evaluate the effect of umbelliferone on erectile function and nitrergic relaxation responses in the CC. However, umbelliferone treatment decreased in the diabetes-induced renal damage related to the diabetic nephropathy (Garud and Kulkarni 2017). In addition, the treatment improved the activities of enzymatic and non-enzymatic antioxidants (Ramu et al. 2016), as well as in vitro increased the glycolytic activities (Gao et al. 2015).

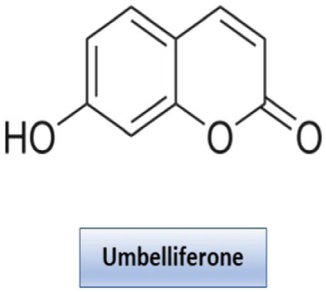

Fig. 1 Chemical structure of umbelliferone

In the present study, the relaxation response to umbelliferone was lower after the pre-contraction of KCl in control rats than after pre-contraction of Phe ($p < 0.05$, Fig. 4a). The decreased response by umbelliferone in high K^+ medium indicating that relaxation to umbelliferone does not significantly change in a Ca^{+2} channel antagonistic property.

There was no difference between umbelliferone-induced maximum relaxation responses in CC from control and diabetic rats (Fig. 4b). The intracavernosal administration of umbelliferone contributed to being increased erectile responses in the present data. It seems that umbelliferone responses serve as the normal activity in vivo and in vitro under diabetic conditions.

In the present study, we investigated the underlying mechanism of umbelliferone effects on erectile responses that can be mediated by the NO/cGMP-dependent pathway which is damaged in the diabetic condition. In the presence of L-NAME and ODQ, umbelliferone-induced maximum relaxation responses were inhibited 53% and 76% in CC from control rats ($p < 0.001$, Fig. 4c, d). Umbelliferone is likely to have a role for the NO-cGMP signaling pathway in mediating CC relaxation responses. Also, the previous study demonstrated that umbelliferone caused a rapid, transient relaxation in the splenic artery which was inhibited by L-NAME (Roberts et al. 2013).

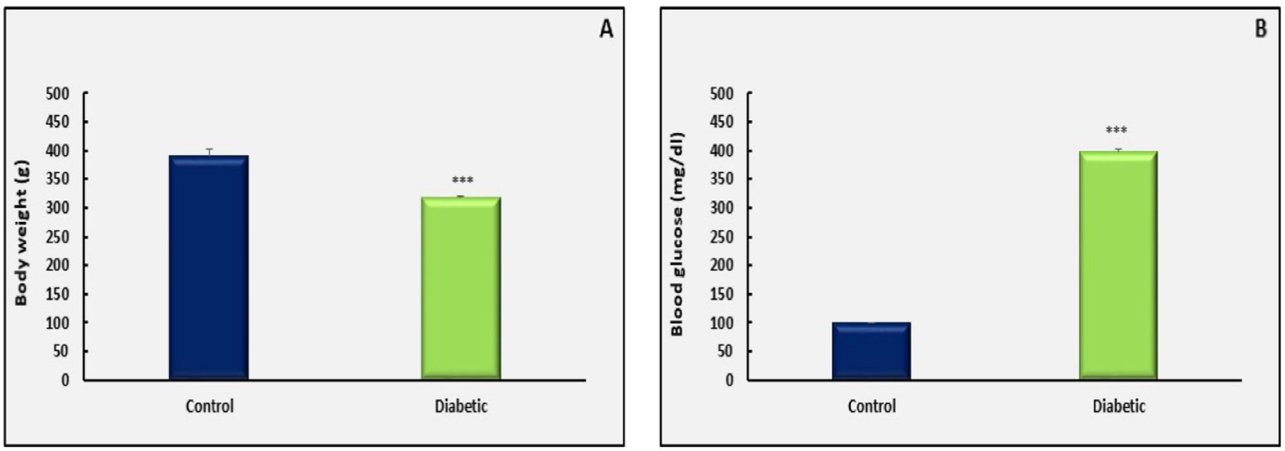

Fig. 2 Bar graphs showing body weight **a** in control and diabetic groups and glucose levels **b** in control and diabetic groups. Data represent the mean ± SEM ($n = 6$) and ***$p < 0.001$ vs control

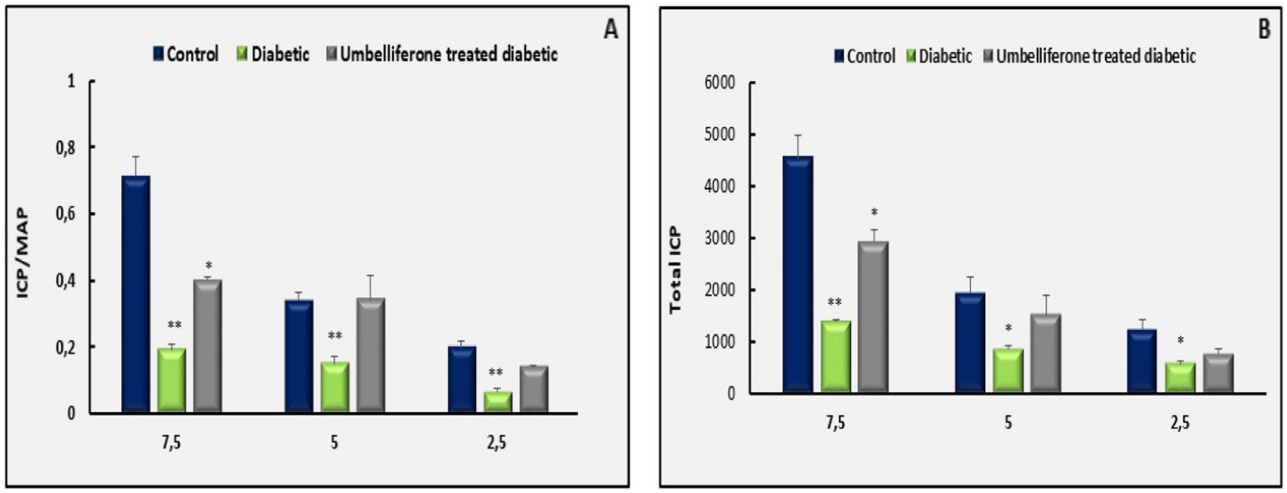

Fig. 3 In vivo intracavernosal effect of umbelliferone on control and diabetic rat penile erection. Bar graphs showing ICP/MAP **a** and total ICP **b**. Data represent the mean ± SEM of 6–8 observations. $*p < 0.05$, $**p < 0.01$ vs control value

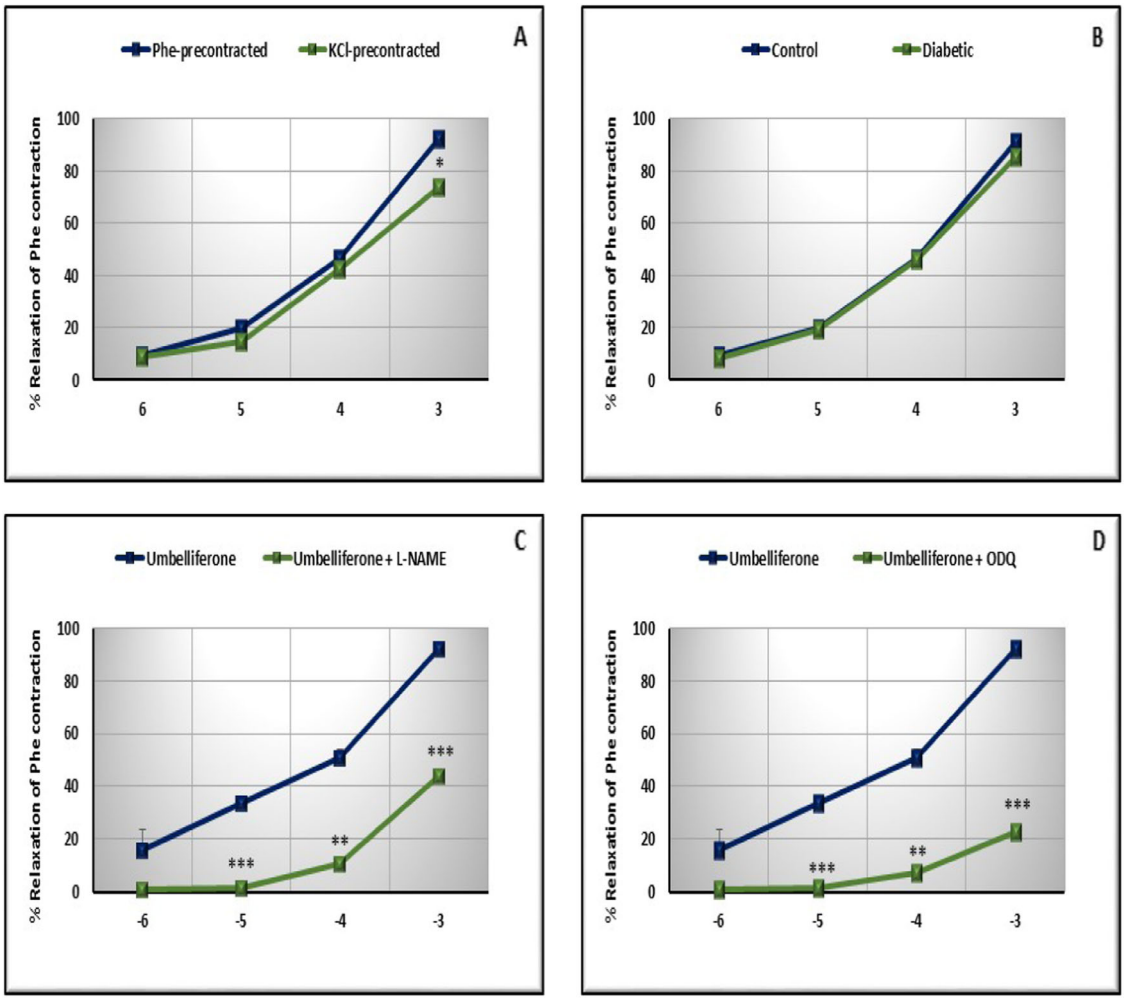

Fig. 4 Concentration–response curves to umbelliferone (10^{-6}–10^{-3} M) in corpus cavernosum after pre-contraction with KCl (60 mM, **a**) in control rat corpus cavernosum, and pre-contraction with phenylephrine (Phe, 10 µM, **b**) in control and diabetic rat corpus cavernosum. Concentration–response curves to umbelliferone (10^{-6}–10^{-3} M) in corpus cavernosum in the presence of L-NAME (100 µM, **c**) and ODQ (30 µM, **d**). Data represent the mean ± SEM of 6–8 observations. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs control value

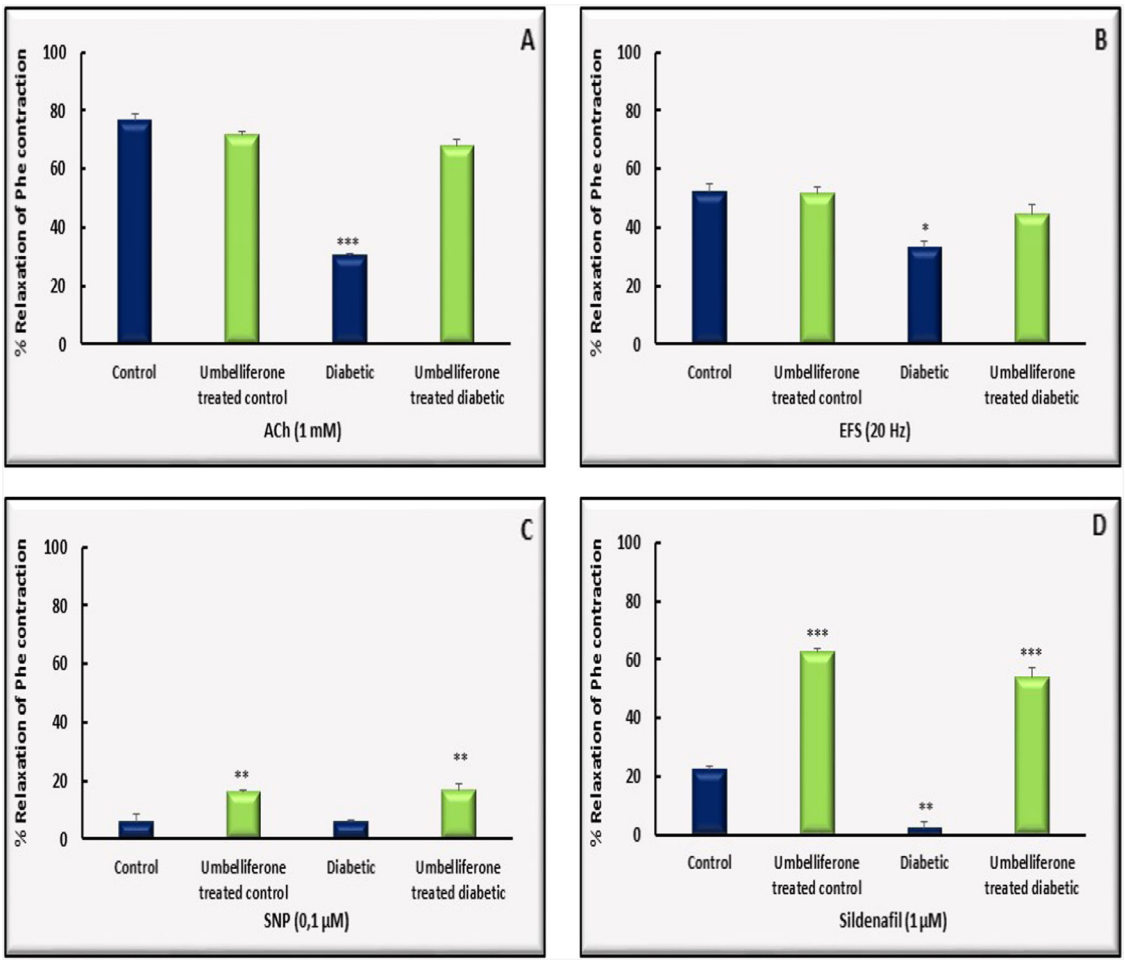

Fig. 5 Relaxation responses to single doses of ACh (1 mM, **a**), EFS (20 Hz, **b**), SNP (0.1 µM, **c**), and sildenafil (1 µM, **d**) in the presence of umbelliferone (100 µM). Data represent the mean ± SEM of 6–8 observations. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs control value

The alteration of the cGMP-NO pathway in diabetic men with impaired vascular relaxation was linked to endothelial dysfunction (Angulo et al. 2010). In the isolated CC from the diabetic group, endothelium-dependent relaxation response to ACh was remarkably decreased which was potentialized in the presence of umbelliferone (100 µM) in control and diabetic groups (Fig. 5a). An earlier study showed that the relaxation to umbelliferone was inhibited by removing the endothelium in porcine isolated blood vessels (Roberts et al. 2013). Decreased NO availability is a common factor in diabetes-induced ED, cavernosal relaxation in an in vivo situation would be extremely beneficial, leading to or improving the quality of an erection (Nunes et al. 2015). In addition, this result supports umbelliferone may interact with endogenous NO synthesis.

There was no difference between the endothelial-independent relaxation response to SNP (at 0.1 µM dose) in diabetic and control rats. In previous studies, SNP-induced relaxant responses were not altered in diabetic rats when compared with control rats (Yilmaz et al. 2014; Cengiz et al. 2017). The incubation of umbelliferone was increased in SNP responses of CC in both groups (Fig. 5c). However, relaxation responses to SNP were augmented after incubation with umbelliferone (100 µM) in control and diabetic rats ($p < 0.01$, Fig. 5c).

We found that sildenafil-induced relaxation response at 1 µM dose was significantly decreased in diabetic rats when compared with control rats ($p < 0.01$ Fig. 5d). After incubation with umbelliferone (100 µM), relaxation responses to sildenafil were markedly increased in control and diabetic rats (Fig. 5d). There was no difference relaxant response to sildenafil between diabetic and control rat CC after incubation with umbelliferone. This result suggests a strong rationale for further studies using combinations of umbelliferone and PDE-5 inhibitors in diabetes-induced ED. In another previous study, a coumarin compound osthole had a relaxant effect in rabbit CC via inducing NO-cGMP pathway, as well as inhibiting the common PDE (especially PDE-5) signal pathway (Chen et al. 2000).

Conclusions

The study firstly revealed that the beneficial effect of intracavernosal administration of umbelliferone on ED in diabetic rats. The improvement in ED induced by diabetes related to in vivo and ACh, EFS and sildenafil-induced relaxation of CC may likely involve in NO/cGMP pathway. The preclinical findings should extend our knowledge of the successful effect of umbelliferone on the penile function to develop therapeutic or preventive agents. Combinations of umbelliferone and PDE-5 inhibitors may be a reasonable therapeutic alternative for diabetes-induced ED.

Compliance with ethical standards

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

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