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The beneficial effect of clove essential oil and its major component, eugenol, on erectile function in diabetic rats

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Abstract

Diabetic men are at a higher risk of erectile dysfunction (ED). A tropical plant, clove (Syn. *Eugenia caryophyllata*, *Caryophyllus aromaticus* L., Syzygium aromaticum (L.) Merr. & L.M. Perry) from the Myrtaceae family has displayed aphrodisiac activity. The present research aimed to investigate the impacts of clove essential oil (CEO) and the ingredient of CEO, eugenol (E) on ED in diabetic rats. We divided Sprague-Dawley rats into control and diabetic groups. Erectile function was evaluated before and after CEO and E intracavernosal injection. CEO- and E-induced relaxation responses were investigated in isolated corpus cavernosum (CC) using various inhibitors. The intracavernous administration of CEO and E restored erectile responses in diabetic rats. CEO and E induced remarkable relaxation in all groups. CEO- and E-induced relaxation responses were partially inhibited after pre-contraction with KCl. Tetraethylammonium and glibenclamide inhibited the relaxation response to CEO. Glibenclamide inhibited maximum relaxation to E. The inhibitors of nitric oxide synthase (NOS), soluble guanylyl cyclase and nifedipine did not change CEO- and E-induced relaxation responses. The current results suggest that CEO and the major compound of the essential oil, E improved diabetes-induced ED in rats, and CEO caused CC relaxation via K^+ channels independently NO signalling pathway.

KEYWORDS

clove, corpus cavernosum, diabetes, erectile dysfunction, eugenol, *Syzygium aromaticum*

1 | INTRODUCTION

Diabetes is a common problem that has been closely related to erectile dysfunction (ED) in men (Lue, Brant, Shindel, & Bella, 2000). In men with diabetes, ED prevalence ranges from 20% to 70%, as well as the incidence and severity of ED raise with diabetes duration (El Taieb, Hegazy, Maklad, & Khairy, 2019). The cause of diabetes-induced ED involves multifactorial alterations in diabetic neuropathy, oxidative stress and endothelial dysfunction (Maiorino, Bellastella, & Esposito, 2014). Additionally, diabetic men poorly respond to phosphodiesterase type 5 inhibitors (PDE-5i) due to the complete pathogenesis of ED (Ruan et al., 2016). Many plants and plant-derived drugs have contributed to human health for a long time. The popular options for ED are *Gingko biloba*, *Ginseng* sp., *Schisandra chinensis*, *Rubus coreanus*, *Lepidium meyenii*, *Epimedium koreaum*, *Ferulago* sp. and *Ferula* sp., etc. (Ayuob, Al-Harbi, & Abdulhadi, 2014; Karakaya, Yilmaz Oral, & GÜR, S., Duman, H., & KiliÇ, C. S., 2019; Shin, Zhao, Zhang, & Park, 2015; Zenico, Cicero, Valmorri, Mercuriali, & Bercovich, 2009). Therefore, alternative therapy may be a significant approach in diabetic patients with ED.

Syzygium aromaticum (L.) Merr. & Perry (Syn: *Eugenia caryophyllata* Thunb., *Caryophyllus aromaticus* L.) from Myrtaceae

family is commonly called clove in India, Sri Lanka and Indonesia in cultivated coastal areas (Cortes-Rojas, Souza, & Oliveira, 2014; Tajuddin, Latif, & Qasmi, 2003, 2004). In fact, it is a particularly famous spice used for medicinal and agricultural purposes for centuries in Europe and Asia (Atawodi, 2011). Clove is a good source of essential oil and phenolic components, such as flavonoids, hydroxyphenyl propens, hydroxycinnamic acids, hydroxybenzoic acids, tannins and phytosterols (Cortes-Rojas et al., 2014; Singh, Dhamanigi, & Asad, 2009). Previous studies have been reported that clove had aphrodisiac activity (Tajuddin et al., 2004), antioxidant, stomachic, and antimicrobial effects (Radunz et al., 2019). In addition, a major component of the essential oil, eugenol (E), possesses potent antioxidant activity and relaxant effect on vascular smooth muscle (Cortes-Rojas et al., 2014; Tajuddin et al., 2003). Furthermore, various formulations including clove have been used for sexual-gynaecological disorders as a sexual invigorator in India (Modak, Gorai, Dhan, Mukherjee, & Dey, 2015). In earlier studies, the *S. aromaticum* flower buds restored sexual performance in experimental animals (Mishra & Singh, 2008; Singh, Ali, Gupta, Ali, Gupta, Semwal, & Jeyabalan, 2013; Singh, Ali, & Singh, 2013). In addition, clove caused an increase in sexual behaviour without side effects in male rats (Tajuddin et al., 2003, 2004). However, there is no report for the effects of clove and its major component on diabetes-induced ED. Thus, the present investigation aimed to examine the possible therapeutic impacts of clove essential oil (CEO) as well as its major ingredient, E on the corpus cavernosum (CC) tissue.

2 | MATERIALS AND METHODS

2.1 | Plant/Oil material

CEO (Product number: 4820174890186, Flora Secret, Kyiv, Ukraine) and E (Product number: 06-02077, Keystone Industries Myerstown) were supplied from commercial companies and kept in a freezer until experiments. The oil has been subjected to gas chromatography (GC)/mass spectroscopy (MS) for determination and confirmation of CEO components.

2.2 | GC/MS analyses

Isolation and identification of the volatile compounds were performed using a GC coupled with a mass spectrometer detector (Shimadzu Corporation). The GC/MS system was equipped with a TRACSIL Meta X5 (95% dimethylpolysiloxane and 5% diphenylpolysiloxane) column (60 m × 0.25 mm, 0.25 μm film thickness; Teknokroma S. Coop. C. Ltd.).

Analyses were carried out using helium as carrier gas at a flow rate of 0.3 ml/min in a split ratio of 1:11. The following program was used: (a) 80°C for 0 min; (b) increase of 3°C/min from 80 to 210°C and hold for 1 min; and (c) increase of 25°C/min from 210 to 300°C and hold for 3 min. The temperatures of the injector and detector were 230 and 300°C, respectively. Most compounds were identified using three different analytical methods: (a) Retention indices; (b) GC/MS retention times (authentic standards of all compounds reported were used for identification purposes) and (c) mass spectra (authentic chemicals and NIST05 spectral library collection). Semi-quantification of the volatile compounds was performed on a gas chromatograph (Shimadzu 2010) with a flame ionisation detector. The column and chromatographic conditions were the same as those described above for GC/MS analyses. The injector temperature was 250°C, and N_2 was used as carrier gas (1 ml/min). Data handling was carried out by means of GC solution 2.3 (Shimadzu Corporation). For the semi-quantification of volatile compounds, benzyl acetate was added at a concentration of ~IgL − 1 in chloroform (50 μl) and was used as an internal standard after checking that it was absent in the extracts of dill, and under the proposed conditions, it separates other volatile compounds effectively. Data were calculated semi-quantitatively due to no standard curves constructed for any of the quantified volatile compounds. However, relative values were useful to compare differences among farming treatments.

2.3 | Animal studies

Male Sprague-Dawley rats (n = 20, 300–350 g) were split into two groups: control (n = 10) and diabetic (n = 10). Diabetes was induced by a single intraperitoneal injection with streptozotocin (STZ; 40 mg/kg) in citrate buffer (pH = 5.5). Seventy-two hours after the administration of STZ, diabetes was verified by the assessment of blood glucose levels higher than 300 mg/dl with a blood glucose meter (Accu-Chek; Roche Diagnostics). Animals were located under environmental conditions (12/12 hr light: dark, at 22 ± 1°C) with water and food ad libitum. The protocol was approved by Ankara University Institutional Animal Care and Use Committee (2017-7-61).

2.4 | In vivo erectile response evaluation

Eight weeks after diabetes induction, intracavernosal pressure (ICP) was evaluated in anaesthetised rats (ketamine, 100 mg/kg; xylazine, 10 mg/kg, i.p.). Polyethylene tubing was inserted into the trachea and the carotid artery to maintain the constant airway and determine mean arterial pressure (MAP) using a transducer (Statham) with a data acquisition system (Biopac MP 100 System). The right crura was cannulated with the polyethylene tubing with a 25-gauge needle to measure ICP using the transducer. After the identification of the cavernous nerve (CN) and the major right pelvic ganglion, the CN was stimulated (2.5, 5 and 7.5 V, 15 Hz, 30-s pulse width) with a stainless steel bipolar hook electrode and a square pulse stimulator (Grass Instruments). The measurements were again obtained after CEO (1 μM) and E (1 μM) intracavernosal administration in the diabetic group (Onder, Yilmaz-Oral, Jarkovic, Akdemir, & Gur, 2019).

2.5 | Organ bath studies in vitro

In vitro experiments were executed to evaluate cavernosal tissues' isometric tension in control and diabetic rats (n = 6–8). Following in vivo rectile function evaluation, rats were euthanised by cardiac exsanguination under anaesthesia. The cavernosal tissue was removed as well as dissected free of connective tissue. Isolated CC strips (1 × 1 × 8 mm) were placed in an organ bath (20 ml) under an initial isometric tension (1 g) within Krebs solution (containing NaCl: 118.1, KCl: 4.7, $NaHCO_3$: 25.0, $MgSO_4$: 1.0, $CaCl_2$: 2.5, KH_2PO_4: 1.0 and glucose: 11.1 mM, pH: 7.4, 37°C) and constantly bubbled with a mixture of O_2/CO_2 (95%/5%). The CC strips were equilibrated for 1 hr, and the solution was changed every 15 min. All alterations in tension were recorded using an isometric force transducer connected to a PC-based Data acquisition system (Biopac System). CEO- and E (26–104 mg)-induced relaxant responses were obtained after phenylephrine (Phe, 10 μM) and KCl (60 mM)-induced pre-contraction(Halder et al., 2011; Hannigan et al., 2017; Onder et al., 2019; Salahdeen, Idowu, Yemitan, Murtala, & Alada, 2015). After Phe-induced pre-contraction (10 μM), CEO- and E-induced relaxation responses were obtained before and after the incubation with nitric oxide synthase (NOS) blocker, L-*N*(G)-nitroarginine methyl ester (L-NAME, 100 μM); soluble guanylate cyclase (sGC) blocker, 1H-[1,2,4]-oxadiazolo[4,3-a] quinoxalin-1-one (ODQ, 30 μM); L-type Ca^{2+} channel inhibitor, nifedipine (10 μM); non-selective K^+ channel inhibitor, tetraethylammonium (TEA, 100 μM); and K_{ATP} channel inhibitor, glibenclamide (10 μM). The measurements of relaxation responses in the cavernosal tissues were repeated after the incubation with inhibitors for 20 min.

2.6 | Data analysis

All results are presented as mean ± *SEM*. Statistical differences were measured using one-way analysis of variance (ANOVA) and Bonferroni post-test (GraphPad Software). A p-value of less than .05 was regarded as statistically significant.

3 | RESULTS

3.1 | Essential oil components identification

CEO components (100%) were identified based on the results of GC/MS. E (90.45%) was observed as a major component in CEO (Table 1). Also, 1,8-Cineole (5.72%) was found as an another main compound (Table 1).

3.2 | Body weight and blood glucose levels in animals

The body weight in diabetic rats (319.1 ± 5.8 g, p < .01) was decreased when compared with controls (394.5 ± 12.8 g, Table 2). Blood glucose level in diabetic animals (405.1 ± 20.8 mg/dL, p < .001) was considerably higher than in controls (99.3 ± 4.0 mg/dL, Table 2).

3.3 | Effects of intracavernosal CEO and E on erectile function

Erectile responses were decreased in diabetic animals (p < .01; Figure 1). Following CEO intracavernosal injection (Figure 1a,b), increase in the ICP/MAP (p = .82 versus controls) and the total ICP values (p = .34 versus controls) were observed in diabetic animals (Figure 1a,b). The ICP/MAP (p = .45 versus controls) and the total ICP (p = .71 versus controls) values in diabetic rats were recovered after the intracavernosal administration of E (Figure 1c,d).

TABLE 1 The essential oil composition of the clove oil

				Dilution 1:50	Dilution 1:100	
Peak #	Compound	RT (min)	Retention index	Relative abundance (%)		Mean
1	α-Pinene	10.604	947	0.3	0.25	0.28
2	Limonene	14.698	1045	0.37	0.32	0.35
3	*p*-Cymene	14.842	1048	0.27	0.21	0.24
4	1,8-Cineole	15.253	1057	5.8	5.64	5.72
5	Phenethyl alcohol	21.442	1186	0.71	0.64	0.68
6	Benzyl acetate	22.584	1210	1.07	1.03	1.05
7	Methyl chavicol = Estragole	23.933	1238	0.03	0.16	0.10
8	α-Terpineol	24.318	1246	0.91	0.89	0.90
9	Linalyl acetate	26.100	1282	0.05	0.05	0.05
10	Nerol	27.052	1302	0.19	0.22	0.21
11	Eugenol	32.775	1423	90.3	90.59	90.45
				100.0	100.0	100.0

3.4 | In vitro responses of CC strips

The highest relaxation to CEO in CC obtained from control rats was 97.8 ± 0.2%, which was not changed in diabetic CC (100.0 ± 0.1%, p = .66 versus controls; Figure 2a). Moreover, maximum relaxation responses induced by E in control and diabetic rats were 97.9 ± 1.8% and 99.5 ± 0.4%, respectively (p = .35 versus controls; Figure 2b).

CEO caused 23% (p < .001) relaxation after KCl-induced pre-contraction, which was 77% less than after Phe-induced pre-contraction (Figure 3a). The relaxant responses to CEO were not affected by L-NAME (94.6 ± 4.0%, p = .79; Figure 3b) and ODQ (93.8 ± 4.2%, p = .26; Figure 3c). Nifedipine did not affect CEO-induced relaxation responses (p = .12; Figure 3d). TEA inhibited 57% of CEO-evoked maximal relaxation in control cavernosal tissue (p < .01; Figure 3e). K_{ATP} channel blocker glibenclamide inhibited 45% of CEO-induced maximum relaxation in controls (p < .001; Figure 3f).

E induced 32% (p < .001) relaxation after KCl-induced pre-contraction, which was 67% lower than after Phe-induced pre-contraction (Figure 4a). The relaxation response to E did not alter after the incubation with NOS (94.8 ± 2.4%, p = .43; Figure 4b) and sGC inhibitor (91.9 ± 0.4%, p = .15; Figure 4c). Nifedipine did not change E (p = .07)-induced relaxation responses (Figure 4d), but the relaxation response to E was increased after the presence of nifedipine at 26 mg (p < .05). The incubation with TEA did not affect relaxation response to E in control cavernosal tissues (p = .34, Figure 4e).

TABLE 2 Characteristics of animals

	Control	Diabetic
Body weight (g)	394.5 ± 12.8	319.1 ± 5.8**
Blood glucose (mg/dL)	99.3 ± 4.0	405.1 ± 20.8***

Note: Values are the mean ± *SEM* from n = 8–10 rats per group.
**p < .01,
***p < .001 versus control group.

Glibenclamide decreased 22% of E (p < .01)-induced maximum relaxation in controls (Figure 4f).

4 | DISCUSSION

The current data demonstrate that (a) CEO and E improve erectile responses in the diabetic group; (b) CEO and E cause relaxation in the cavernosal smooth muscle obtained from both groups; (c) CEO- and E-induced relaxation responses are independent to NO/sGC/cyclic guanosine monophosphate (cGMP) pathway; and (d) K^+ channels may have an essential role in CEO- and E-induced relaxation responses.

We demonstrated that erectile function responses in diabetic rats were lessened compared to healthy rats. Following the intracavernosal injection of CEO and E, the ICP/MAP and the total ICP values were significantly improved in diabetic rats. Also, CEO and E remarkably relaxed both control and diabetic rat cavernosal tissue after α1-adrenergic receptor agonist, Phe-induced pre-contraction. There are no previous data regarding evaluating the impacts of CEO and E on ED. Previous studies demonstrated that in male rats, the treatment with the flower buds of *S. aromaticum* ethanolic extract increased sexual behaviour (Tajuddin et al., 2003, 2004). In addition, oral treatment with *S. aromaticum* hexane extract in male mice enhanced the serum level of testosterone (Mishra & Singh, 2008). Furthermore, the methanolic extract of *S. aromaticum* displayed a modest inhibitor of Rho-kinase 2 (Goswami et al., 2012). Moreover, the treatment with E in rats had positive effects on diabetes-induced defects in conduction velocities of motor and sensory nerve, as well as blood flow of nerve due to the improvement in NO, and endothelium-mediated alterations (Nangle, Gibson, Cotter, & Cameron, 2006). The current data indicate a rationale for pre-clinical and clinical studies with combinations of CEO and/or E and PDE-5i in diabetic ED.

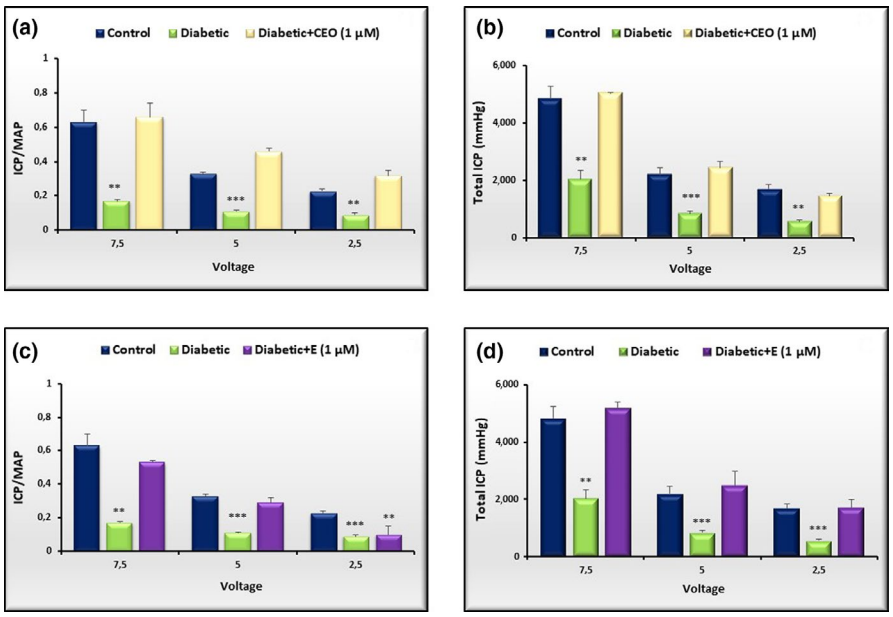

FIGURE 1 In vivo intracavernosal effect of CEO (a and b) and E (c and d) on the penile erection of diabetic and control rats. Data represent the mean ± standard error of the mean of 6–8 observations. **p<.01, ***p<.001 versus control value

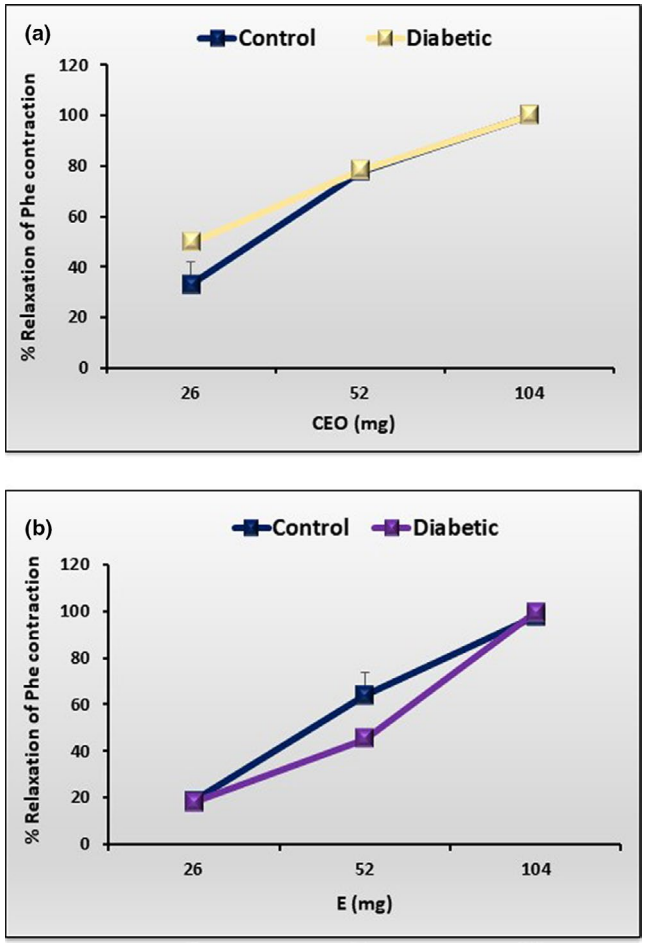

FIGURE 2 Concentration-response curves to CEO (a) and E (b) after pre-contraction with Phe (10^{-5} M) in control and diabetic rat CC

The current data showed CEO- and E-induced CC relaxation that cannot be dependent on the NO-cGMP pathway. There have been no studies on the mechanism of CEO and E in the penile tissue. Damiani et al. showed that the incubation with NOS inhibitor and methylene blue, sGC inhibitor, did not change the maximal relaxation to E in rat thoracic aorta (Damiani, Rossoni, & Vassallo, 2003). Furthermore, the intravenous injection of E induced dose-dependent hypotension (Lahlou, Interaminense, Magalhaes, Leal-Cardoso, & Duarte, 2004) and relaxation in the mesenteric vascular bed, that did not affect via NOS as well as voltage-dependent calcium channel inhibition in anaesthetised animals (Criddle, Madeira, & Soares de Moura, 2003). Also, the current data demonstrated that nifedipine, L-type calcium channel inhibitor, did not affect the maximum relaxation to CEO and E in cavernosal tissue of rats. Similarly, E relaxed the isolated ileum from rats, as well as inhibited contractions via a direct effect on the ileum smooth muscle (Leal-Cardoso et al., 2002). Furthermore, earlier data demonstrated that Phe-induced contractions were inhibited by either E or nifedipine in the aortic rings (Damiani et al., 2003). Interestingly, the inhibitory effect was increased after the incubation with E plus nifedipine (Damiani et al., 2003). It could be recommended that K^+ channels direct effect on cavernosal smooth muscle might have a more significant function. As supportive data, our results showed ATP-sensitive K^+ channel blocker; glibenclamide blocked 45% of CEO and 22% of E-induced relaxation responses in rat CC. In addition, voltage-dependent K^+ channel blocker, TEA blocked 57% of CEO-induced maximum relaxation in rat CC. Similarly, Criddle et al. (2003) showed that TEA did not affect E-induced vasodilation in the mesenteric vascular bed. Taken together, the relaxation effects of CEO and E on cavernosal smooth muscle are likely to depend on K^+ channels and be independent of the NO signalling pathway. CEO and E had different relaxant effects on CC smooth muscle in the presence of TEA. Many plant species have pharmacological activities attributable to their phytoconstituents, for instance, glycosides, saponins, flavonoids, steroids, tannins, alkaloids and terpenes (Batiha et al., 2020). CEO includes many components such as flavonoids, alkaloids and monoterpenes, and has broad pharmacological efficacy (Osanloo, Sedaghat, Esmaeili, & Amani, 2018; Salim, 2017). Furthermore, the pharmacological effects of mentioned constituents are different, for example, the main site action of flavonoids is acetylcholinesterase (Perumalsamy, Jang, Kim, Kadarkarai, & Ahn, 2015), as well as alkaloids and monoterpenes target Na-K-ATPase or Na^+ and K^+ channels (Lucia, Zerba, & Masuh, 2013; Rajashekar & Shivanandappa, 2017). The differences between the pharmacological activities of CEO and its isolated compound, E may result from the synergistic or additive effects of the secondary compounds. Furthermore, alterations in the structure/function/activity of K^+ channels may underlie at least some aspects of observed diabetes-related differences in tissue sensitivity to K^+ channel modulators (Venkateswarlu et al., 2002). The period of diabetes mellitus may play a key role in the K_{ATP} channels function in rat CC (Ghasemi, Sadeghipour, Asadi, & Dehpour, 2007). A previous study showed that the relaxation responses to K_{ATP} channel modulators were attenuated in human isolated corporal tissue strips from diabetic patients with ED (Venkateswarlu et al., 2002). These results may be supported further studies using combinations of these compounds and PDE-5i for the treatment of diabetic ED.

The KCl-induced contraction is produced as a result of membrane depolarisation, which induces an increase in the influx of Ca^{2+} (Ebeigbe & Aloamaka, 1987). In the current study, KCl-contracted cavernosal tissue caused concentration-dependent relaxations after cumulative additions of CEO (22.6%) and E (31.9%). The pre-contraction of the CC with 60 mmol KCl markedly reduced CEO- and E-induced relaxations when compared with the pre-contraction of the CC with Phe. Our data indicate that high K^+ caused inhibition on CEO- and E-induced relaxations in rat cavernosal tissue, suggesting this reduction could be mediated by K^+ conductance channels such as K_{ATP} channels. There are no previous data evaluating the impacts of CEO and E on ED. The previous study showed that E caused a reversible and dose-dependent vasorelaxation in rat mesenteric vascular bed after KCl-induced contraction (Lahlou et al., 2004). The relaxant effects of E on Phe and KCl (75 mM) pre-contracted aorta were resembled, without considerable difference in both maximal response and sensitivity (Damiani et al., 2003). We further suggest that the relaxant effects of E may be dependent on the given dose and types of tissue.

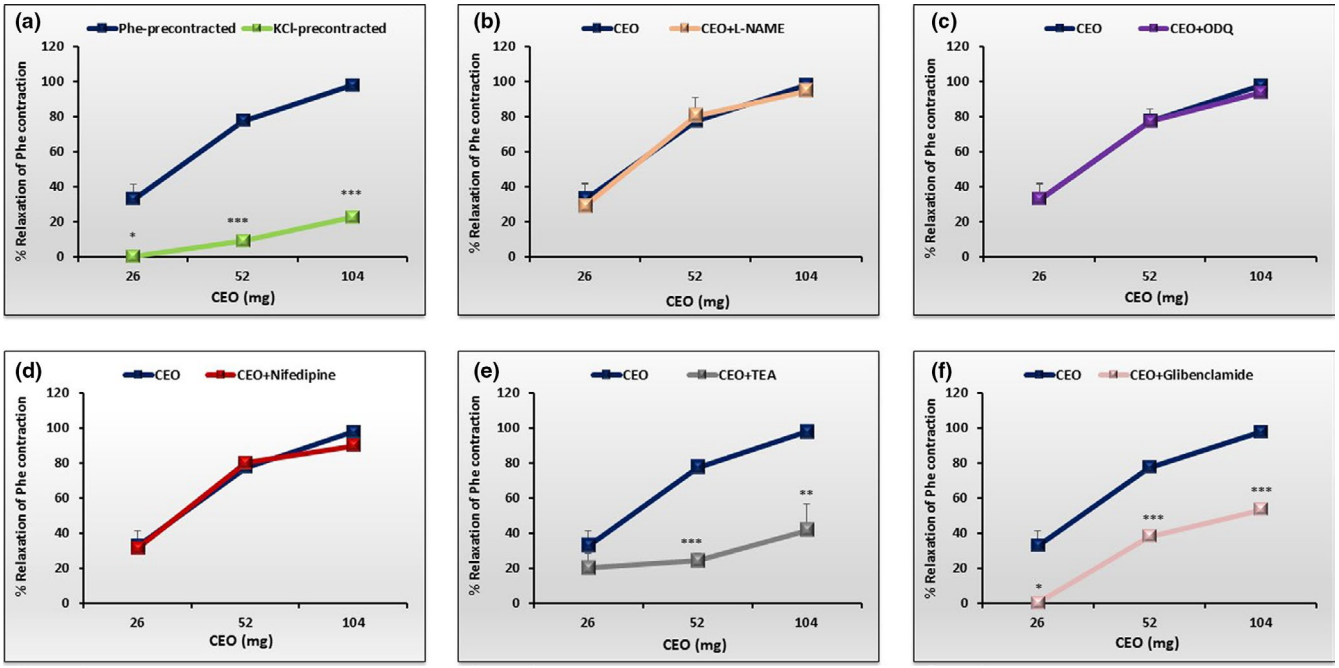

FIGURE 3 Concentration-response curves to CEO (26-104 mg) in CC after pre-contraction with KCl (a) and Phe (10^{-5} M) in the incubation with L-NAME (100 μM; b), ODQ (30 μM; c), nifedipine (10 μM; d), TEA (100 μM; e) and glibenclamide (10 μM; f).*p<.05, **p<.01, ***p<.001 versus control value (n = 6–8)

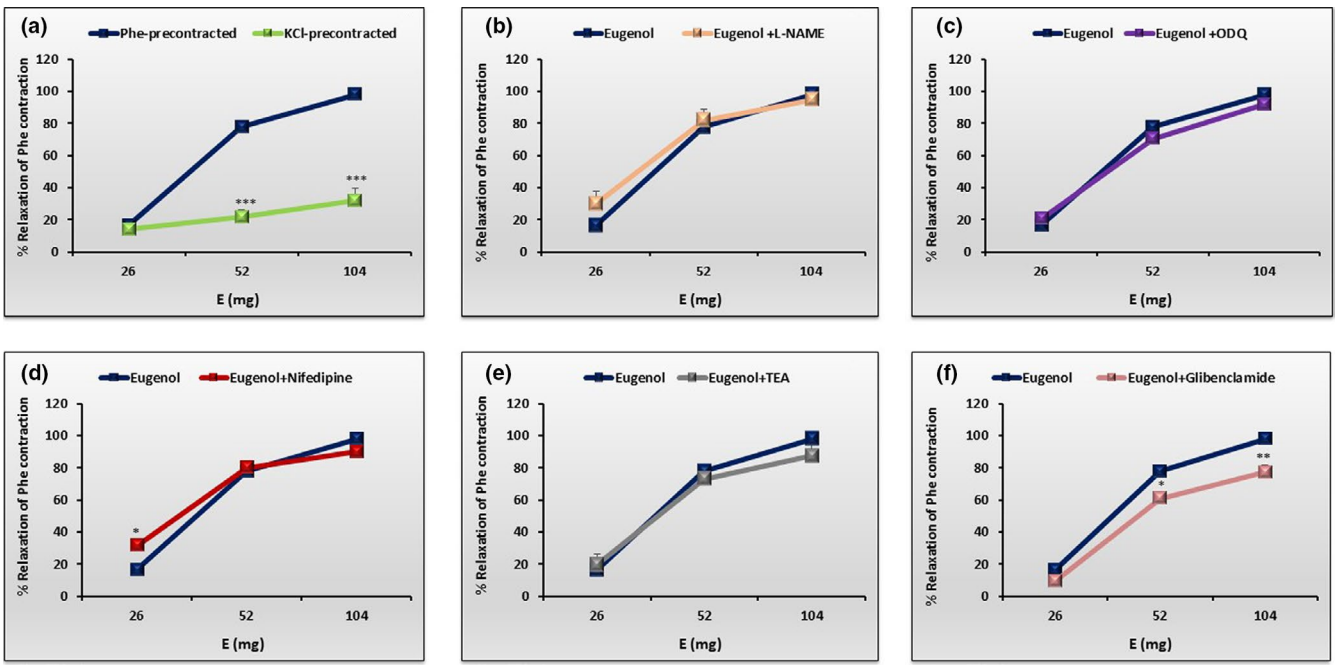

FIGURE 4 Concentration-response curves to E (26-104 mg) in CC after pre-contraction with KCl (a) and Phe (10^{-5} M) in the presence of L-NAME (100 μM; b), ODQ (30 μM; c), nifedipine (10 μM; d), TEA (100 μM; e) and glibenclamide (10 μM; f).*p<.05, **p<.01, ***p<.001 versus control value (n = 6–8)

In conclusion, the present data firstly revealed that CEO and E improve erectile function in the diabetic group, and cause relaxation in rat CC in a NO/cGMP-independent manner. The pre-clinical trials should broaden our knowledge of the beneficial effect of CEO and E on erectile function, and more research is required to address the combined therapy with PDE-5i plus CEO or/and E for diabetic ED.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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