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RESEARCH ARTICLE

Investigation of the Effects of Oleuropein on Mouse Detrusor Muscle Contractility

Elif Nur BARUT[*°], Ahmet Can OZTURK[**], Seckin ENGIN[***], Gulin RENDA[****]

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SUMMARY

Oleuropein (OLE) is an important bioactive compound isolated from Olea europaea L. (olive), the best-known species of the genus Olea, which grows in the Mediterranean region and has many biological activities. In this study, we aimed to investigate the effect of OLE on isolated mouse detrusor smooth muscle (DSM) contractility. OLE was isolated from Olea europaea L and the effects of OLE on the contractility of DSM strips were investigated using in vitro isolated organ bath systems. The relaxant effect of OLE (10^{-8}-10^{-3} M) cumulatively on KCl-precontracted strips was evaluated. Moreover, the effect of OLE (10^{-4} and 10^{-3} M) incubation on KCl-, carbachol (CCh)- or electrical field stimulation (EFS)-induced contractile responses of detrusor strips were assessed concentration-dependently. OLE at higher concentration (10^{-3} M) relaxed KCl-precontracted detrusor strips significantly. Moreover, the contractile responses to KCl, CCh, and EFS were significantly decreased in the strips preincubated with OLE (10^{-3} M) ($p<0.05$). Our results showed that OLE decreased the contractility of isolated mouse detrusor strips in a concentration-dependent manner and thus it would be a promising agent in pathological conditions related to increased bladder contractility such as overactive bladder. Additionally, further studies are needed to elucidate the exact mechanisms of these effects of OLE.

Key Words: *Carbachol, detrusor smooth muscle, isolated organ bath, mice, oleuropein*

Oleuropeinin Fare Detrusor Kası Kasılması Üzerine Etkilerinin Araştırılması

ÖZ

Oleuropein (OLE), Akdeniz bölgesinde yetişen ve birçok biyolojik aktiviteye sahip olan Olea cinsinin en iyi bilinen türü olan Olea europaea L.'den (zeytin) izole edilen önemli bir biyoaktif bileşiktir. Bu çalışmada Olea europaea L.'den izole edlen OLE'nin izole fare detrusor düz kas kontraktilitesine etkisini incelemeyi amaçladık. OLE, Olea europaea L'den izole edildi ve in vitro organ banyosu sistemi kullanılarak izole fare detrusor düz kas şeritlerinin kontraktilitesi üzerine etkisi incelendi. Kümülatif OLE'(10^{-8}-10^{-3} M) uygulamasının KCl ile ön-kasılma oluşturulmuş detrusor şeritleri üzerinde gevşetici etkisi araştırıldı. Ayrıca OLE (10^{-4} ve 10^{-3} M) inkübasyonunun KCl, karbakol (CCh) ve elektriksel alan stimülasyonu (EAS) ile indüklenen kasılma yanıtları üzerine konsantrasyona-bağımlı etkisi değerlendirildi. KCl ile ön kasılma oluşturulmuş detrusor şeritlerinde OLE, yüksek konsantrasyonda (10^{-3} M) belirgin gevşeme yanıtına neden oldu. Ayrıca OLE (10^{-3} M) inkübasyonu KCl, CCh ve EAS ile indüklenen kasılma yanıtlarını anlamlı olarak azalttı ($p<0.05$). Elde ettiğimiz sonuçlar, OLE'nin izole fare detrusor düz kas kontraktilitesini konsantrasyona-bağımlı olarak azalttığını ve böylece aşırı aktif mesane gibi mesane kontraktilitesinin arttığı patolojik durumlarda umut vaat edici bir ajan olabileceğini göstermektedir. OLE'nin bu etkilerinin mekanizmasının aydınlatılabilmesi için ileri çalışmaların yapılması gerekmektedir.

Anahtar Kelimeler: *Karbakol, detrusor düz kası, izole organ banyosu, fare, oleuropein*

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[*] ORCID: 0000-0003-3284-848X, Department of Pharmacology, Faculty of Pharmacy, Karadeniz Technical University Trabzon, Türkiye.
[**] ORCID: 0009-0001-6803-4018, Department of Pharmacology, Faculty of Pharmacy, Karadeniz Technical University Trabzon, Türkiye.
[***] ORCID: 0000-0002-1982-7820, Department of Pharmacology, Faculty of Pharmacy, Karadeniz Technical University Trabzon, Türkiye.
[****] ORCID: 0000-0001-6323-0338, Department of Pharmacognosy, Faculty of Pharmacy, Karadeniz Technical University Trabzon, Türkiye.

[°] Corresponding Author; Elif Nur Barut
E-mail: elifgazioglu@ktu.edu.tr

INTRODUCTION

The Oleaceae family includes about 600 species of deciduous trees and shrubs. The genus *Olea*, belonging to the Oleaceae family, consists of 30 species, but *Olea europaea L.* also known as olive tree is the best-known species of the *Olea* genus, used as food and grown in the Mediterranean region. *Olea europaea* is generally grown in tropical and warm temperate regions of the world. It is generally spread in the coastal regions of the Eastern Mediterranean region. Today, 97% of olive trees are located in Mediterranean countries, including Türkiye (Ghanbari et al., 2012; Ozturk et al., 2021). Mediterranean countries have been cultivating olives since ancient times to obtain both oil and medicinal compounds (Topuz & Bayram 2021). The olive leaf extracts have been used for centuries to protect human health against various diseases (Ghanbari et al., 2012). The components responsible for the biological activities of the leaves and fruits of the olive tree are considered phenolic compounds and it is known that their concentrations vary between species. *Olea europaea*, which is widely distributed in Türkiye, contains dominant phenolic oleosides such as oleuropein (OLE), demethyl-oleuropein, ligstroside and oleoside (Omar 2010).

OLE is a phenolic iridoid glycoside, formed by the combination of hydroxytyrosol, elenolic acid, and glucose molecules, predominantly found in the oil and leaves of the plant and it is responsible for the diverse pharmacological effects of *Olea europaea* (Hassen et al., 2015; Rahiman et al., 2019). OLE is a phenolic secoiridoid formed by the combination of hydroxytyrosol, elenolic acid, and glucose molecules (Hassen et al., 2015). The antioxidant, anti-inflammatory, antimicrobial, antifungal, anticancer, hypoglycemic, antihyperlipidemic and cardioprotective effects of OLE have been shown in several *in vivo* and *in vitro* studies (Carrera-González et al., 2013; Sepporta et al., 2014; Hassen et al, 2015). OLE treatment also decreases blood glucose and improves glucose tolerance in animal models of alloxan or streptozotocin-induced diabetes. It has also been reported that OLE reduces the amount of serum malondialdehyde, increases the amount of reduced glutathione, and has an antioxidant effect by increasing the activity of superoxide dismutase (Qadir et al., 2016; Khalil et al., 2017).

The effects of OLE on vascular smooth muscle contractility have also been investigated. Gilani et al. determined that the leaf extracts of *Olea europaea* had a hypotensive effect due to Ca^{2+} channel blocking action and Scheffler et al. showed that OLE was responsible for this effect (Gilani et al., 2005; Scheffler et al., 2008). In addition, OLE was shown to have a relaxant effect on the contractions induced by phenylephrine in rat thoracic aorta preparations, and endothelial-derived nitric oxide was found to mediate the vasorelaxant effect of OLE (Ilic et al., 2021). In rat gastric fundus, it was shown that OLE caused a marked relaxation in the fundus muscle than other dietary polyphenols, and nitric oxide (NO) formation was responsible for this effect (Rocha et al., 2009). However, the information about the effects of OLE on urinary tract smooth muscle contractility is very limited, and there is no study available reporting its effect on detrusor smooth muscle (DSM) contractility.

Acetylcholine (ACh) is the main neurotransmitter that regulates detrusor contractility. During the micturition phase, ACh stimulates muscarinic receptors that mediate bladder contraction (Merril et al., 2016). DSM relaxation is also mediated by the release of catecholamines from sympathetic nerve terminals and catecholamine-mediated activation of β-adrenoreceptors (Propping et al., 2015). Various ion channels play a major role in the activity of bladder DSM such as voltage dependent calcium and different types of potassium channels. It has been found that voltage-dependent calcium channels allow the calcium influx that results in bladder contraction and it has also been reported that nitric oxide causes relaxation by reducing intracellular calcium levels in DSM (Andersson & Arner 2004; Petkov et al., 2011).

Disturbances in the regulation of detrusor contractility can cause bladder dysfunction, ranging from bladder atony to overactive bladder related to poor quality of life in patients. Currently, medical treatment options for bladder dysfunctions are limited and patient compliance problems may occur due to the side effects of therapeutic agents (Marinkovic, 2019). Therefore, there is an urgent need for potential therapeutics that can regulate bladder contractility with adequate efficacy and appropriate safety profile.

In this study, we aimed to identify the potential effect of OLE on the contractility of the mouse DSM.

MATERIALS AND METHODS

Chemicals

CCh (muscarinic agonist) was purchased from Sigma Aldrich (St. Louis, Missouri, USA). *n*-hexane (104368, Merck), methanol (24229, Riedel-De-Haën), ethyl acetate (100864, Merck), TLC (silica gel aluminum plates, SiO_2 60 F_{254}, 5554, Merck) were used. Silica gel 60 (Kieselgel 60, 70-230 mesh, 107734, Merck) was employed for open column chromatography (CC). Reversed-phase material (LiChroprep C18) (25-40 µm, 09303, Merck) was utilized for vacuum liquid chromatography (VLC). CCh, KCl and OLE were dissolved in distilled water.

Extraction and isolation of OLE

The air-dried and grounded leaves of *O. europaea* (352 g) were extracted with 70% methanol (2 L) at 40 °C by a rotary evaporator under reflux. The procedure was repeated four times, and the extracted materials were filtered. The filtrates had been combined and evaporated to dryness with a rotary evaporator at 40 °C under reduced pressure. The crude extract (69.8 g, yielding 19.8%) was dispersed in a mixture of methanol and water (1:9) and partitioned with *n*-hexane and ethyl acetate, respectively, by using a separatory funnel (Cifa et al, 2018; Otero et al., 2020). The ethyl acetate sub-extract (26.7 g) was subjected to VLC over reversed-phase material (LiChroprep C18), and elution with increasing concentrations of MeOH in H_2O mixtures (in steps of 10% of MeOH, each 200 mL, fraction volumes 200 mL) as eluent, yielded 5 fractions (fractions A-E). The fractions obtained with the elutions were monitored by the TLC system and combined according to the TLC results. Fraction E (11.3 g) was subjected to chromatographic separation on a silica gel (100 g) column using $CHCl_3$/MeOH with increasing polarity (100:0 to 90:10 mixtures and combined fractions afforded OLE (6.35 g). The compound's structure was determined by comparing its spectroscopic data to those described in the literature (Zun-Qiu et al., 2015).

Animals

Adult female Balb/C mice (6-8 weeks old) were used in the study. The animals were housed in the humidity and temperature-controlled (22 ± 1 °C) rooms with access to food and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Care and Ethics Committee (Approval number: 2022/04).

DSM strips preparation

The mice were euthanized by cervical dislocation then bladders were identified with abdominal incision and then quickly transferred in Krebs-Henseleit solution (mmol/L: NaCl 118, KCl 4.7, NaH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.5, $NaHCO_3$ 25 ve glucose 11). The sphincter and trigon were removed and DSM strips (3-4 mm long and 2–3 mm wide) were prepared (Engin et al., 2022; Engin et al., 2023).

Contractility studies

The detrusor strips were placed in 30 mL isolated organ baths with Krebs-Henseleit solution at 37 °C and bubbled with carbogen (95% O_2 and 5% CO_2) during the experiments. To determine the tissue contractility, strip was mounted to an isometric force transducer (MAY FDT10A, Commat, Ankara, Türkiye) attached to data acquisition/recording system (BIOPAC MP 35, Goleta, USA). The strips were placed organ bath under 1 g resting tension then stabilized with the equilibrate period for 60 min.

Strips were wash out with fresh bath solution every 20 min. After the equilibration period, DSM strips were contracted with 80 mM KCl to confirm the viability of the strips. All experimental procedures were set up based on previous studies (Engin et al., 2022; Engin et al., 2023).

Effect of OLE on KCl-precontracted DSM strips

To test the effect of OLE on DSM strips precontracted with KCl, KCl (80 mM) was added to the organ bath and the contractile response was waited until it reached a plateau. Then cumulative concentration-dependent responses of OLE (10^{-8}-10^{-3} M) on DSM strips were recorded. The relaxant response was reported as the percentage of precontraction with KCl.

Effect of OLE on KCl-induced contractions of DSM strips

To investigate the effect of OLE on KCl-induced contractions of DSM strips, the contractile control response was obtained with 80 mM KCl. After a washout period, the strips were incubated with OLE at 10^{-4} M or 10^{-3} M for 20 min and then KCl-induced contractile responses were repeated. The contractile responses were reported as the percentage of maximal control response.

Effect of OLE on CCh-induced contractions of DSM strips

To understand the effect of OLE on CCh-induced contractions of DSM strips, a cumulative response to CCh (10^{-8}-10^{-4} M) was obtained as a control. Following an equilibration period, DSM strips were incubated with OLE at 10^{-4} M and 10^{-3} M for 20 min and then concentration-dependent contractile responses to cumulative CCh were repeated. The responses were expressed as the percentage of maximal control contraction induced by CCh.

Effect of OLE on nerve-evoked contractions of DSM strips

To determine the effect of OLE on the nerve-evoked contraction of DSM strips, the contractile responses were assessed by EFS before and after the incubation with OLE (10^{-3} M) for 20 min at the frequencies of 8, 16 and 32 Hz (50 V, 0.2 msec duration) (Engin et al., 2023).

Statistical analysis

Data were expressed as the mean ± standard error. Statistical analysis was performed using GraphPad Prism (Version 5.01; GraphPad Software, San Diego, CA, USA). The differences between groups were compared with student's *t* test or one-way ANOVA, followed by Bonferroni multiple comparison test and $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Effect of OLE on KCl-precontracted DSM strips

OLE at low concentrations (10^{-8}-10^{-4} M) did not produce a marked relaxation response in KCl-precontracted DSM strips, while the highest concentration of OLE (10^{-3} M) produced a significant relaxation response (Figure 1.).

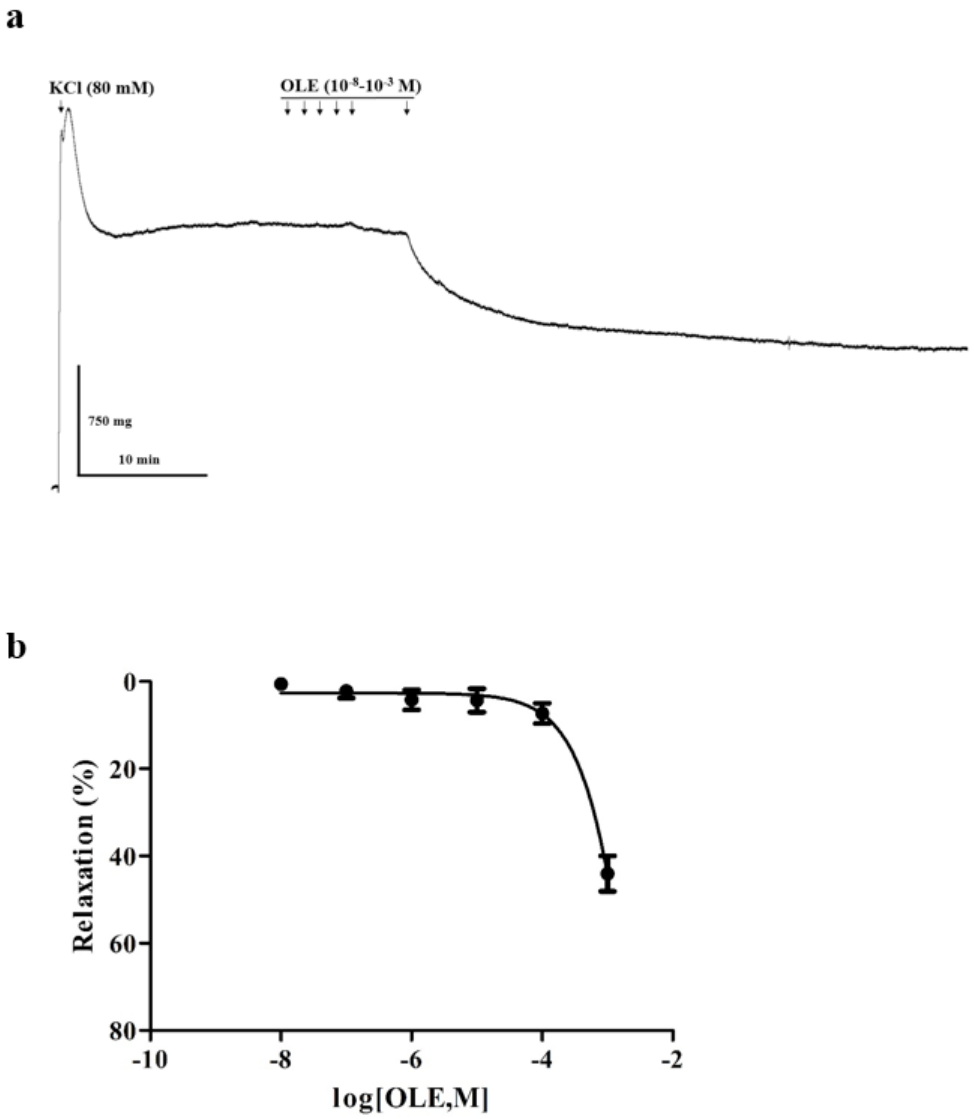

Figure 1. Effect of OLE on the contractility of DSM strips pre-contracted with KCl (80 mM) Data were expressed as mean±standard error (n=4). (a) Representative trace showing the relaxant effect of OLE on DSM strips pre-contracted with KCl. (b) Cumulative concentration-response curve of the inhibitory response induced by OLE (10^{-8}-10^{-3} M) in KCl pre-contracted DSM strips.

Effect of OLE on KCl-induced contractions of DSM strips

Preincubation with OLE at 10^{-4} M did not alter KCl-induced contractions of DSM strips. However, there was a statistically marked decrease in the KCl-induced contractions after the incubation with OLE at 10^{-3} M (Figure 2., $p<0.001$).

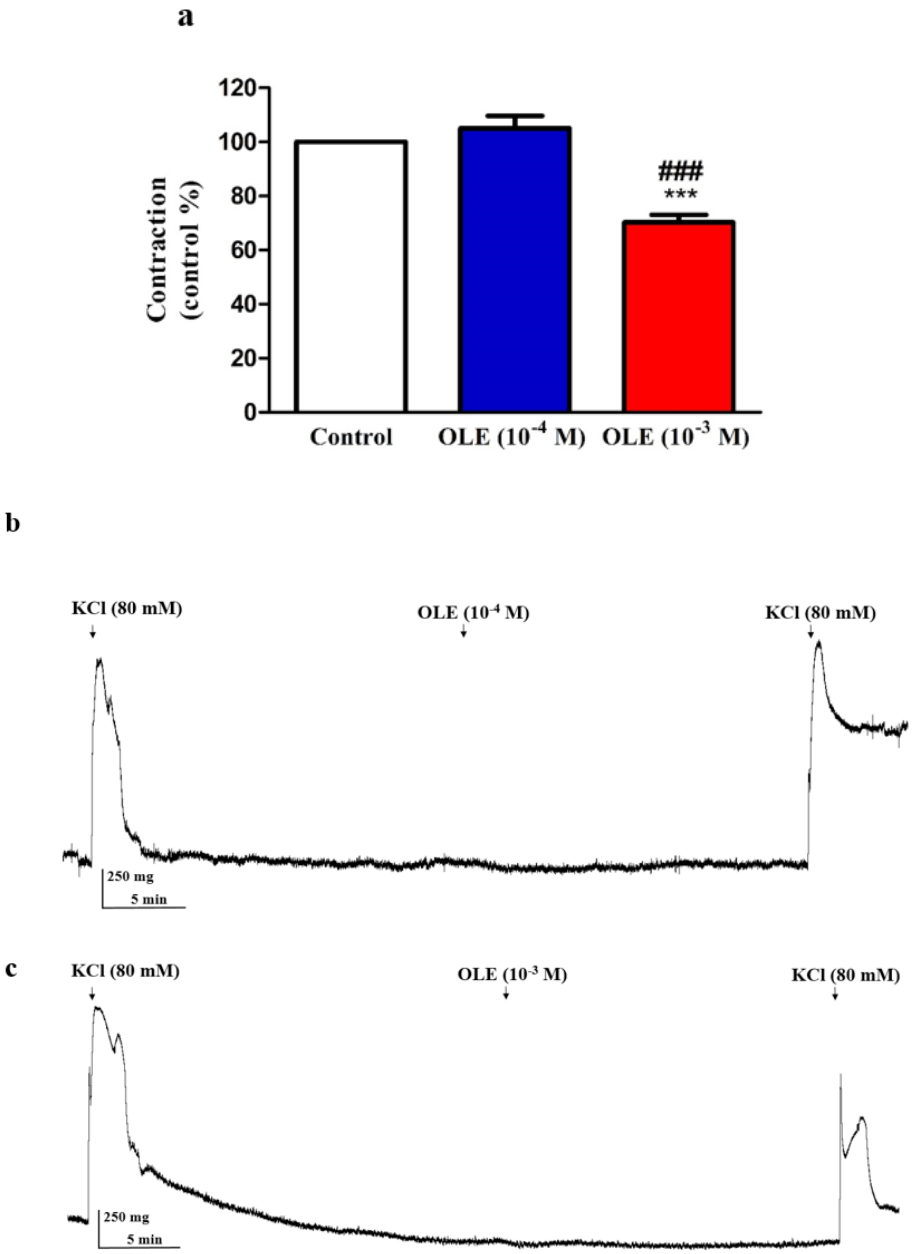

Figure 2. Effect of OLE incubation on KCl-induced contractions of DSM strips. Data were expressed as mean±standard error (n=4). (a) Effect of OLE (10^{-4}, 10^{-3} M) incubation on the maximum contractile response induced by KCl in DSM strips. Representative trace showing the effect of OLE incubation at (b) 10^{-4} M and (c) 10^{-3} M on the maximal contractile response induced by KCl in DSM strips. ***$p<0.001$ significantly different from the control, $^{###}p<0.001$ significantly different from the OLE (10^{-4} M).

Effect of OLE on CCh-induced contractions of DSM strips

CCh-induced contractile responses of DSM strips obtained following the incubation with 10^{-4} M OLE were similar to the that of control, but a statistically significant decrease was found in the contractions induced by higher concentrations of CCh (10^{-5} and 10^{-4} M) after the incubation with 10^{-3} M OLE (Figure 3., $p<0.001$).

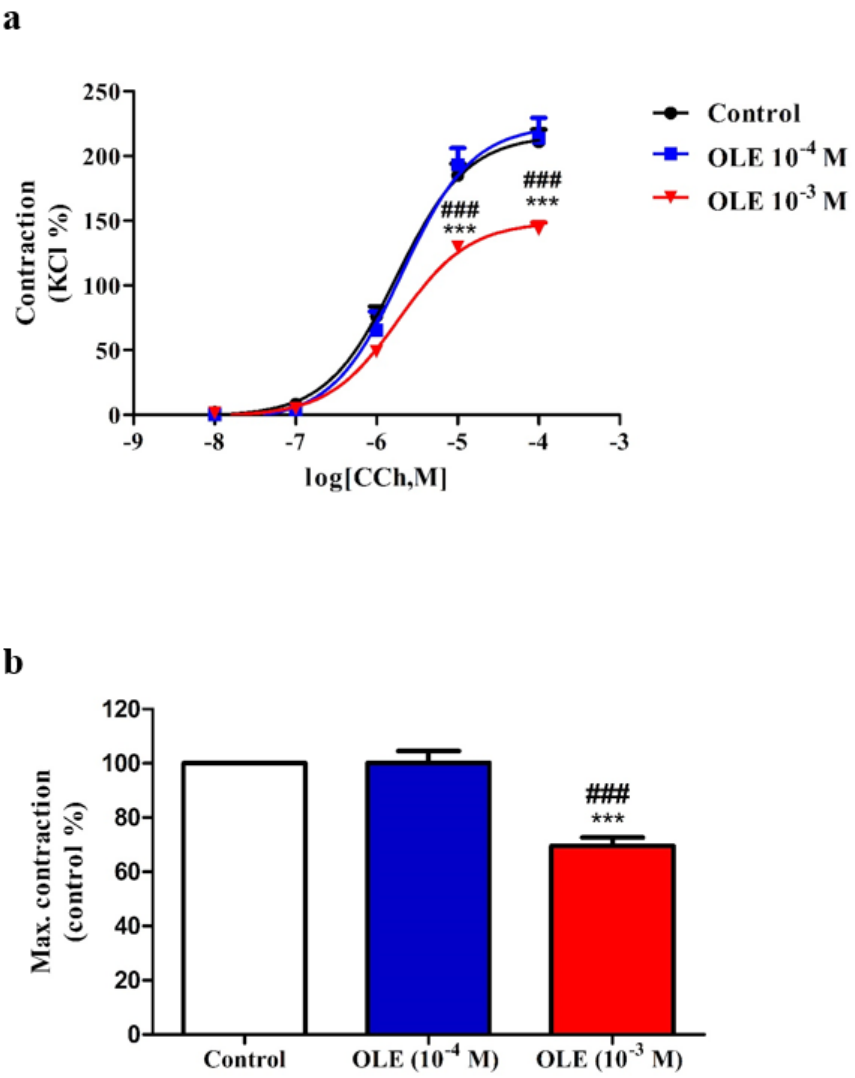

Figure 3. Effect of OLE incubation on CCh-induced contractions of DSM strips. Data were expressed as mean±standard error (n=4). (a) Concentration-response curves showing the inhibitory effect of OLE (10^{-4}, 10^{-3} M) incubations on CCh-induced contractions in DSM strips. (b) Effect of OLE (10^{-4}, 10^{-3} M) incubation on the maximum contractile response induced by CCh in DSM strips. ***p<0.001 significantly different from the control, ###p<0.001 significantly different from the control OLE (10^{-4} M).

Effect of OLE on nerve-evoked contractions of DSM strips

The contractile responses induced by EFS (8, 16 and 32 Hz) were evaluated in DSM strips after incubation with the highest concentration of OLE tested, which was observed to reduce contractile responses in previous experiments. After the incubation period with 10^{-3} M OLE, there was a statistically significant decrease in 32 Hz-induced contractions in EFS-induced contractions of DSM strips (Figure 4., p<0.05).

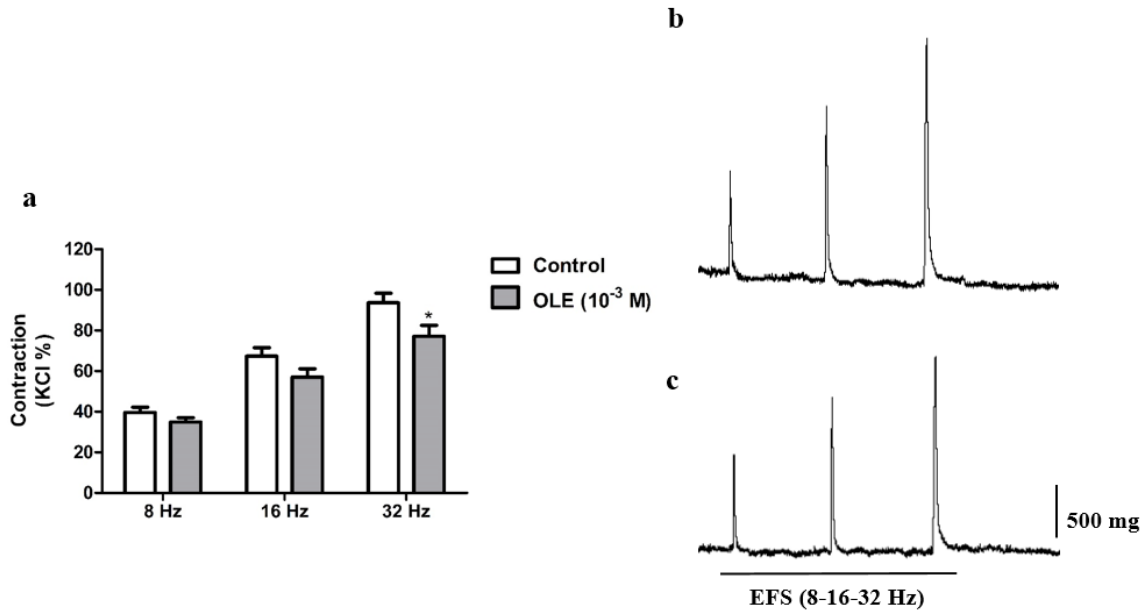

Figure 4. Effect of OLE incubation on EFS-induced neurogenic contractions of DSM strips. Data were expressed as mean±standard error (n=4). (a) Effect of OLE (10^{-3} M) incubation on the maximum contractile response induced by EFS (8-16-32 Hz) in DSM strips. (b) Representative trace showing EFS-induced contractions of DSM strips (b) in the absence (control) and (c) in the presence of OLE (10^{-3} M). *p<0.05 significantly different from the control group.

In our study, we found that the highest concentration of OLE (10^{-3} M) produced a significant relaxation in KCl-precontracted DSM strips. In addition, while there was no significant change in KCl responses following OLE incubation at 10^{-4} M compared to the control group, a significant decrease was found in the KCl responses after the OLE incubation at 10^{-3} M. Previous studies have shown that leaf extract of *Olea europaea* mediated vasorelaxation via inhibiting Ca^{2+} channel in aortic smooth muscle, and OLE has been shown the primary bioactive compound responsible for this effect. (Gilani et al., 2005; Scheffler et al., 2008). In line with previous studies, we concluded that high concentrations of OLE may reduce DSM contractility via inhibiting the voltage-sensitive Ca^{2+} channels. We also showed that the CCh-induced contractile responses following the preincubation with OLE at 10^{-4} M were not different from the control, but a significant decrease was found in CCh-induced contractile responses of DSM strips after the preincubation with OLE at 10^{-3} M. The reduction of CCh-induced contractions in the presence of OLE may be due to its direct muscarinic receptor antagonist activity or its blocking of intracellular signaling activated by the stimulation of muscarinic receptors.

Detrusor neurogenic contractions are controlled by neurotransmitters such as ACh and ATP via muscarinic and purinergic receptor activation (Fowler et al., 2008). In our study, we observed that incubation with the highest concentration of OLE reduced EFS-induced neurogenic contractions of DSM strips. The mechanisms of the decrement in neurogenic contractions caused by OLE in DSM should be investigated in the presence of various antagonist agents.

CONCLUSION

Disturbances in the regulation of detrusor contractility can cause bladder dysfunction, ranging from bladder atony to overactive bladder. Antimuscarinic drugs and β-agonists are the major therapeutics used

in the pharmacological treatment of overactive bladder, a common bladder dysfunction. However, current pharmacotherapy for overactive bladder has insufficient efficacy and several side effects (Marinkovic, 2019). Therefore, studies are underway to develop potential therapeutics that can regulate bladder contractility with adequate efficacy and appropriate safety profile. In light of our findings, we exhibited that OLE reduces DSM contractility and may be a promising agent in overactive bladder-like bladder dysfunction following the exact mechanism of action clarified.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Conceptualization: ENB, ACO. Methodology: ENB, SE, GR. Data curation, investigation: ENB, ACO, SE, GR. Funding acquisition: ENB, ACO. Writing original draft: ENB, GR. Review & Editing: ENB, ACO, SE, GR.

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