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The Effects of *Rosmarinus officinalis* (Rosemary) Aqueous Extract on Smooth Muscle Contractions in Isolated Rat Urinary Bladder

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Absract: *Rosmarinus officinalis* (Rosemary) is widely used in traditional medicine and it has diuretic and spasmolytic effects on the ileum, trachea, and stomach smooth-muscle. However, a study the related effects of Rosemary on the myogenic activities of urinary bladder smooth-muscles has not been founded. The present study investigated the effects of Rosemary extract on the smooth-muscle contractility of isolated rat urinary bladder. Muscle strips were prepared from urinary bladder of male Sprague Dawley rats. Prepared muscle strips were placed in an isolated organ bath containing Krebs solution. The muscle strips were pre-contracted by inducing with Ach (10^{-4}M) and KCl (60mM), and these values were considered to be reference values. The effects of the different doses (150, 300, 600 and 1200µg/mL) of Rosemary extract on ACh and KCl-induced contractions were determined. It was determined that no different from references values of the contractions induced by ACh and KCl in the presence of Rosemary doses in bath (P>0.05). In the present study, the various doses of Rosemary extract had no effect on myogenic activity in rat urinary bladder smooth-muscle. It may be conceive that the use of Rosemary for different purposes does not result in an undesirable effect on the physiological activities of bladder smooth-muscle.

Keywords: Aqueous extract, Rat, *Rosmarinus officinalis,* Smooth muscle, Urinary bladder.

İzole Edilmiş Rat Mesane Düz Kas Kontraksiyonları Üzerine *Rosmarinus officinalis* (Rosemary) Aköz Ekstraktının Etkileri

Öz: *Rosmarinus officinalis* (Rosemary) geleneksel tıpta yaygın olarak kullanılmaktadır ve o diüretik etkiye ve ileum, trakea, mide düz kasları üzerine gevşetici etkilere sahiptir. Fakat Rosemary'nin idrar kesesi düz kaslarının miyojenik aktiviteleri üzerine herhangi bir çalışmaya rastlanılmamıştır. Bu çalışmada izole edilmiş rat mesane düz kas kontraktilitesi üzerine Rosemary ekstraktının etkileri araştırılmıştır. Kas şeritleri Sprague Dawley cinsi erkek ratların mesanesinden hazırlandı. Hazırlanan kas şeritleri Krebs solüsyonu içeren izole organ banyosuna yerleştirildi. Kas şeritler ACh (10^{-4} M) ve KCl (60 mM) ile uyarılarak kastırıldı ve bu değerler referans değer olarak kabul edildi. ACh ve KCl ile indüklenen kontraksiyonlar üzerine Rosemary ekstraktının farklı dozlarının (150, 300, 600 ve 1200 µg/mL) etkileri belirlendi. Banyoda Rosemary'nin dozlarının varlığında ACh ve KCl ile indüklenen kontraksiyonların referans değerlerden farklılık arz etmediği belirlendi (P>0.05). Mevcut çalışmada, Rosemary ekstraktının çeşitli dozlarının rat idrar kesesi düz kaslarının miyojenik aktivitesi üzerine etkisinin olmadığı görüldü. Çeşitli amaçlar için Rosemary kullanımının idrar kesesi düz kaslarının fizyolojik aktivitesi üzerine istenmeyen etkilerinin olmadığı düşünülebilir.

Anahtar Kelimeler: Düz kas, İdrar kesesi, Rat, *Rosmarinus officinalis*, Sulu ekstrakt.

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INTRODUCTION

Interest in alternative medicine has increased in recent years, and the use of various plants in this area has gained momentum. *Rosmarinus officinalis* (Rosemary) is grown in many the regions of the world (1), and is commonly used in alternative medicine. Important compounds, such as rosmarinic acid, carnosic acid, carnosol, luteolin, kaempferol, and genkwanin, are isolated from Rosemary leaves (2,3), and Rosemary extract has numerous pharmacological activities, such as hepatoprotective (4), antithrombotic (5,6), anti-ulcerogenic (1), antidiabetic, antioxidant (7), antinociceptive (8), and anti-inflammatory effects (9). In addition, Rosemary has potential benefits in the prevention and treatment of several illnesses, such as atherosclerosis, ischemic heart disease, cataracts, and cancer (10), and is also used as an ethopharmacology agent in treating depression (11). Rosemary hydro-alcoholic extract cures ulcerative stomach lesions created with reserpine, ethanol, and indomethacin. However, it has been reported that Rosemary has no antisecretory effect on gastric acid (1). Rosemary volatile oil inhibits contractions in the tracheal smooth-muscles of rabbits and guinea pigs (12), and Rosemary ethanol extract has a spasmolytic effect on guinea-pig ileum (13). Haloui et al. (14) also showed that Rosemary has a diuretic effect. The effects of Rosemary on the various smooth-muscles have been determined in numerous previous studies (12,13,15), but we have found no articles related to its effects on the myogenic activities of bladder smooth-muscles. Plants used with treatment aims may have undesirable side effects on physiological systems. Therefore, the specific effects that they exert on a variety of tissues should be well established. In the present study, we determined the effects of Rosemary on myogenic activities in rat urinary bladder smooth-muscles.

MATERIALS and METHODS

The study was approved by the Local Ethics Committee on Animal Experiments of Atatürk University (Decision no: 2012/70)

Animals

A total of 24 samples from adult male Sprague Dawley rats, weighing 200–250 g, were used. All animals were purchased from the Veterinary Control and Research Institute Management, Erzurum, Turkey. The care of the animals was ensured by the provision of adequate moisture and light, room temperature, and water and food ad libitum until the day of the experiment. The experiments were conducted in accordance with the national guidelines for the use and care of laboratory animals and were approved by the local animal care committee of Atatürk University, Erzurum, Turkey.

Plant Material

Aerial parts of Rosemary were collected in Erzurum in July 2012. The collected leaves were dried in the shade and the dried materials were appropriately preserved in storage boxes in the dark.

Preparation of the Plant Extract

The Rosemary leaves was powdered by grinding with a blender. A total of 25 grams were then taken, to which 500 mL of pure water was added. The mixture was blended in a magnetic stirrer at boiling point for 15 minutes, then cooled at room temperature and filtered with Whatman No. 1 paper. The filtrate was frozen and lyophilized in lyophilizator at 5 µm-Hg pressure at -50 °C. The extract was placed in a glass bottle and then stored at -20 °C up to used (16).

Tissue Preparation

The rats were euthanized under ketamine hydrochloride (Ketalar: 75mg/kg) and xylazine (Rompun: 15 mg/kg) anesthesia by the humane cervical dislocation method. The abdominal cavity was cut open, and the whole bladder was immediately removed and placed in a Petri dish containing Krebs solution (composition: NaCl, 119; KCl, 4.75; $CaCl_2$, 2.5; $MgSO_4$, 1.5; KH_2PO_4, 1.2;

$NaHCO_3$, 25; glucose, 11 mM; and pH 7.4). The fatty tissues and serosal forms in the external parts of the bladder were then cleaned; the bladder was made planar by making a longitudinal incision in a vertical direction from the bladder neck toward its apex and opening the bladder. The bladder mucosa was cleared from mucosal forms by sharp dissection, and 2x10 mm longitudinal sections were taken from the bladder neck toward the apex, in a vertical direction (17,18). Two muscle strips were prepared from each bladder and then placed in a 20 mL organ bath (Emka Technologies, France) containing Krebs solution and gassed with 95% O2/5% CO2 at a stable temperature of 37°C (19). The high end of the muscle strips was bonded to an isometric transducer (ELJ-S045C-EMKA-R04003 ve R04004) and the lower end was bonded to the bath floor by a metal hook. After the muscle strips were stretched for 1 g, they were left to equilibrate for 60 minutes and were washed with fresh Krebs solution every 15 minutes. The contractile activities of the muscles strips were constantly recorded.

The $LogEC_{50}$ Values of the Acetylcholine (ACh) and Potassium Chloride (KCl)

Dose response curves were created using ACh (10^{-10}-10^{-3} M-Figure 1) and KCl (20, 40, 60, and 80 mM-Figure 2). These doses were used for twelve different tissues by repeating them three times. Consequently, the $LogEC_{50}$ doses were determined as -4.85 for ACh and -2.74 for KCl using the Graphpad Prism computer program. Submaximal doses were identified as 10^{-4} for ACh and 60 mM for KCl.

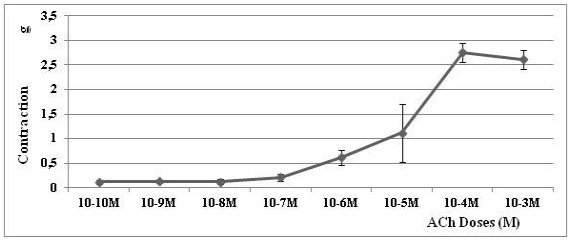

Figure 1. The dose-response curve of ACh.
Şekil 1. ACh'nın doz cevap eğrisi.

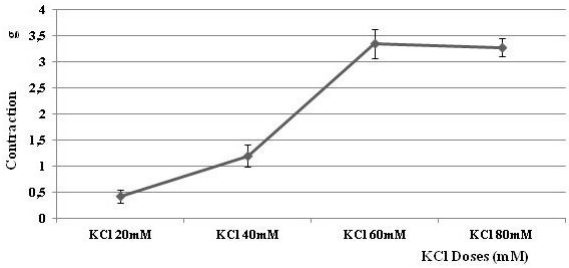

Figure 2. The dose-response curve of KCl.
Şekil 2. KCl'nin doz cevap eğrisi.

Muscle Strips Pre-contracted with ACh

Amplitudes of contractions, stimulated by ACh at a final concentration of 10^{-4} M, were recorded, after which a final concentration of 150 µg/mL Rosemary extract was given to the bath. After 10 minutes of exposure, the amplitudes of the muscular contractions, generated by the application of 10^{-4} M ACh, were recorded. This process was used for twelve different tissues by repeating it three times. The entire protocol was also carried out for the 300, 600, and 1200 µg/mL doses of Rosemary extract.

Muscle Strips Pre-contracted with KCl

Amplitudes of contractions, stimulated by KCl at a final concentration 60 mM, were recorded, after which a final concentration of 150 µg/mL Rosemary extract was given to the bath. After 10 minutes of exposure, the amplitudes of the muscular contractions, generated by the application of 60 mM KCl, were recorded. This process was used for twelve different tissues by repeating it three times. The entire protocol was also carried out for the 300, 600, and 1200 µg/mL doses of the Rosemary extract.

Statistical Analysis

The data were analyzed by using the SPSS version 20.0 statistical software. The data were evaluated by ANOVA, Tukey test. $P<0.05$ was considered as significant.

RESULTS

The Effects of Rosemary Extract on ACh-Induced Bladder Contractions

The contraction values obtained from bladder muscles at a 10^{-4} M dose of ACh were accepted as 100%. In the presence of 150, 300, 600 and 1200 µg/mL doses of Rosemary in the bath was obtained contractions induced with submaximal dose (10^{-4} M) of ACh (Figure 3). These contraction values were compared with the response induced with ACh accepted as 100% (Table 1).

At 150, 300, 600 and 1200 µg/mL doses of Rosemary extract, the responses to 10^{-4} M ACh were 106.75±11.12%, 104.64±12.14%, 97.63±11.42%, and 98.28±10.97%, respectively (Figure 3). According to the indications, it was determined that the 150, 300, 600, and 1200 µg/mL doses of Rosemary extract had no statistically significant effects on smooth-muscle contractions induced by ACh (n: 12, P>0.05).

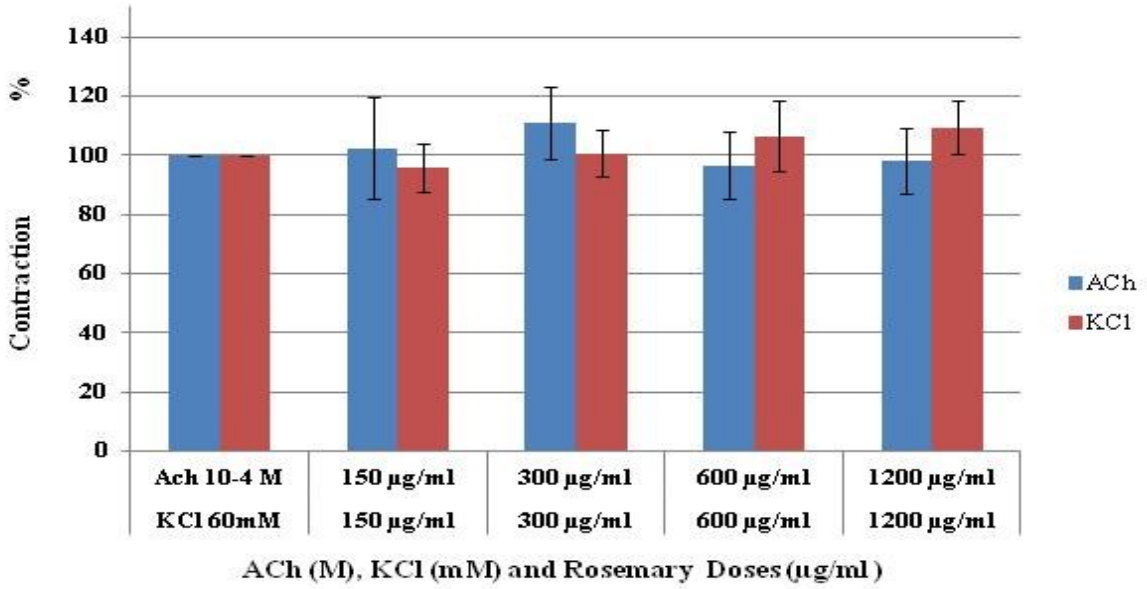

Figure 3. The effects of Rosemary extract (150, 300, 600 and 1200µg/mL) on the smooth muscles contractions of rat urinary bladder induced by ACh and KCl. The among applied doses could not be found any statistically significant difference (n: 12, P<0.05).
Şekil 3. ACh ve KCl ile indüklenen rat mesane düz kas kontraksiyonları üzerine Rosemary ekstraktının (150, 300, 600 and 1200 µg/mL) etkileri. Uygulanan dozlar arasında herhangi önemli istatistiksel farklılık bulunmadı (n: 12, P<0.05).

Table 1. The effect of the Rosemary extract on rat urinary bladder smooth muscles contractions with ACh-induced (n: 12).
Tablo 1. ACh ile indüklenen rat mesane düz kas kontraksiyonları üzerine Rosemary ekstraktının etkileri (n: 12).

Rosemary Doses	Contractions (g)		Contractions (%)		P values (n: 12)
	10^{-4} M ACh	Extract+10^{-4} M ACh	10^{-4} M ACh	Extract+10^{-4} M ACh	
	$\bar{X}$±SEM	$\bar{X}$±SEM	$\bar{X}$±SEM	$\bar{X}$±SEM	
150 µg/mL	1.4±0.31	1.43±0.44	100±0.00	102.14±17.08	>0.05
300 µg/mL	1.49±0.33	1.65±0.30	100±0.00	110.23±12.14	>0.05
600 µg/mL	1.17±0.2	1.13±0.16	100±0.00	96.58±11.43	>0.05
1200 µg/mL	1.26±0.17	1.23±0.21	100±0.00	98.17±10.97	>0.05

The Effects of Rosemary Extract on KCl-Induced Bladder Contractions

The contraction values acquired from urinary bladder muscles at a 60 mM dose of KCl were accepted as 100%. In the presence of 150, 300, 600 and 1200 µg/mL doses of Rosemary in the bath was obtained contractions induced with submaximal dose (60 mM) of KCl (Figure 3). These values were compared with the response induced with KCl accepted as 100% (Table 2).

At 150, 300, 600, and 1200 µg/mL doses of Rosemary extract, the responses to 60mM KCl were 95.75±8.21%, 99.27±7.83%, 106.44±11.97%, and 109.44±8.90%, respectively (Figure 3). According to the indications, it was determined that the 150, 300, 600, and 1200 µg/mL doses of Rosemary extract had no statistically significant effects on smooth-muscle contractions induced by KCl (n: 12, P>0.05).

Table 2. The effect of the Rosemary extract on rat urinary bladder smooth muscles contractions with KCl-induced (n: 12).
Tablo 2. KCl ile indüklenen rat mesane düz kas kontraksiyonları üzerine Rosemary ekstraktının etkileri (n: 12).

Rosemary Doses	Contractions (g)		Contractions (%)		P values (n: 12)
	60 mM KCl	60 mM KCl+Extract	60 mM KCl	60 mM KCl+Exract	
	$\bar{X}$±SEM	$\bar{X}$±SEM	$\bar{X}$±SEM	$\bar{X}$±SEM	
150 µg/mL	1.32±0.52	1.26±0.42	100±0.00	95.75±8.21	>0.05
300 µg/mL	1.37±0.26	1.36±0.18	100±0.00	99.27±7.83	>0.05
600 µg/mL	1.58±0.59	1.68±0.66	100±0.00	106.44±11.97	>0.05
1200 µg/mL	1.74±0.65	1.9±0.72	100±0.00	109.44±8.90	>0.05

DISCUSSION and CONCLUSION

The present study examined the effects of Rosemary aqueous extract on the contractility of rat urinary bladder smooth-muscles. To induced the urinary bladder smooth muscle contractions were used as contractions inductive agent the ACh (10^{-4} M) and KCl (60 mM). KCl and ACh are agents that induce contraction, and are commonly used in the study of plant extracts, chemical compounds, or the effects of drugs on *in vitro* muscle contractility (20-22). When contractions were induced with KCl, voltage-sensitive L-type Ca^{2+} channels in the cell membrane were opened as a consequence of depolarization, which resulted from the increase of K+ in the muscle cells. The increase of intracellular ion concentration led to induction of contractions (23). When contractions were induced with ACh, intracellular Ca^{2+} release was triggered (24), and the activation of myosin fibers increased. This resulted in an increase in the density of depolarization and the action potential in the smooth-muscle cells (25). In addition, with the help of G protein, ACh results in contractions via its connection with the postsynaptic M_3 receiver on smooth-muscle (26). Beer et al. (15) stated that high doses of Rosemary oil have a spasmolytic effect, while low doses of Rosemary oil increase contractions *in vitro* in stomach circular smooth-muscles. Zhu BT et al. (27) showed that Rosemary inhibits the uterotopic effects of these compounds on the uterus by increasing microsomal oxidation and glucuronidation of estrone and estradiol in the liver of female rats. To the best of our knowledge, no study of Rosemary's direct effects on uterus smooth-muscle contraction has been published. Ventura-Martínez R et al. (13) stated that Rosemary extract inhibited ileum contractions induced with ACh, KCl, and electrical field stimulation. They showed that contractions induced with ACh were inhibited by Rosemary oil in rabbit trachea smooth-muscle. In the same study, it was reported that contractions induced with histamine in trachea smooth-muscle were inhibited by this oil. It was found that Rosemary

oil dose-dependently and reversibly inhibited the contractions induced by high-dose potassium solution in rabbit smooth-muscles (12). In our study, the effects of 150, 300, 600, and 1200 µg/mL doses of Rosemary extract on smooth-muscle contractions induced by ACh and KCl in the rat urinary bladder were examined. When we evaluated our findings, we found that the Rosemary extract did not alter the contractions induced with ACh and KCl. In accordance with these results, it is may be considered that the compounds found in Rosemary extract have no effect on the mechanism that governs bladder smooth- muscle contractions.

In conclusion, compounds in Rosemary extract may not have an effect on mechanisms related to bladder smooth-muscle contractions. We believe that the use of Rosemary for different purposes will not result in an undesirable effect in pathological conditions, such as overactive bladder and bladder atony. In addition, we assert that the use of Rosemary by patients with such conditions will have neither a positive nor a negative effect.

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