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RESEARCH ARTICLE

The Effect of Sainfoin *(Onobrychis viciifolia)* Extract on Acethylcholine, Bethanechol and Potassium - Evoked Responses on Jejunum and Ileum of Sheep [1]

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Summary

The aim of this study was to clarify the effect of *Onobrychis viciifolia* Scop. *(O. viciifolia)* extract on jejunum and ileum of sheep The contractile response as E_{max}, pD_2 and EC_{50} of acetylcholine (10^{-3}-10^{-8} M, Ach), bethanechol (10^{-3}-10^{-8} M) and potassium (10 - 80×10^{-3} M, KCl) were determined in the absence and presence of extract (1.6 mg/ml). The contractile response to Ach in the presence of verapamil (10^{-6} or 10^{-8} M) or in calcium-free Tyrode's solution was also determined in the absence and presence of extract. Cumulative treated of extract significantly reduced the response to KCl-evoked (10^{-3} M) contraction. Extract did not affect contractile response to Ach and bethanechol but decreased the contractile response to potassium. The atropine-resistant component of Ach-evoked contraction and 4-diphenyl-acetoxy-N-methyl-piperidine methiodide-resistant component of bethanechol-evoked contraction were not inhibited in the presence of 1.6 mg/ml extract. The contractile response to Ach was reduced in calcium-free Tyrode's solution and verapamil 10-8 M had no additional effect. In contrast to 1.6 mg/ml extract was added together with verapamil 10^{-8} M, the contractile response to Ach was inhibited. In conclusion, extract inhibits jejunum and ileum muscle contractions through the inhibition of calcium influx and the modulation of calcium movement.

Keywords: *Contraction, Inhibition, Intestinal smooth muscle, Sheep, Onobrychis viciifolia Scop.*

Korunga Bitkisi *(Onobrychis viciifolia)* Ekstraktının Koyun Jejunum ve İleumunda Asetilkolin, Betanekol ve Potasyum ile Uyarılan Cevaplar Üzerine Etkisi

Özet

Bu çalışmada, *Onobrychis viciifolia* Scop. *(O. viciifolia)* ekstraktının koyun jejunum ve ileum üzerindeki etkisinin ortaya konması amaçlandı. Ekstrakt (1.6 mg/ml) varlığı ve yokluğunda, asetilkolin (10^{-3}-10^{-8} M, Ak), betanekol (10^{-3}-10^{-8} M) ve potasyumun (10 - 80×10^{-3} M, KCl) kontraktil cevapları Emax, pD_2 ve EC_{50} olarak belirlendi. Ayrıca ekstrakt varlığında ve yokluğunda, verapamil (10^{-6} veya 10^{-8} M) ve kalsiyumsuz Tyrode solüsyonunda Ak'nin kontraktil cevapları belirlendi. Ekstraktın kümülatif uygulaması, KCl (10^{-3} M) ile uyarılmış kontraksiyonları önemli derecede azalttı. Ekstrakt, Ak ve betanekolle oluşan kontraktil cevapları etkilemedi fakat KCl ile oluşan kontraktil cevapları azalttı. 1.6 mg/ml ekstrakt atropine direnç gösteren Ak ile uyarılmış kontraksiyon ve 4-diphenyl-acetoxy-N-methyl-piperidine methiodide (4-DAMP) direnç gösteren betanekol ile uyarılmış kontraksiyonları engellemedi. Kalsiyumsuz Tyrode solüsyonunda Ak'nin kontraktil cevapları azaldı ve 10^{-8} M verapamil buna ilave bir etki oluşturmadı. Ayrıca 1.6 mg/ml ekstrakt 10^{-8} M verapamil ile birlikte uygulandığında Ak'nin kontraktil cevaplarını engelledi. Çalışma sonunda ekstraktın jejunum ve ileum kas kontraksiyonlarını engellediği, bu etkisini de kalsiyumun taşınımında değişikliğe yol açarak ve kalsiyumun hücre içine girişini kısıtlayarak gösterdiği belirlendi.

Anahtar sözcükler: *Kontraksiyon, İnhibisyon, Bağırsak düz kası, Koyun, Onobrychis viciifolia Scop.*

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INTRODUCTION

Onobrychis viciifolia Scop. (*O. viciifolia*), also known as sainfoin, is a perennial forage legume and it has an early growth habit, sprouting earlier than alfalfa in spring to give good forage yields. While the availability of early fresh forage for stock is appreciated by farmers it is the ability of the feed to reduce incidence of bloat and increase animal performance that provided the main incentive for its incorporation to farm management [1].

Lu et al.[2] reported that *O. viciifolia* included phenolic compounds as seven cinnamic acid derivatives and nine flavonoid glycosides all of which were identified by NMR spectroscopy. Ince and Filazi [3] also reported that ethanolic extract of *O. viciifolia* contained phenolic acid, flavonoids and condensed tannins. The presence of the condensed tannins in feeds can exert beneficial effects on protein metabolism in sheep, slowing degradation of dietary protein to ammonia by rumen microorganisms and increasing protein outflow from the rumen, thus increasing absorption of amino acids in the small intestine of the animal. This was shown to result in increases in lactation, wool growth and live weight gain, without changing voluntary feed intake [4,5]. At low level of condensed tannins in ruminant diets a number of beneficial effects have been reported such as reducing the effects of parasites in the gastrointestinal tract [6]. However, flavonoids are absorbed from the gastro-intestinal system of ruminant by resorption and by bacterial metabolism in the rumen and intestine [7]. Also, *O. viciifolia* has great produced as animal feed by farmers, so it has high consumed from ruminants. Therefore, we aimed to determinate the effect of *O. viciifolia* extract in organ bath on jejunum and ileum motility of sheep.

MATERIALS and METHODS

Chemicals: Acetylcholine (Ach), bethanechol, 4-diphenyl-acetoxy-N-methyl-piperidine methiodide (4-DAMP), atropine, verapamil, ethylene glycol tetraacetic acid (EGTA) and potassium chloride (KCl) purchased from Sigma-Aldrich (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) were used as test compounds. All the other chemicals and reagents were purchased from commercial sources.

Plant Material and Extraction Method*: O. viciifolia* was collected from department of Haymana Agriculture Education and Research Center, University of Ankara, Turkey in May, 2008. It was identified by Prof. Dr. Saime Unver, Faculty of Agriculture, University of Ankara, Turkey. The voucher specimen was kept at the Crop Science Herbarium, University of Ankara, Turkey. The aerial part was collected, cut into small pieces and dried in a room condition at 25°C for 12 h. The dried plant material was coarsely powdered. The dried plant material (150 g) was extracted with 95% ethanol (500 ml) using a Soxhlet apparatus set at 50°C for 3 h. The extract was filtrated through filter paper (Whatman no. 3) and dried under reduced pressure.

% yield of the extract was calculated using the following equation:

$$\% \text{ yield} = W_{\text{crude extract}}/W_{\text{dried plant}} \times 100$$

$W_{\text{crude extract}}$ = weight of crude extract

$W_{\text{dried plant}}$ = weight of dried plant material

Study Design: Sheep intestinal (jejunum and ileum) tissues were obtained from a local abattoir, incubated in Tyrode's solution and transported to the laboratory within 1 h of slaughter. Jejunal segments (10 cm in length, ending 5 cm from the flexura duodenojejunalis) and distal ileal segments (15-20 cm in length, ending 5 cm from the ileocecal junction) were removed, cleared of contents and placed in Tyrode's solution (NaCl 137 mM, KCl 2.7 mM, $NaHCO_3$ 11.9 mM, glucose 5.6 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, NaH_2PO_4 0.4 mM, in distilled water pH 7.2, aerated with 95% O_2, 5% CO_2 and warmed to 37°C). Intestinal samples were dissected of fat and blood vessels and longitudinal strips (1.2 x 0.4 cm) cut along the mesentery. The muscle strips were placed in an organ bath and one end of the muscle was anchored to stationary clamp and the other end attached to an isometric force transducer (The BioPac system and MP35 Acquisition Box was used with FDT05 finger transducers) chambers were prefilled with 15 ml Tyrode's solution at 37°C and continuously bubbled with 95% O_2 and 5% CO_2. The muscle strip was placed under tension of 1 g for 1 h to equilibrate.

Final concentrations of extract were prepared by first dissolving the solid in ethanol. Atropine, Ach, bethanechol, verapamil, 4-DAMP and KCl were made up in Tyrode's solution on the day of the experiment. Calcium-free Tyrode's solution was made without $CaCl_2$ and with the addition of EGTA 2 mM [8]. Repeated contractile responses of sheep intestine muscle strips over time showed no significant alteration. The experimental protocols were approved by the Animal Care and Use Committee at Afyon Kocatepe University (2008-134).

Effect of Extract on Intestinal Muscle Contractions to Evoked KCl: Extracts (0.1; 0.2; 0.4; 0.8; 1.6; 3.2 mg/ml) were used alone and cumulative concentrations in the experiments. Tissue strips were contracted with 10^{-3} M KCl and then cumulative concentrations of extract were applied in the experiments. A control relaxant response of intestine muscle to a sub-maximal concentration of extract (1.6 mg/ml) was determined.

Effect of Ethanol and Extract on Ach - Evoked Responses: Control dose response curves of Ach were obtained and then repeated in the presence of 0.25% ethanol, 0.5% ethanol and 0.5% ethanol plus extract (1.6 mg/ml). 10^{-3} - 10^{-8} M Ach cumulative concentrations were used in the experiments. These experiments were carried

out to determine the effect of ethanol alone on the contractile response of sheep intestinal muscle.

Effect of Atropine on Ach-Evoked Responses in the Absence and Presence of Extract: Control Ach dose response curves (10^{-3} - 10^{-8}M) were obtained and then repeated after incubation with extract 1.6 mg/ml for 10 min each. Further responses were obtained after incubation with 10^{-6} M atropine alone and then with 10^{-6} M atropine plus 1.6 mg/ml extract.

Effect of Verapamil on Ach-Evoked Responses in the Absence and Presence of Extract: The effect of extract on the contractile response of intestine muscle to Ach was determined in the presence of extract and verapamil. Control contractile responses were obtained to Ach (10^{-3} - 10^{-8} M) and then repeated after incubation with Tyrode's and 10^{-6} M verapamil alone or with 10^{-6} M verapamil plus 1.6 mg/ml extract.

Effect of Extract on Bethanechol-Evoked Responses: Control contractile responses of intestinal muscle of bethanechol (10^{-3} - 10^{-8}M) were determined. Responses were then repeated in the presence of 1.6 mg/ml extract after equilibration for 10 min.

Effect of 4-DAMP on Bethanechol-Evoked Responses in the Absence and Presence of Extract: The effect of extract on the contractile response of intestine muscle to bethanechol was determined in the presence of extract and 4-DAMP. Control contractile responses were obtained to bethanechol (10^{-3}-10^{-8} M) and then repeated after incubation with 10^{-7} M 4-DAMP alone or with 10^{-7} M 4-DAMP plus 1.6 mg/ml extract.

Effect of Extract on Potassium-Evoked Responses: To determine the effect of extracts on contractile response to potassium, a control contractile response curve was obtained to potassium chloride, 10×10^{-3} M - 80×10^{-3} M and then repeated after the strips were treated with 0.8 and 1.6 mg/ml extracts in organ bath including Tyrode's solution for 10 min.

Effects of Extract and Verapamil on Ach-Evoked Responses in Calcium-Free Tyrode's Solution: To determine the effect of extract on intracellular calcium release, Ach-evoked contractile responses were determined in calcium-free Tyrode's solution. Two strips from each intestine muscle used were studied in parallel. Control contractile responses to Ach (10^{-3} - 10^{-8} M) were determined in normal Tyrode's solution and then in Ca^{2+} free Tyrode's solution. The evoked responses to Ach in calcium-free Tyrode's solution were obtained following the disappearance of spontaneous contractions after the Tyrode's solution was changed from calcium containing to calcium-free. The muscle strips were then reincubated with normal Tyrode's solution for 20 min to allow the intracellular calcium stores to be replenished. The normal Tyrode's solution was then switched to calcium-free Tyrode's solution and the samples incubated with either 10^{-8} M verapamil alone or with 10^{-8} M verapamil plus 1.6 mg/ml extract before being stimulated with 10^{-3} - 10^{-8} M Ach again, once the spontaneous contractions had ceased.

Statistical Analyses: Results are presented as mean ± SEM. Statistical analyses were carried out using one-way analysis of variance followed by Bonferroni's correction for multiple comparisons. A *P* value of *<0.05* was considered significant. All analyses were performed using GraphPad Prism software.

RESULTS

Plant Extract: The yield of ethanol extract of dried *O. viciifolia* was obtained by nearly 5% (w/w).

Effect of Extract on Intestinal Muscle Contractions to Evoked KCl: Cumulative concentrations (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml) of extract treatment inhibited jejunum and ileum muscle contractions to evoked 10^{-3} M KCl *(Fig. 1)*. One concentration of extract treatment did not inhibit jejunum and ileum muscle contraction to evoked KCl.

Effect of Ethanol and Extract on Ach - Evoked Responses: Ethanol 0.25% and 0.5% had no effect on the maximum contractile responses of jejunum and ileum muscle to Ach. The addition of 1.6 mg/ml extract did not affect Ach E_{max}, pD_2 and EC_{50} levels compared to control Ach responses *(Table 1)*.

Effect of Atropine on Ach-Evoked Responses in the Absence and Presence of Extract: Incubation of the intestine strips with 10^{-6} M atropine reduced the jejunum and ileum E_{max} ($P<0.001$), jejunum and ileum pD_2 ($P<0.001$), and increased jejunum and ileum EC_{50} ($P<0.001$) compared to Ach control responses *(Table 1)*. The further addition of 1.6 mg/ml extract reduced jejunum and ileum E_{max} ($P<0.05$), jejunum ($P<0.01$) and ileum pD_2 ($P<0.001$) and increased jejunum and ileum EC_{50} ($P<0.01$) compared to Ach control responses *(Table 1)*. Ach E_{max} and pD_2 responses were also found to be low levels in presence of atropine than presence of extract.

Effect of Verapamil on Ach-Evoked Responses in the Absence and Presence of Extract: To investigate the effect of extract on calcium release from intracellular stores, responses to Ach were obtained in the presence of verapamil, a potent inhibitor of Ca^{2+} influx through L-type [9]. Addition of 10^{-6} M verapamil to the organ bath reduced the jejunum ($P<0.01$) and ileum E_{max} ($P<0.05$), jejunum and ileum pD_2 ($P<0.001$), and increased jejunum and ileum EC_{50} ($P<0.01$) compared to Ach control responses *(Table 1)*. The further addition of 1.6 mg/ml extract reduced jejunum and ileum E_{max} ($P<0.001$), and jejunum and ileum EC_{50} ($P<0.01$) compared to Ach control responses *(Table 1)*, suggesting an effect of extract on intracellular calcium mobilization as well as calcium entry mechanisms.

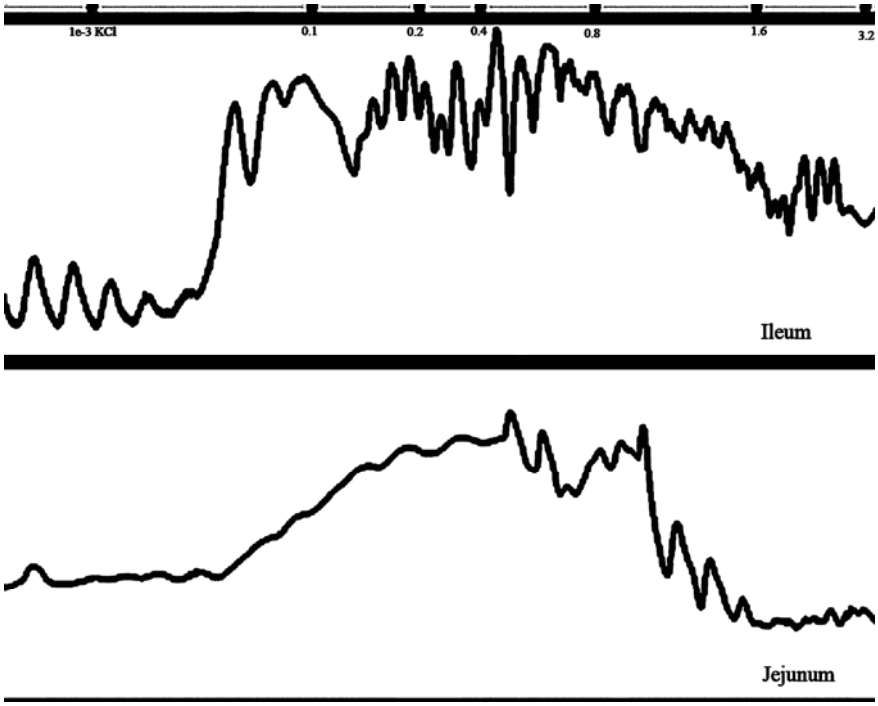

Fig 1. The effect of cumulative *O. viciifolia* extract treatment to KCl-evoked (10^{-3} M) response on jejunum and ileum motility of sheep

Şekil 1. 10^{-3} M KCl ile uyarılmış koyun jejunum ve ileum motilitesi üzerine kümülatif ekstrakt uygulamasının etkisi

Table 1. *The effects of alone and with together treatment of 1.6 mg/ml extract (E), 10^{-8} M atropine (A) and 10^{-6} M verapamil (V) to acetylcholine (Ach) pD_2, E_{max} and EC_{50} levels on jejunum and ileum motility of sheep*

Tablo 1. *Koyun jejunum ve ileum motilitesi üzerine 1.6 mg/ml ekstraktın (E) tek başına, 10^{-8} M atropin (A) ve 10^{-6} M verapamil (V) varlığında asetilkolin (Ak) pD_2, E_{max} and EC_{50} düzeyleri üzerine etkisi*

Treatment (n:12)	E_{max}		pD_2		EC_{50} (Value x 10^{-6} M)	
	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
Ach	112.1±2.4	110.4±2.5	5.2±0.2	5.4±0.2	4.2± 2.0	4.1±1.1
E+Ach	106.3±1.8	105.2±1.9	5.0±0.2	4.9±0.1	5.8±1.4	4.9±1.1
A+Ach	96.4±2.4 ***	100.7±1.6 ***	4.3±0.1 ***	4.3±0.1 ***	75±13 ***	110±12 ***
A+E+Ach	100.6±0.5 ***	102.1±0.4 *	4.4±0.1 ***	4.4±0.1 ***	38±6.7 **	22±7.0 **
V+Ach	101.1±1.0 **	102.9±0.7 *	4.3±0.2 ***	4.3±0.2 ***	32±5.8 **	34±9.0 **
V+E+Ach	100.8±0.8 ***	96.9±2.1 ***	4.9±0.2	4.9±0.2	22±4.6 **	21±7.4 **

In the same column values with stars show statistically significant differences
** P<0.05; ** P<0.01; *** P<0.001*

Effect of Extract on Bethanechol-Evoked Responses: The addition of 1.6 mg/ml extract to 0.5% ethanol did not affect the jejunum and ileum E_{max}, jejunum and ileum EC_{50} levels compared to control bethanechol responses. 1.6 mg/ml extract reduced the jejunum and ileum pD_2 ($P<0.05$) compared to bethanechol control responses *(Table 2)*.

Effect of 4-DAMP on Bethanechol-Evoked Responses in the Absence and Presence of Extract: Incubation of the intestine strips with 10^{-7} M 4-DAMP, a muscarinic-3 receptor antagonist, reduced the jejunum and ileum E_{max} ($P<0.001$), jejunum and ileum pD_2 ($P<0.001$), and increased jejunum and ileum EC_{50} ($P<0.001$) compared to bethanechol control responses *(Table 2)*. 1.6 mg/ml extract and addition of 10^{-7} M 4-DAMP reduced the jejunum and ileum E_{max} ($P<0.001$), jejunum ($P<0.001$) and ileum pD_2 ($P<0.01$) and increased jejunum and ileum EC_{50} ($P<0.001$) compared to bethanechol control responses *(Table 2)*.

Effect of Extract on Potassium-Evoked Responses: Potassium induced intestinal muscle contraction was inhibited by removal of extracellular Ca^{2+} or a suppression of transmembrane calcium flux and utilization of the intracellular stored calcium [10]. To investigate whether extract affects calcium influx an experiment was carried out to determine the effect of extract on contractile responses to depolarization-evoked calcium influx. The addition of 0.8 mg/ml extract did not affect but 1.6 mg/ml extract reduced the jejunum ($P<0.05$) and ileum E_{max} ($P<0.01$), jejunum and ileum pD_2 ($P<0.05$) and increased jejunum and ileum EC_{50} ($P<0.01$) compared to control KCl responses *(Table 3)*. These results suggest that 1.6 mg/ml extract has an inhibitory effect on excitation-coupling mechanisms and may inhibit calcium influx through voltage-sensitive calcium channels.

Table 2. *The effects of alone and with together treatment of 1.6 mg/ml extract (E), 10^{-7} M 4-DAMP (D) to bethanechol (Bet) pD_2, E_{max} and EC_{50} levels on jejunum and ileum motility of sheep*

Tablo 2. *Koyun jejunum ve ileum motilitesi üzerine 1.6 mg/ml ekstraktın (E) tek başına, 10^{-7} M 4-DAMP (D) varlığında betanekol (Bet) pD_2, E_{max} and EC_{50} düzeyleri üzerine etkisi*

Treatment (n:12)	E_{max}		pD_2		EC_{50} (Value x 10^{-6} M)	
	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
Bet	115±3.8	115±2.2	4.5±0.1	4.6±0.1	34±7.1	31±7.7
E+Bet	112±2.9	111±2.5	4.2±0.1 *	4.1±0.1 *	44±8.2	88±20
D+Bet	99±0.7 ***	100±0.2 ***	3.6±0.1 ***	3.6±0.7 ***	140±3.7 ***	290±49 ***
D+E+Bet	104±1.4 ***	102±0.8 ***	3.8±0.2 ***	3.9±0.2 **	160±9.8 ***	390±79 ***

In the same column values with stars show statistically significant differences
** P<0.05; ** P<0.01; *** P<0.001*

Table 3. *The effects of 0.8 and1.6 mg/ml extracts treatment to potassium chloride (KCl) pD_2, E_{max} and EC_{50} levels on jejunum and ileum smooth muscle motility of sheep*

Tablo 3. *Koyun jejunum ve ileum motilitesi üzerine 0.8 and1.6 mg/ml ekstraktın potasyum klorid (KCl) pD_2, E_{max} and EC_{50} düzeyleri üzerine etkisi*

Treatment (n:12)	E_{max}		pD_2		EC_{50} (Value x 10^{-3} M)	
	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
KCl	103.5±2.1	110±1.9	2.6±0.3	2.2±0.3	22±5.6	32±4.4
0.8 mg + KCl	100.1±2.2	106±2.0	2.0±0.3	1.6±0.1	40±8.8	49±3.3
1.6 mg + KCl	94.71±1.8 *	101±0.3 **	1.2±0.1 *	1.3±0.1 *	59±2.3 **	63±2.7 **

In the same column values with stars show statistically significant differences
** P<0.05; ** P<0.01*

Effect of Extract and Verapamil on Ach - Evoked Responses in Calcium-Free Tyrode's Solution: The experiment above was repeated in calcium-free Tyrode's solution to further investigate the effect of extract on responses involving intracellular calcium stores only. A lower concentration of 10^{-8} M verapamil was used to reduce the possibility of non-specific effects, and a shorter incubation period (until spontaneous contractions ceased) was used to minimize loss of calcium from intracellular stores. The addition of 10^{-8} M verapamil to calcium-free Tyrode's solution had no additional inhibitory effect on the contractile responses to Ach. When 1.6 mg/ml extract was added together with 10^{-8} M verapamil reduced the jejunum and ileum E_{max} ($P<0.05$), jejunum and ileum pD_2 ($P<0.05$) and increased jejunum ($P<0.05$) and ileum EC_{50} ($P<0.001$) compared to Ach control responses *(Table 4)*.

DISCUSSION

The results of this study demonstrate that *O. viciifolia* extract has not an inhibitory action on the contractile response of sheep jejunum and ileum strips to Ach and bethanechol, but it has an inhibitory action to potassium. One treatment of extract did not affect intestinal motility but application to cumulative concentration of extract inhibited motility of jejunum and ileum. 1.6 mg/ml extract treatment did not affect to Ach evoked responses on jejunum and ileum. Sub-maximal dose treatment of atropine which is an antagonist agent against to muscarinic effects, significantly reduced E_{max} and pD_2 levels of Ach in strips. It also increased EC_{50} levels of Ach in both jejunum and ileum. However, 1.6 mg/ml extract did not change the atropine-resistant response to Ach, indicating that

Table 4. *The effects of alone and with together treatment of 1.6 mg/ml extract (E), 10^{-8} M verapamil (V) to Ach pD_2, E_{max} and EC_{50} levels on jejunum and ileum smooth muscle motility of sheep in Ca free Tyrode's solution*

Tablo 4. *Koyun jejunum ve ileum motilitesi üzerine, kalsiyumsuz Tyrode çözeltisi içerisinde, 1.6 mg/ml ekstraktın tek başına ve 10^{-8} M verapamil (V) varlığında Ak pD_2, E_{max} and EC_{50} düzeyleri üzerine etkisi*

Treatment (n:12)	E_{max}		pD_2		EC_{50} (Value x 10^{-4}M)	
	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
Cafree-Ach	86±2.5	87±1.8	5.5±0.2	5.6±0.2	64±12	29±13
Cafree+V+Ach	83±1.2	81±3.4	5.2±0.4	5.3±0.2	80±18	55±17
Cafree+V+E+Ach	77±2.0 *	73±4.3 *	4.9±0.1 *	5.0±0.1 *	130±13 *	140±15 ***

In the same column values with stars show statistically significant differences
** P<0.05; ** P<0.001*

extract was not effect on muscarinic receptors binding Ach in jejunum and ileum. In contrast to, Ince and Filazi [11] reported that *O. viciifolia* extract (6.4 mg/ml) inhibited intestinal smooth muscle contraction and its effect also inhibited presence of atropine in mice jejunum and ileum and they suggested that *O. viciifolia* extract may act as an antimuscarinic agent. The discrepancy between our study and that of Ince and Filazi [11] may be attributed to the procedures used to high concentration of the extract. Verapamil is L-type calcium channel blocker and sub-maximal dose treatment of verapamil reduced E_{max} and pD_2, and increased EC_{50} levels of Ach in both jejunum and ileum. 1.6 mg/ml extract and verapamil treatment also reduced E_{max} and pD_2, and increased EC_{50} levels of Ach in both jejunum and ileum. These results suggest that inhibiting effect of verapamil on motility of jejunum and ileum was not attenuated by 1.6 mg/ml extract.

Pharmacological studies demonstrated that selective muscarinic receptor subtype antagonists indicated that bethanechol-mediated longitudinal muscle contractile responses are primarily mediated via muscarinic-3 receptor [12]. This receptor immunoreactivity in the intestinal muscle is consistent with cholinergic-induced contraction of this muscle layer, which is known to modulate adjacent epithelial secretory responses [13]. In this study, sub-maximal dose treatment of 4-DAMP inhibited contractions of bethanechol, but 1.6 mg/ml extract only inhibited pD_2 of bethanechol in both jejunum and ileum. This suggested that extract may affect Ca^{2+} transition through the membrane and potent of bethanechol may be inhibited. 1.6 mg/ml extract with 4-DAMP treatment also inhibited bethanechol E_{max}, pD_2 and EC_{50} levels. These results indicate that sub-maximal dose (1.6 mg/ml) of *O. viciifolia* extract did not affect the binding of Ach and bethanechol to the receptors. Therefore, *O. viciifolia* extract may be not acting via muscarinic pathways on jejunum and ileum of sheep.

The contractions of smooth muscles which are induced by the presence of high K^+ are dependent upon ingress of calcium into the cells through voltage operated Ca^{2+} channels [14]. Stimulation by potassium results in depolarization of the sarcolemma and activation of calcium channels permitting calcium entry and contraction to be initiated independent of muscarinic or purinergic receptor activity [15]. 0.8 mg/ml extract treatment did not change E_{max}, pD_2 and EC_{50} levels of KCl in both jejunum and ileum. 1.6 mg/ml extract treatment reduced E_{max} and pD_2, and increased EC_{50} levels of KCl in both jejunum and ileum. So, the extract suppressed potassium chloride-induced responses non-competitively in jejunum and ileum reflecting functional antagonism. Dar and Channa [16] reported that the ethanol extract of *B. monniera* which contained many compound(s) such as flavonoides, triterpenoid saponins, and alkaloids caused inhibition of potassium chloride- and barium chloride-induced contractions on guinea-pig ileum. They also indicated that the extract may have directly affected the smooth muscles since the compounds in the extract are responsible for spasmolytic activity.

The inhibition of KCl-stimulated contractile responses by 1.6 mg/ml extract suggested that extract affected downstream signaling or contractile mechanisms. In intestinal smooth muscle, contractile responses to receptor activation are mediated by the release of intracellular calcium stores, in addition to the entry of extracellular calcium [17]. To investigate possibility inhibition by 1.6 mg/ml extract of Ach-evoked contractile responses of intestinal strips perfused with calcium free Tyrode's solution containing verapamil, to remove the calcium entry component, was investigated. Contractile responses were reduced in the absence of extracellular calcium. These responses were not inhibited further by the calcium entry blocker, verapamil, indicating that the residual contractile activity in these experiments was due to mobilization of intracellular calcium stores. The addition of 1.6 mg/ml extract to calcium free, verapamil-containing Tyrode's solution was inhibited the E_{max}, pD_2 and increased EC_{50} levels of Ach thus suggesting that the relaxant effect could be mediated through the inhibition of transmembrane calcium influx and/or inhibition of release of intracellular calcium from stores in the sarcoplasmic reticulum. Together, the data are suggestive of actions of extract to inhibit both calcium entry and release of internal calcium stores, possibly by the modulation of the Ca^{2+}-induced Ca^{2+} release initiation of muscle contraction. Similarly, previous studies have reported that plants especially containing flavonoids have decreased the intestinal motility via Ca^{2+} channels [18-22]. Generally, calcium antagonistic activity has been reported in various groups of natural products containing alkaloids [23], terpenes [24] and flavonoids [25,26]. It is known that the extract used in the present study contains many compounds such as flavonoids, phenolics and condensed tannins [2-6]. Likewise, these compounds in the extract of *O. viciifolia* might be responsible for the spasmolytic activity on jejunum and ileum of sheep.

In conclusion, *O. viciifolia* has showed an inhibitory effect via Ca^{2+} channel on the intestinal smooth muscle of sheep. For these reasons, use of *O. viciifolia* itself as a beneficial feed for ruminants cannot be ruled out but feeding ruminants with *O. viciifolia* in excessive amounts may cause to the reduction of intestinal motility.

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