

Spasmolytic effect of 1,8 cineole is mediated through calcium channel blockade in the bovine ileum

Yaser Nozohour, Masoud Maham*, Bahram Dalir-Naghadeh

Department of Internal Medicine and Clinical Pathology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

Article Info	Abstract
Article history: Received: 25 April 2021 Accepted: 14 July 2021 Available online: 15 September 2022	<p>Gastrointestinal motility disorders include increased or decreased movements. Other studies have shown that herbal components, for example, essential oils can modify the increase and decrease of gastrointestinal movements of ruminants. The 1,8-cineole being obtained from the essential oil of many plants has several effects. The present study has investigated the effect of 1,8-cineole on the contractility of bovine ileum smooth muscle. The experiment was performed on the circular smooth muscle of ileum samples taken from slaughtered cows in the organ bath. Seven cumulative concentrations of 1,8-cineole from 1.00 to 1,000 $\mu\text{g mL}^{-1}$ were added to tissue samples. The used solution was Tyrode's solution aerated with a mixture of 95.00% oxygen and 5.00% carbon dioxide, and the temperature was set at 37.00 °C. The effects of 1,8-cineole on baseline contractions and three induced contractions with barium chloride, potassium chloride, and carbachol were investigated. The effects of 1,8-cineole, and verapamil (standard calcium channel blocker) on calcium channels were assessed. The results revealed that 1,8-cineole significantly inhibited spontaneous contractions as well as all spasmogen-induced contractions. The 1,8-cineole exerts its myorelaxant properties by inhibiting calcium channels in smooth muscle. It seems that 1,8-cineole has a good potential for producing antispasmodics or gastrointestinal motility modulators in veterinary medicine.</p>
Keywords: 1,8-cineole Anti-spasmodic Calcium channel blockade Ileum Smooth muscle	

© 2022 Urmia University. All rights reserved.

Introduction

The alimentary tract is one of the essential organs of ruminants.¹ The bovine gastrointestinal disorders are included disruption in movement, digestion, secretion, and absorption.¹ Gastrointestinal motility disorders are included hyper-motility and hypo-motility. The increased gastrointestinal movements followed by the spasm of smooth muscle of the bovine intestine cause abdominal pain.² Also, increased intestinal movements and reduced intestinal transfer time may lead to dyspepsia, mal-absorption, and diarrhea due to insufficient time for digestion and absorption of consumed food.^{3,4} Therefore, the use of a spasmolytic agent is essential in such cases to reduce gastrointestinal movements. Given the increasing tendency to use medicinal plants in modern animal husbandry to produce organic products, it has been focused on medicinal plants as essential alternative substances for traditional chemicals (especially antibiotics).⁵ Former studies have shown that ingredients of medicinal

plants, for example, essential oils, tannins and saponins, can decrease or increase the gastrointestinal movements of ruminants.⁶ Due to the importance of the subject, several studies have been conducted in this field. In a recent study, the effects of *Bidens tripartita* on pig jejunum movements were examined, suggesting that different plant extracts have an excellent ability to increase intestine movements.⁷ In another study, *Artemisia dracunculus* essential oil inhibited acetylcholine-induced contractions in ruminal and abomasal tissues, indicating that some plants also reduce gastrointestinal motility.⁸

The 1,8-cineole is obtained from the essential oil of plants such as *Coriandrum sativum* L, *Mentha longifolia* L., *Rosmarinus officinalis* L., *Zingiber officinale* Rosc, *A. dracunculus* L. and *Origanum vulgare* L.^{9,10} Cineole has anti-inflammatory and anti-oxidant effects¹¹ and effectively prevents the exacerbation of chronic obstructive pulmonary disease and controls asthma.¹¹ It has been shown that 1,8-cineole has a potential value in preventing ulcers and gastrointestinal inflammation.¹² The 1,8-cineole in low

*Correspondence:

Masoud Maham. DVM, DVSc

Department of Internal Medicine and Clinical Pathology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

E-mail: m.maham@urmia.ac.ir



This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International (CC BY-NC-SA 4.0) which allows users to read, copy, distribute and make derivative works for non-commercial purposes from the material, as long as the author of the original work is cited properly.

concentrations has a pronounced stimulating effect on the spontaneous contractile activity, and higher concentrations of 1,8-cineole lead to an inhibition of the spontaneous contractile activity of smooth muscle fibers.¹³ A recent study has shown that 1,8-cineole has anti-spasmodic and anti-secretory effects being able to treat diarrhea.⁹

It seems that medicinal plants have good potential for the prevention and treatment of gastrointestinal problems. Their effects on gastrointestinal motility in ruminants are unknown, and dysmotility is a vital part of the ruminant gastrointestinal diseases pathophysiology. Thus, natural treatment of these problems is essential in animals. In line with that, the present study investigated the effect of 1,8-cineole on the contractility of bovine ileum smooth muscle.

Materials and Methods

Chemicals. 1,8-cineole, carbachol (CCh), verapamil hydrochloride, and acetylcholine chloride were obtained from Sigma (St. Louis, USA). Potassium chloride (KCl), sodium dihydrogen phosphate (NaH_2PO_4), calcium chloride (CaCl_2), sodium chloride (NaCl), glucose, magnesium chloride (MgCl_2), sodium bicarbonate (NaHCO_3), ethylenediaminetetraacetic acid (EDTA), barium chloride (BaCl_2) and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany).

Tissue samples collection. Ileal tissue samples were prepared from fifty slaughtered Holstein bulls between two and four years old from Urmia Industrial Slaughterhouse, Urmia, Iran, with no previous history of gastrointestinal disorders. The ileum was prepared immediately after the animal slaughter and a segment of the ileum (15.00 cm in length) was taken. To evacuate fecal matter, a longitudinal incision was made to open the lumen of ileum. Tyrode's solution containing NaHCO_3 (11.90mM), NaCl (136.90 mM), KCl (2.70 mM), glucose (5.60 mM), MgCl_2 (1.10 mM), NaH_2PO_4 (0.40 mM) and CaCl_2 (1.80 mM) was used as a rinse, transportation and incubation medium. The serous and mucosal surfaces of ileal tissue samples were immediately rinsed with cooled (4.00 °C) and aerated Tyrode's solution to remove the digestive contents.⁸ Specimens were immersed in 4.00 °C Tyrode's solution and kept at this temperature until reaching the laboratory (in the shortest possible time). The solution was exchanged 10 min after the initial collection to remove digestive contents and supply the necessary material to the tissue. In the laboratory, whole ileal tissue pieces were placed on the dissected board filled with Tyrode's solution. Mucosa and submucosa were carefully separated from the ileal smooth muscle. The tissue strips (5.00 × 20.00 mm) were cut in the path of the circular muscle layer.⁸

Smooth muscle activity registration. The ileal smooth muscle strips were housed in separate chambers, each containing 25.00 mL of Tyrode's solution at 37.00 °C, and gassed without interruption with a mixture of 5.00%

CO_2 and 95.00% O_2 . In order to fix the samples in chambers, one end of each tissue was connected to an isometric transducer with a thread, and the other end was fixed to the down hook of the chambers (Model TRI 202p; PanLab, Barcelona, Spain). Six transducers were linked to an amplifier (model ML224; AD Instruments, Castle Hill, Australia) and Power Lab data acquisition system (model ML870; AD Instruments) was used for data collection. Lab chart software (version 6.0; AD Instruments) was employed to view and record data.⁸

Experiments design. At First, the ileal muscle samples were rested in the Tyrode's medium for one h to adapt to the new environment. In this stage, the Tyrode solution was replaced every 15 min, and two 1.00 g tensions were applied to the tissues at 15-min intervals. All specimens were tested before the main experiment to evaluate the viability and contractile function by adding 10.00 μM of acetylcholine. After that, tissues were washed with Tyrode's solution to achieve basal contraction of muscles. This way was repeated three times and, if the results were the same, the tissue was considered acceptable for testing.⁸ The effect of 1,8-cineole on the contractions of bovine ileal circular smooth muscle was investigated in four groups, each containing six tissue samples. In the first group, the effects of cumulative concentrations of 1,8-cineole were examined on the basal tonus. When the tissue samples reached a balance point in the tissue bath and followed a fixed baseline, the incremental concentrations of 1,8-cineole in separate (1.00 to 1,000 $\mu\text{g mL}^{-1}$) were added to bath cumulatively. In the second group, after the tissue samples reached equilibrium, they first underwent contraction under the influence of BaCl_2 (3.00 mM). Then, the incremental concentrations of the studied substance (1.00 to 1,000 $\mu\text{g mL}^{-1}$) were added cumulatively to the bath. In the third group, the tissue samples were initially contracted under the influence of CCh (1.00 μM) and then, the incremental concentrations of 1,8-cineole (1.00 to 1,000 $\mu\text{g mL}^{-1}$) were added cumulatively to the bath. In the fourth group, the tissue samples were initially contracted under the influence of KCl (20.00 and 60.00 mM) and then, the incremental concentrations of 1,8-cineole (1.00 to 1,000 $\mu\text{g mL}^{-1}$) were added cumulatively to the bath. The 1,8-cineole in separate was dissolved in DMSO 5.00% and dilutions of 1.00, 3.00, 10.00, 30.00, 100, 300 and 1,000 $\mu\text{g mL}^{-1}$ were prepared by adding the Tyrode's solution. All concentrations of 1,8 cineole in separate groups were added to the medium at 2-min intervals. At the end, the tissue samples were washed with Tyrode's solution. The viability of muscles was confirmed by their response to the addition of 10.00 μM acetylcholine.

Mechanism of action. The effect of 1,8-cineole on calcium channels was investigated in bovine ileal smooth muscle. Tissue strip samples were treated with the concentrations of 100 and 300 $\mu\text{g mL}^{-1}$ 1,8-cineole, and the concentration-response curves (CRCs) were then obtained

by adding calcium to the calcium-free medium. Also, CRCs of 0.10 and 0.30 μM verapamil concentrations as a standard calcium channel blockers were evaluated in distinct groups.^{14,15} The ileal samples were allowed to stabilize in typical Tyrode's solution, which was then replaced with calcium-free Tyrode's solution containing: NaHCO_3 (11.90 mM), glucose (5.60 mM), NaCl (136.90 mM), KCl (2.70 mM), MgCl_2 (1.10 mM), EDTA (0.10 mM) and NaH_2PO_4 (0.40 mM) for 30 min to remove calcium from the ileal tissues and then to replace it with the depolarizing solution containing: NaH_2PO_4 (0.42 mM), KCl (60.00 mM), EDTA (0.10 mM), NaHCO_3 (11.90 mM), NaCl (91.04 mM), glucose (5.55 mM) and MgCl_2 (1.05 mM) for 30 min. After preparing the K^+ -rich and calcium-free (depolarizing) solutions, two control CRCs were recorded with calcium concentrations. After each period, washing of tissues with the depolarizing solution restores the contractions to the baseline. In the third round, the rates of calcium CRCs were recorded 10 min after adding the cineole (100 and 300 $\mu\text{g mL}^{-1}$) and verapamil (0.10 and 0.30 μM).^{14,15} Cumulative concentrations of 0.003, 0.01, 0.03, 0.10, 0.30, 1.00 and 3.00 μM verapamil were added to the induced contractions of 60.00 mM KCl (K60) to investigate the effect of standard calcium channel blocker. Cumulative concentrations of 1,8 cineole were added to the KCl 20.00 mM (K20) induced contractions in separate groups to assess their effects on low dose KCl (K20) and compare it with high dose KCl (K60).^{14,15}

Statistical analysis. The assumption of data normality was tested by the Shapiro-Wilkes test. Data were graphically assessed using histogram and box plots for assumptions of normal distribution and homogeneity of variance. Since the assumptions were not met, the Nonparametric Friedman Repeated Measures Analysis of Variance on Ranks was employed to compare the results. Pairwise comparisons between each concentration and the control were identified using Dunnett's test. The significance level was set at $p < 0.05$. Results were expressed as medians and interquartile ranges (25th - 75th percentiles). The IBM SPSS Statistics for Windows, (version 25.0; IBM Corp., Armonk, USA) was used for statistical analysis of data.

Results

In general, this study showed that 1,8-cineole in different concentrations, significantly decreased the basal (spontaneous) and stimulated (spasmogenic) contractions in the bovine ileal smooth muscle. The investigation of the mechanism of action revealed that, like verapamil, 1,8-cineole applies its relaxing effect via blocking the calcium channels. 1,8-cineole solvent (i.e., DMSO) did not affect ileal smooth muscle isolates' contraction. At all periods of the tests, the effects created by 1,8-cineole were eliminated after the tissue rinse. At the end of the test, the tissues

showed a normal reaction to acetylcholine, indicating no damage to the tissue due to the presence of this substance.

Effect of 1,8-cineole on the contraction of ileal tissues. Cumulative concentrations of 1,000 $\mu\text{g mL}^{-1}$ 1,8-cineole caused a significant reduction in the spontaneous basal contraction of the ileal smooth muscle. The CCh-induced contractions were significantly relaxed at 10.00 to 1,000 $\mu\text{g mL}^{-1}$ concentrations of 1,8 cineole. The BaCl_2 -induced contractions were significantly inhibited at 100 to 1,000 $\mu\text{g mL}^{-1}$ concentration of 1,8 cineole ($p < 0.05$; Fig. 1).

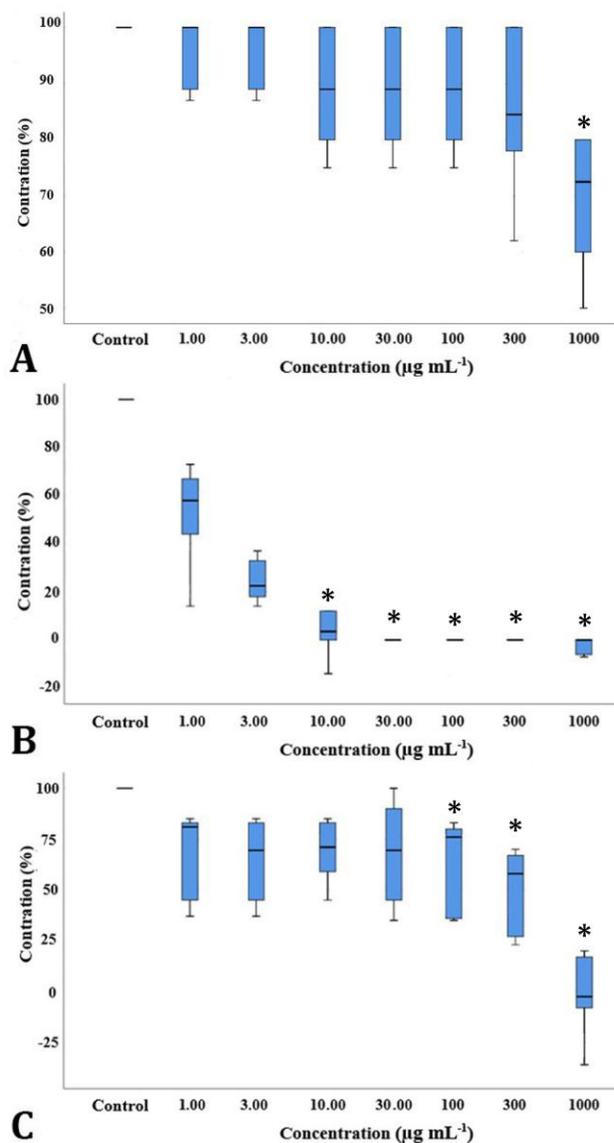


Fig. 1. The effect of different concentrations of 1,8 cineole on **A)** basal tonus, **B)** contraction induced by carbachol, and **C)** barium chloride induced contraction in the circular smooth muscles of bovine ileum. Results have been reported as the percentage of stable initial maximum contraction of each spasmogen and the percentage of stable initial basal contractions about spontaneous contractions. * indicates a significant difference compared to the control group ($p < 0.05$).

The 1,8 cineole relaxed the contractions produced by k20 and k60 at concentrations of 100 to 1,000 $\mu\text{g mL}^{-1}$ and 30 to 1,000 $\mu\text{g mL}^{-1}$, respectively, which was statistically significant (Fig. 2). Different concentrations of verapamil caused a significant reduction in KCl contractions (Fig. 3).

Blocking effect on calcium channels. The analysis of the ileal smooth muscle responses to different concentrations of calcium before and after incubation with 1,8-cineole revealed that the tissue response to various concentrations of calcium was decreased after treatment.

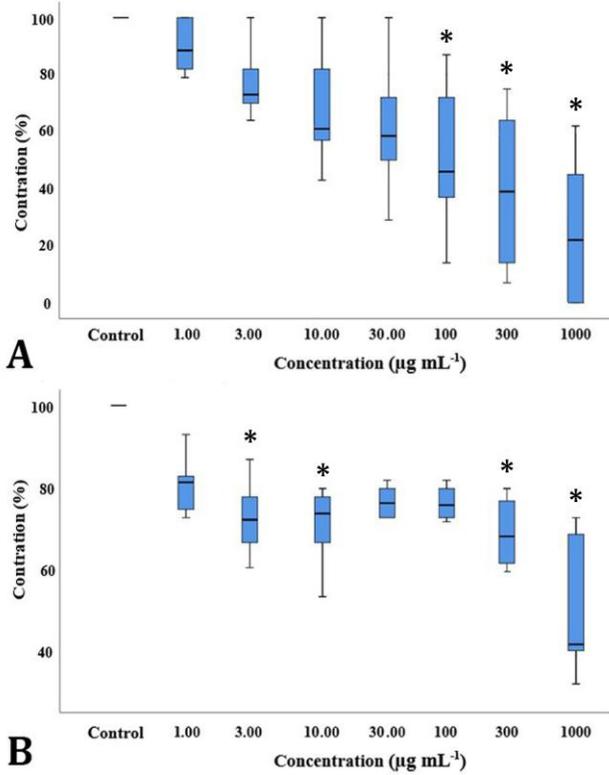


Fig. 2. The effect of different concentrations of 1,8 cineole on contractions induced by potassium chloride **A)** K20, and **B)** K60, in the circular smooth muscles of bovine ileum. * indicates a significant difference compared to the control group ($p < 0.05$).

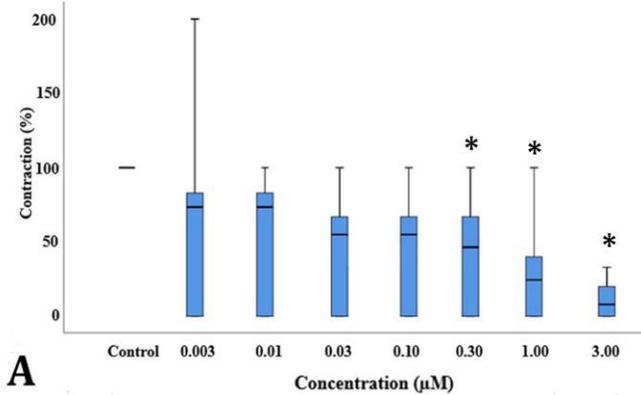


Fig. 3. The effect of different concentrations of verapamil on contraction induced by potassium chloride: **A)** K20, and **B)** K60, in the circular smooth muscles of bovine ileum. * indicates a significant difference compared to the control group ($p < 0.05$).

The results were also compared with those from verapamil-treated samples (standard calcium channel blocker) and found to be similar (Fig. 4).

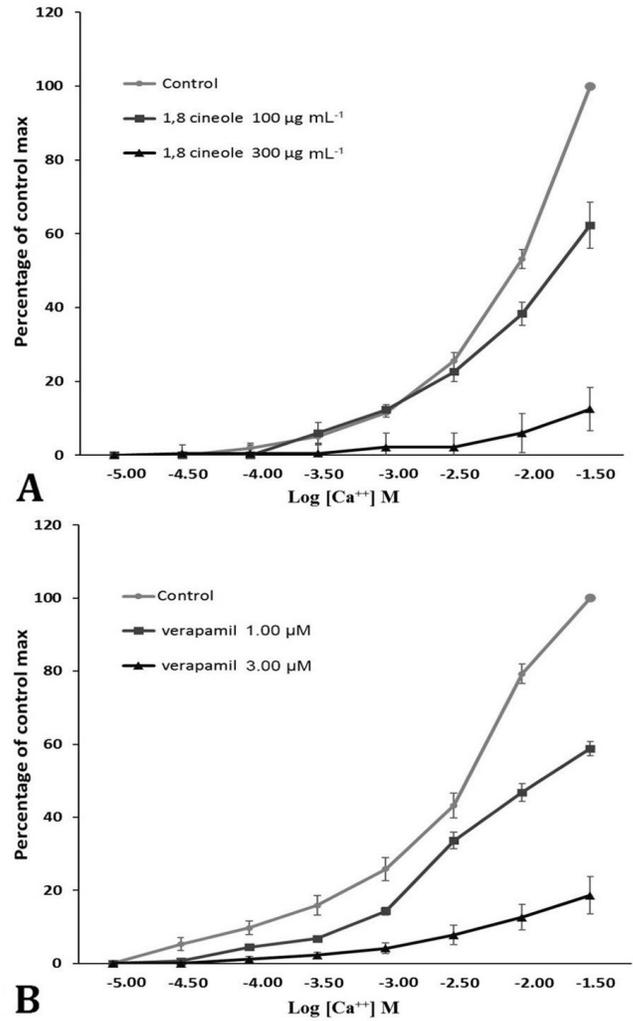


Fig. 4. Concentration-response curves of bovine ileal smooth muscle to calcium concentration increase in the absence and presence of two different concentrations of each of **A)** 1,8-cineole, and **B)** verapamil.

Discussion

The present study investigated the *in vitro* effect of 1,8-cineole on contractions induced by various spasmogens in bovine ileal smooth muscle. Several *in vitro* studies have performed on animals gastrointestinal motility, and it is believed that these experiments may reflect *in vivo* conditions.⁷ The ileum is used to evaluate intestine motilities in most species, and there are few studies regarding bovine ileum motilities.^{16,17} However, cattle ileum is one of the sites involved in diarrhea syndromes, Bovine Viral Diarrhea Virus, and Johne's disease.^{18,19} Numerous studies have performed to identify the anti-spasmodic and intestinal contraction depressor effects of essential oils and extracts of different plants. However, the exact effect of 1,8-cineole, and its mechanism of action in bovine ileum motility have remained undefined.

It has been reported that 1,8-cineole has muscle relaxant properties that is in agreement with the present study findings. The evaluation of the effects of 1,8-cineole on the castor oil-induced diarrhea in rats showed that 1,8-cineole has anti-spasmodic and anti-secretory activities.⁹ In another study, it was also found that 1,8-cineole has spasmolytic effects on the smooth muscle fiber of guinea pig stomach.¹³

A study of 1,8-cineole effect on guinea pig airway smooth muscle showed that 1,8-cineole could have spasmolytic effect in the guinea pig tracheal smooth muscle.¹⁵ Prior studies have shown that greater Ca⁺⁺ entry is involved in sensitized tissues hyper-reactivity to spasmogens. It has been reported that 1,8-cineole depresses the development of force in rat ventricular papillary muscles probably through acting as a Ca⁺⁺ channel blocker.^{20,21} The results of this study are consistent with previous studies on 1,8-cineole. In this study for the first time, the spasmolytic effect of 1,8 cineole on contractions induced by CCh, KCl, and BaCl₂ in bovine ileal smooth muscle was proved. Muscle contraction relaxants may have beneficial clinical uses and *in vitro* myorelaxant effect of herbal extracts could be considered in the diarrhea treatment.²² Using intestinal motility modulators may lead to better results than using antibiotics alone. Intestinal smooth muscle spasm is one of the causes of acute abdominal syndrome in cattle²³ and 1,8 cineole can be a natural alternative for anti-spasmodics such as hyoscine butyl bromide in this syndrome.

The study of mechanism of action determined that when the ileal tissue is affected by different concentrations of calcium in a calcium-free and potassium-rich environment before and after incubation with 1,8 cineole, the tissue response to various concentrations of calcium after treatment with the mentioned substance decreases, which is similar to the response of a tissue treated with verapamil (standard calcium channel blocker). In the present study, before treating the target tissue with

1,8 cineole, or verapamil, a strong contractile response was observed in the tissue when different concentrations of calcium added; after exposing the tissue sample to the 1,8 cineole substances, the contractile response created by adding different concentrations of calcium was weaker than previous stage, i.e., before treatment with 1,8 cineole or verapamil.

In conclusion, the *in vitro* ability of 1,8-cineole in reduction of spontaneous and induced contractions in the bovine ileum was demonstrated in this study. The 1,8 cineole was able to relax the contractions caused by CCh at 10.00 µg mL⁻¹, BaCl₂, and KCl at 100 µg mL⁻¹. The 1,8-cineole relaxed smooth muscles through the calcium channel blocking activity. Considering that, 1,8-cineole has a good potential to produce anti-spasmodic or gastrointestinal motility modulators in veterinary medicine. The products of 1,8-cineole may be used to treat ruminant gastrointestinal disorders such as acute abdomen or intestinal hypermotility. However, further research should be done to evaluate the *in vivo* effect of 1,8-cineole on gastrointestinal motility.

Acknowledgments

We thank all the technical staff of the Veterinary Hospital of Urmia University, and Urmia Industrial Slaughterhouse, Urmia, Iran.

Conflict of interest

The authors declare no competing financial interest.

References

1. Constable PD, Hinchclif KW, Done SH, et al. *Veterinary medicine: A Textbook of the diseases of cattle, horses, sheep, pigs and goats*. 11th ed. St. Louis, USA: Elsevier Health Sciences 2017; 176-908.
2. Fecteau G, Desrochers A, Francoz D, et al. Diagnostic approach to the acute abdomen. *Vet Clin North Am Food Anim Pract* 2018; 34(1): 19-33.
3. Navarre CB, Roussel AJ. Gastrointestinal motility and disease in large animals. *J Vet Intern Med* 1996; 10(2): 51-59.
4. Heller M, Chigerwe M. Diagnosis and treatment of infectious enteritis in neonatal and juvenile ruminants. *Vet Clin North Am Food Anim Pract* 2018; 34(1): 101-117.
5. Ugbogu EA, Elghandour MM, Ikpeazu VO, et al. The potential impacts of dietary plant natural products on the sustainable mitigation of methane emission from livestock farming. *J Clean Prod* 2019; 213: 915-925.
6. Mendel M, Chłopecka M, Dziekan N, et al. Phytogetic feed additives as potential gut contractility modifiers-A review. *Anim Feed Sci Technol* 2017; 230: 30-46.

7. Mendel M, Chłopecka M, Latek U, et al. Evaluation of the effects of *Bidens tripartita* extracts and their main constituents on intestinal motility - An *ex vivo* study. *J Ethnopharmacol* 2020; 259:112982. doi: 10.1016/j.jep.2020.112982.
8. Jalilzadeh-Amin G, Maham M, Dalir-Naghadeh B, et al. In vitro effects of *Artemisia dracunculus* essential oil on ruminal and abomasal smooth muscle in sheep. *Comp Clin Path* 2012; 21: 673-680.
9. Jalilzadeh-Amin G, Maham M. The application of 1,8-cineole, a terpenoid oxide present in medicinal plants, inhibits castor oil-induced diarrhea in rats. *Pharm Biol* 2015; 53(4): 594-599.
10. Van Vuuren SF, Viljoen AM. Antimicrobial activity of limonene enantiomers and 1,8-cineole alone and in combination. *Flavour Fragr J* 2007; 22(6): 540-544.
11. Juergens UR. Anti-inflammatory properties of the monoterpene 1,8-cineole: current evidence for co-medication in inflammatory airway diseases. *Drug Res (Stuttg)* 2014; 64(12): 638-646.
12. Santos FA, Silva RM, Campos AR, et al. 1,8-cineole (eucalyptol), a monoterpene oxide attenuates the colonic damage in rats on acute TNBS-colitis. *Food Chem Toxicol* 2004; 42(4): 579-84.
13. Sagorchev P, Lukanov J, Beer AM. Effects of 1,8-cineole (eucalyptol) on the spontaneous contractile activity of smooth muscles fibre. *J Med Plant Res* 2015; 9(14): 486-493.
14. Farre AJ, Colombo M, Fort M, et al. Differential effects of various Ca²⁺ antagonists. *Gen Pharmacol* 1991; 22(1): 177-181.
15. Bastos VP, Brito TS, Lima FJ, et al. Inhibitory effect of 1,8-cineole on guinea-pig airway challenged with ovalbumin involves a preferential action on electro-mechanical coupling. *Clin Exp Pharmacol Physiol* 2009; 36(11): 1120-1126.
16. Pfeiffer JB, Mevissen M, Steiner A, et al. In vitro effects of bethanechol on specimens of intestinal smooth muscle obtained from the duodenum and jejunum of healthy dairy cows. *Am J Vet Res* 2007; 68(3): 313-322.
17. Ansia I, Stein HH, Brøkner C, et al. Short communication: A pilot study to describe duodenal and ileal flows of nutrients and to estimate small intestine endogenous protein losses in weaned calves. *J Dairy Sci* 2020; 103(10): 9102-9109.
18. Khodakaram-Tafti A, Farjanikish GH. Persistent bovine viral diarrhea virus (BVDV) infection in cattle herds. *Iran J Vet Res* 2017; 18(3): 154-163.
19. Albarrak SM, Waters WR, Stabel JR, et al. Evaluating the cytokine profile of the WC1+ $\gamma\delta$ T cell subset in the ileum of cattle with the subclinical and clinical forms of MAP infection. *Vet Immunol Immunopathol* 2018; 201: 26-31.
20. Perpiña M, Cortijo J, Fornás E, et al. Hyperreactivity and ⁴⁵Ca movements in sensitized guinea-pig tracheal muscle. *Eur Respir J* 1991; 4(4): 450-457.
21. Wang Z, Ma Z, Zhou J. Changes of Ca²⁺ channel behaviors in bronchial smooth muscle cells of asthma guinea-pig model [Chinese]. *Zhonghua Jie He He Hu Xi Za Zhi* 2001; 24(9): 539-541.
22. Aleem A, Janbaz KH. Dual mechanisms of anti-muscarinic and Ca⁺⁺ antagonistic activities to validate the folkloric uses of *Cyperus niveus* Retz. as anti-spasmodic and antidiarrheal. *J Ethnopharmacol* 2018; 213: 138-148.
23. Mullooney PC, Patterson WH. Therapeutic agents used in the treatment of calf diarrhea. *Vet Clin North Am Food Anim Pract* 1985; 1(3): 563-579.