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RESEARCH ARTICLE

Evaluation of Peppermint (*Mentha piperita* L.) Essential Oil as a Digestive Tract Regulator in Broilers

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ABSTRACT

The aim of this study was to investigate the effects of peppermint *(Mentha piperita L.)* essential oil on duodenal and colonic contractions of broilers *in vitro*. For this purpose, 10 broiler intestines (42 days old) obtained from a slaughterhouse were brought to the laboratories by immersed in Krebs solution (NaCl, 118 mmol/L; KCl, 4.7 mmol/L; $CaCl_2$, 2.5 mmol/L; $MgSO_4$, 1 mmol/L; KH_2PO_4, 1 mmol/L; glucose, 11 mmol/L; $NaHCO_3$, 25 mmol/L) at +4°C. Isolated duodenal and colon tissue strips were placed in a four-channel, isolated organ bath system, where they were exposed to Krebs solution aerated with a gas mixture of 95% O_2 - 5% CO_2, at 39°C. The effect of peppermint essential oil (PEO) obtained by hydrodistillation method on spontaneous duodenum and colon contractions was evaluated in the concentration range of 0.1-1000 µg/mL. Subsequently, the effects of 300 and 1000 µg/mL PEO were reevaluated on acetylcholine-induced contractions after incubation with Nω-Nitro-L-arginine (L-NNA, NOS inhibitor) and Methylene Blue (MB, cGC inhibitor). It was determined that the PEO dose-dependently decreased the amplitude of spontaneous and acetylcholine-induced contractions in both isolated tissues. This relaxant effect persisted after L-NNA and MB incubations. In conclusion, PEO induced relaxation in isolated duodenum and colon intestinal smooth muscles not through the nitric oxide-sGMP pathway.

Keywords: Broiler, Colon, Contraction, Duodenum, Peppermint essential oil

INTRODUCTION

Antibiotics are prohibited from being used as feed additives in poultry nutrition in the European Union because they cause a decrease in beneficial microorganisms in the intestinal microflora of animals, create residual risk in animal products, and adversely affect the health of people fed with these products [1]. Accordingly, studies on the development of alternative feed additives to antibiotics have been accelerated. In this context, the use of natural and reliable herbal products (aromatic plants and essential oils extracted from these plants) for poultry nutrition has gained importance [2,3]. Essential oils obtained by steam distillation or squeezing from the leaves, flowers, bark, seeds, and roots of plants have volatile and fragrant properties at room temperature [4]. Many studies have reported that these products protect the endocrine and immune systems [3,5], increase the digestibility and absorption of nutrients [6-8] and improve performance [9-11], as well as antioxidant, anti-inflammatory, and anti-microbial effects [12].

Mentha plants are one of the most popular plants used since ancient times for their medicinal and aromatherapy properties [13]. The essential oil of peppermint, which is the most important Mentha specie, has been reported to have antioxidant, antidiabetic, antibacterial, anti-mutagenic, antifungal, and anticarcinogenic effects [14]. Studies conducted on the use of Mentha plants in poultry nutrition have evaluated the effects on performance [13,15], carcass traits [16,17], immunity, and some serum biochemistry parameters [18] during the growth period in broilers and on performance, egg quality and serum parameters in laying hens [19,20]. It is also stated that peppermint has a beneficial effect on the absorption surface area by improving the small intestine length and villi structure in quails [18]. These plants are used for the treatment of gastrointestinal (GI) system diseases [21-23].

The regulation of intestinal motility is significant as its adequate digestion of food and minimizes the risk of colonization by intestinal pathogens in the lower GI tract [24,25]. Prior studies have provided mixed results regarding the effect of essential oils and extracts derived from aromatic plants on intestinal contractions, as noticed in studies conducted on rats [22,23] and broilers [26]. This study was designed to evaluate the effect of various levels of POE on contractions occurring in the duodenum and colon of poultry. Assessing the potential impact of POE on contractions in the chicken GI tract will contribute to the development of potential uses for POE, which serves as an alternative to synthetic agents, such as antibiotics, in the field of poultry nutrition.

Material and Methods

Ethical Statement

This study was approved by the Afyon Kocatepe University Animal Experiments Local Ethics Committee (Approval no: AKÜ-HADYEK-272-13).

Extraction of PEO

Fresh mint (*Mentha piperita L.*) was obtained from a local herbal market in Afyonkarahisar. Turkey. After being cleared of weeds and parts, it was dried at room temperature without sunlight and then ground into powder using a mechanical grinder. 50 g of ground mint were transferred to a glass flask filled with 500 mL of distilled water. Hydrodistillation was carried out in a Clevenger apparatus for 180 min [26]. Before starting the distillation process, 1 mL of n-hexane was added to the water surface of the side arm of the Clevenger apparatus, and the condensed essential oil droplets were collected, reducing their distribution with water, and increasing efficiency. The resulting essential oil was dried over anhydrous Na_2SO_4 (Sigma-Aldrich, 238597) and stored in a closed dark glass bottle in a refrigerator at 4°C until use [25].

Animal and Tissue Preparation

This study was carried out on 10 broilers (Ross 308; 42 days old). They were obtained from the local private poultry slaughterhouse with standard regulations; the birds were sacrificed via a neck cut and bled for 120 sec. The birds were eviscerated manually [27]. The tissue strips were processed approximately 10 min after the animal was sacrificed, 2 cm-long strips of the mid-duodenum and colon were obtained. Then, these were immersed in a cold, freshly prepared Krebs solution (NaCl, 118 mmol/L; KCl, 4.7 mmol/L; $CaCl_2$, 2.5 mmol/L; $MgSO_4$, 1 mmol/L; KH_2PO_4, 1 mmol/L; glucose, 11 mmol/L; $NaHCO_3$, 25 mmol/L) and transferred to the laboratory. After removing fat and connective tissue from the intestinal segments, longitudinal strips (0.6 cm long and approximately 3-4 mm wide) were placed in a four-chamber organ bath (IOBS 99 Isolated Tissue Bath Stand Set; Commat) with 20 mL Krebs solution (pH 7.4) in 95% O_2 and 5% CO_2 at 39°C. Longitudinal smooth muscle strips were attached to platinum ring electrodes along one edge using 2:0 silk ligatures. The opposite edge of the tissue was connected to a force-displacement transducer (model 10-A; MAY; Commat, Ankara, Türkiye). The isometric smooth muscle activity of the intestine samples was monitored and recorded by the computer using the force transducer and an acquisition system (model MP30 WSW with Biopac Student Lab, PRO Software, Biopac Systems; Commat). To maintain the contractile activity of the tissue, optimal tension relationships for the strips were achieved with resting tensions of 1 g. It was allowed to equilibrate for an hour and the Krebs solution was changed every fifteen minutes. The viability of the strips was verified by adding Acetylcholine (Ach) at a concentration of 10^{-5} M at the beginning and/or end of the experiment.

Monitoring the GI Tract Muscle Activity

Subsequently, the effectiveness of the doses (300 and 1000 µg/mL) of PEO that cause inhibition in duodenal and colonic contractions was reevaluated after incubation with the NOS inhibitor L-NNA (10^{-5} M) and the cGC blocker MB (10^{-2} M). In addition, the effect of 8-bromo-cyclic GMP (8-Br-cGMP; 10^{-8}, 10^{-7} and 10^{-6} M), a cGMP analog, on the amplitude of isolated duodenal and colon spontaneous contractions was evaluated.

Statistical Analysis

Statistical analysis was done using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The Shapiro–Wilk test was used to test the data for normality. All values are presented as mean ± S.E.M. One-way ANOVA was used for the statistical evaluation of the data. The Tukey test was performed to compare individual means of treatment groups. Differences were considered significant at $P<0.05$.

Results

Effect of PEO on the Amplitude of Spontaneous Duodenum and Colon Contractions

It was determined that the cumulative application of PEO at concentrations of 0.1, 0.3, 1, 3, 10, 30 100, 300 and 1000 µg/mL caused inappropriate relaxation to the gradual concentration response in the duodenum *(Fig. 1-A)* and colon *(Fig. 1-B)* tissue samples.

The amplitude of spontaneous duodenal and colonic contractions was reduced by PEO at concentrations of 300 µg/mL ($P<0.01$) and 1000 µg/mL ($P<0.001$). However, no statistically significant influence on these contractions was seen at concentrations from 0.1 to 100 µg/mL. It was

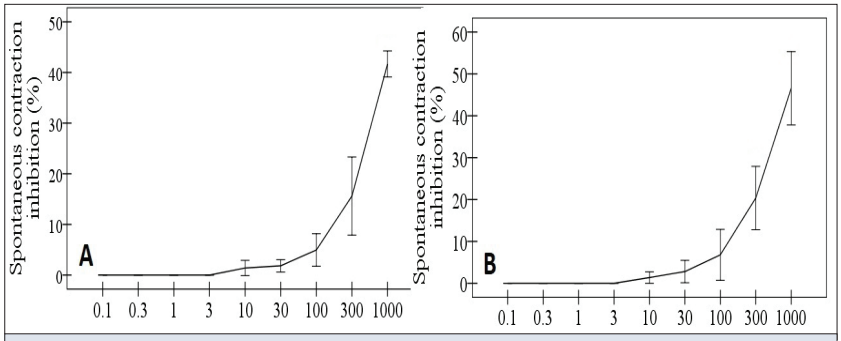

Fig 1. The dose-response curves of PEO in isolated broiler duodenum and colon (A: Duodenum, B: Colon, PEO: Peppermint essential oil)

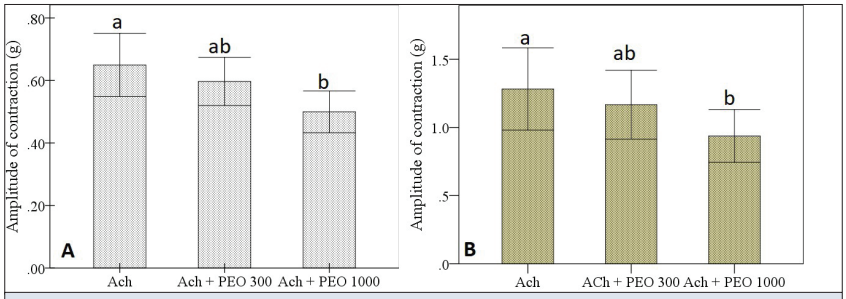

Fig 2. Effect of 300 and 1000 µg/mL PEO on Ach-induced duodenum and colon contractions. (A: Duodenum, B: Colon; PEO: Peppermint essential oil). Amplitude values of contractions in groups induced by EFS in the presence of PEO (300 and 1000 µg/mL. Letters (a and b) indicate significant differences between them in each column (P<0.05)

observed that the contractions returned to normal after washing the tissues with Krebs solution.

Effect of PEO on Ach-Induced Duodenum and Colon Contractions

The effects of 300 and 1000 µg/mL PEO on duodenal and colonic contractions induced by Ach (10^{-4} M), respectively, are shown in *Fig. 2*. It was determined that 1000 µg/mL PEO reduced the contraction effect of Ach in both tissues (P<0.05).

Effect of PEO on Duodenum and Colon Contractions Induced by Ach Following L-NNA and MB Incubation

The effects of 300 and 1000 µg/mL PEO on the amplitude of duodenal and colonic contractions induced by Ach (10^{-4} M) following L-NNA and MB incubations, respectively, are given in *Table 1* and *Table 2*. It was determined that 1000 µg/mL PEO decreased the amplitude of contraction in both tissues (P<0.05), and a similar effect was observed after MB incubation (P<0.05).

Effect of 8-Br-cGMP on Spontaneous Duodenum and Colon Contractions

The effect of 8-Br-cGMP (10^{-8} - 10^{-6} M) on spontaneous duodenal and colonic contractions is shown in *Fig. 3*. It was observed that 10^{-8} M dose of 8-Br-cGMP had no effect on the contraction intensity of both tissues, whereas 10^{-7}

Table 1. *Effect of PEO on the amplitude of Ach-induced contractions following L-NNA incubation (g)*

Tissue	L-NNA + Ach	L-NNA + Ach + PEO 300	LNNA ± Ach + PEO 1000	P-value
Duodenum	0.661±0.052[a]	0.608±0.036[ab]	0.511±0.026[b]	0.048
Colon	1.301±0.151[a]	1.190±0.135[ab]	0.955±0.092[b]	0.032

L-NNA: *Nω-Nitro-L-arginine;* ***Ach:*** *Acetylcholine;* ***PEO:*** *peppermint essential oil*
Letters (a, b) in the same line indicate significant differences between different letters

Table 2. *Effect of PEO on the amplitude of Ach-induced contractions following MB incubation (g)*

Tissue	MB + Ach	MB + Ach + PEO 300	MB ± Ach + PEO 1000	P-value
Duodenum	0.676±0.053[a]	0.608±0.037[a]	0.496±0.021[b]	0.010
Colon	1.313±0.152[a]	1.208±0.146[ab]	0.945±0.093[b]	0.042

MB: *Methylene Blue;* ***Ach:*** *Acetylcholine;* ***PEO:*** *peppermint essential oil*
Letters (a, b) in the same line indicate significant differences between different letters

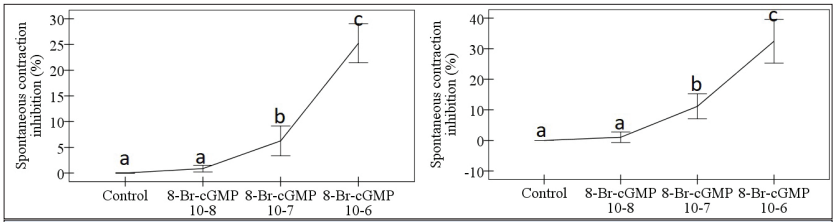

Fig 3. The dose-response curves of 8-Br-cGMP in isolated broiler duodenum and colon (A: Duodenum, B: Colon). Letters (a, b) in the same line indicate significant differences between different letters

M and 10^{-6} M doses depressed the contraction intensity ($P<0.001$).

Discussion

In this study, PEO decreased the amplitude of spontaneous duodenum and colon contractions in broilers in a dose-dependent manner. Similarly, we have previously demonstrated that myrtle, rosemary, and thyme essential oils inhibit contractions of the small intestine [26]. Essential oils have been added to poultry diets for several purposes. Recently, they have also been used to increase the digestibility and absorption of nutrients in poultry [7,28,29]. Essential oils have been demonstrated to increase the secretion of some digestive enzymes [6] and stimulate the appetite center due to their aroma-enhancing properties [30,31]. Since essential oils obtained from different plants regulate the contractions in the GI tract of rodents, the use of these plants is recommended to support the digestive tract [32,33]. Mentha plants have been used in traditional medicine for many years in the treatment of GI disorders [13]. In this study, the spasmolytic effect created by the essential oil of peppermint, known as Mentha spicata, was demonstrated in isolated duodenum and colon smooth muscle.

In the GI tract, Ach is an important neural mediator of parasympathetic innervation in poultry, similar to in other animals. It binds to muscarinic receptors and induces smooth muscle contraction [34,35]. Our previous study showed which Ach concentrations of 10^{-4} M induce maximal contractions in the duodenum of broilers [26]. In this study, duodenum and colon contractions were induced by using Ach (10^{-4} M), and the effectiveness of PEO (300-1000 g/mL) was reevaluated. The spasmolytic activity of PEO has been demonstrated by reducing the amplitude of Ach-induced contractions, similar to spontaneous duodenal and colonic contractions.

In this study, after determining the relaxation effect of PEO, we hypothesized that it may be efficient through the NOS-NO-cGC. The smooth muscle layers are innervated by enteric inhibitory and excitatory motor neurons that directly regulate GI motility [36]. Nitric oxide (NO), which is released in response to nerve stimulation of the myenteric plexus, causes relaxation of smooth muscle in addition to the protection of the digestive tract mucosa as a result of the regulation of gastric mucosal blood flow and fluid secretion. Thus, it plays an important role in regulating stomach and intestinal motility [37,38]. In the myenteric plexus, neuronal NO synthase (nNOS) generates NO from L-arginine [36]. Due to their ability to inhibit NOS, L-arginine analogs, specifically N-nitro-L-arginine (L-NNA) and N-ω-Nitro-L-arginine methyl ester, are frequently used to prevent the production of NO [40,41]. In contraction trials, when the nNOS inhibitor L-NNA is at a concentration of 1 mM, it blocks the NOS enzyme [41,42]. In the present study, it was determined that the relaxing effect of PEO continued in the duodenum and colon, induced by Ach, after inhibition of the NOS enzyme with L-NNA. Therefore, the results of the study show that PEO does not create a relaxant effect by increasing NO formation.

The primary receptor for NO is soluble guanylate cyclase (sGC) [40]. Nitric oxide activation of sGC leads to the formation of cGMP. As a second messenger, cGMP interacts with protein kinases, phosphodiesterases, and ion channels, leading to the cellular effects of NO. The cGMP induces relaxation in smooth muscle tissue by two mechanisms [44]. First, it increases the permeability of K^+ channels by lowering the level of Ca^{++} in the cell. Thus, it hyperpolarizes the plasma membrane [45,46]. Second, cGMP blocks the myosin/actin interaction by activating cGMP-dependent protein kinase (PFG), which causes the dephosphorization of myosin light chains [47]. Moreover, some of the NO-induced smooth muscle relaxations were also reported to be independent of cGMP [36,48]. However, there are no previous studies in which the effects and dose were demonstrated in the anatomical formations of the small and large intestines, although the effect of cGMP was shown in different smooth muscle cells in poultry. In this study, it was determined that the amplitude of duodenum and colon contractions decreased. In this context, a cGMP-dependent relaxation was observed in the poultry intestine.

Methylene blue prevents cGMP-dependent relaxation by inhibiting cGC inhibitor and cGMP formation [49]. It is known that the dose of 10^{-5} M blocks cGC in *in vitro*

experiments [42,50]. The present study provided evidence that the intensity of Ach-induced duodenal and colonic contractions was reduced by PEO after inhibition of MB and cGC, whereas MB did not reduce PEO activity. In conclusion, it is considered that the effectiveness of PEO is not a cGMP dependent.

As a result, it was determined that a cGMP-dependent relaxation was observed in the duodenum and colon of broilers, whereas PEO reduced the amplitude of contractions in the same tissues without using the NOS-cGC-cGMP pathway. It is suggested that >10 µg/mL of PEO be added to broiler diets due to its spasmolytic effect, but it is also recommended to conduct trials in terms of *in vivo* activity.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (T. Bülbül) on reasonable request.

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Ethical Statement

This study was approved by the Afyon Kocatepe University Animal Experiments Local Ethics Committee (Approval no: AKÜ-HADYEK-272-13).

Conflict of Interest

There is no conflict of interest.

Author Contributions

TB designed this study, prepared this manuscript, and analyzed experimental data; VÖ analyzed experimental data; AB analyzed experimental data.

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