ORIGINAL ARTICLE

UDC: 615.322:616.344-08-092.9
https://doi.org/10.2298/VSP161001391G

The effect of the aqueous and methanol fennel stem extracts (*Foeniculum vulgare Miller*) on isolated rat ileum contractility

Uticaj vodenog i metanolnog ekstrakta stabla morača (*Foeniculum vulgare Miller*) na kontraktilnost izolovanog tankog creva pacova

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Abstract

Background/Aim. The fennel *(Foeniculum vulgare* Miller, Apiaceae) has a long history of use as traditional herb medicine due to its carminative properties. The study was aimed to investigate the effects of aqueous and methanol fennel stem extracts on intestinal activity. **Methods.** Relaxant activity of aqueous and methanol fennel stem extracts was evaluated *in vitro* in three experimental models: spontaneous contraction, acetylcholine and potassium chloride (KCl)-induced contraction of an isolated rat ileum. The composition of aqueous and methanol fennel stem extracts was qualitatively analyzed using the high performance liquid chromatographic (HPLC) analysis. **Results.** In the presence of an aqueous fennel stem extract at a concentration of 3 mg/mL, the inhibition of the spontaneous contractions of isolated rat ileum was 35.05% ± 3.57%. In presence of a methanol fennel stem extract at the same concentration, the maximum reduction of the spontaneous contractions was 48.91% ± 6.31%. The extracts in a concentration-dependent manner significantly inhibited the acetylcholine and KCl induced contractions of the isolated rat ileum ($p < 0.01$). The following components were identified in fennel methanol stem extract: 3-caffeoylquinic acid, chlorogenic acid, 4-caffeoylquinic acid, 1,3-dicaffeoylquinic acid, rutin, miquelianin, quercetin heterosides, 1,5-dicaffeoylquinic acid, 1,4-dicaffeoylquinic acid, apigenin and rosmarinic acid. In an aqueous extract, their presence is found in trace amounts. **Conclusion.** The results of this study showed that the aqueous and methanol fennel stem extracts have spasmolytic effects on the intestinal smooth muscle and may be used for the control of intestinal motility.

Key words:
ileum; rats; foeniculum; acetylcholine; potassium chloride; treatment outcome.

Apstrakt

Uvod/Cilj. Morač (*Foeniculum vulgare* Miller, Apiaceae) se od davnina koristi u tradicionalnoj medicini kao karminativ. Ova studija je imala za cilj da istraži efekte vodenog i metanolnog ekstrakta stabla morača na aktivnost tankog creva. **Metode.** Relaksantna aktivnost vodenog i metanolnog ekstrakta morača ispitivana je *in vitro* pomoću tri eksperimentalna modela na izolovanom ileumu pacova: spontana aktivost, acetilholinom i kalijum-holridom (KCl)-indukovane kontrakcije. Kvalitativni sastav vodenog i metanolnog ekstrakta morača određivan je pomoću tečne hormotografije visokih performansi (HPLC). **Rezultati.** U prisustvu vodenog ekstrakta stabla morača u koncentraciji od 3 mg/mL maksimalna inhibicija spontanih kontrakcija izolovanog ileuma pacova je iznosila 35.05% ± 3.57%, dok je u prisustvu metanolnog ekstrakta stabla morača iste koncentracije maksimalna redukcija spontanih kontrakcija iznosila 48.91% ± 6.31%. Ekstrakti stabla morača proporcionalno primenjenoj koncentraciji značajno su inhibisali kontrakcije ileuma stimulisane acetilholinom i kalijum-hloridom ($p < 0.01$). U metanolnom ekstraktu morača utvrđeno je prisustvo 3-kafeoilhine kiseline, hlorogenske kiseline, 4-kafeoilhine kiseline, 1,3-dikafeoilhine kiseline, rutina, mikvelianina, kvercetin heterozida, 1,5-dikafeoilhine kiseline, 1,4-dikafeoilhine kiseline, apigenina i rozmarinske kiseline. U vodenom ekstraktu njihovo prisustvo je nađeno u tragovima. **Zaključak.** Rezultati ove studije potvrđuju da vodeni i metanolni ekstrakti stabla morača deluju spazmolitički na glatke mišiće tankog creva i mogu se koristiti za kontrolu intestinalne motorike.

Ključne reči:
ileum; pacovi; foeniculum; acetilholin; kalijum hlorid; lečenje, ishod.

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Introduction

Functional bowel disorders include a group of several functional gastrointestinal syndromes: irritable bowel syndrome (IBS), functional constipation, diarrhea, bloating, and abdominal pain. The symptoms are manifested in the middle or lower gastrointestinal tract [1]. Many drugs used in the treatment of these disorders exhibit adverse effects during long-term use. Therefore, there is an increased use of natural herbal medicines and teas for the treatment of these disorders, especially the herbs of the Apiaceae family [2].

Fennel (*Foeniculum vulgare* Miller) is a member of the Apiaceae family and has been traditionally used for a long time, both as a medicinal plant and as a spice.

Since prehistoric times, fennel fruit has been used in China, India and Pakistan to treat many conditions such as digestive disorders and for the improvement of vision problems. Also, the fennel is effective in treatment of headache, flu and as a brain tonic. This plant has been used as a household remedies for digestive tract spasms release and for the bloating treatment [3].

Digestive disorders like meteorism, colic, stomach ache are successfully treated with the fennel in herbal medicine [4, 5]. It has also been used in the cosmetic formulations, the perfume industry, as well as in liqueur preparation to improve the taste [6–11]. Literature data has reported that the seeds of this plant regulate the menstrual cycle in women, reduce menopausal symptoms and increase libido [12].

Fennel demonstrates *in vitro* and *in vivo* studies antispasmodic, anti-inflammatory, analgesic and diuretic effects. A review of the literature showed antioxidant, antimicrobial, gastroprotective and hepatoprotective activity of the fennel extracts and essential oil [13]. Also, the ingestion of essential oil induced hypoglycaemic effect in diabetic rats [14]. Pradhan et al. [15] showed that the methanol extract of *F. vulgare* induced cytoprotective and anti-tumour activities in cultured cells.

It has been previously reported that in experiments with isolated uterus, the essential oil of *F. vulgare* reduced the intensity and frequency of oxytocin and prostaglandin E2 induced contractions [16].

Till now, there is no report about the effects of the aqueous and methanol fennel stem extracts on the contractility of intestinal smooth muscles. The present study was designed to examine the potential effects of the aqueous and methanol fennel stem extracts on the contractile responses of an isolated rat ileum.

Methods

Chemicals

All reagents and solvents in this study were of analytical and HPLC grade. Acetylcholine chloride, papaverine, atropine, 3-caffeoylquinic acid, chlorogenic acid, 4-caffeoylquinic acid, 1,3-dicaffeoylquinic acid, rutin, 1,5-dicaffeoylquinic acid, miquelianin, rosmarinic acid and apigenin were purchased from Sigma-Aldrich (Sigma-Aldrich Co., St Louis, MO). 1,4-dicaffeoylquinic acid was obtained from Chem Faces (Wuhan, PRC). All chemicals were dissolved in distilled water for each experimental protocol. The composition of Tyrode's solution (in mM) was: NaCl (136), KCl (2.7), $CaCl_2$ (1.8), $NaHCO_3$ (12), NaH_2PO_4 (0.3), $MgCl_2$ (1.8) and glucose (5.6).

Plant material

The stems of fennel were harvested in the region of Niš during August 2012. The plant material was taxonomically identified by assistant professor Bojan Zlatković from the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš. The fennel stems were open-air dried in the shade.

Extraction procedure

Dried and pulverized stems (200 g) were extracted for 30 minutes in an ultrasonic bath with 250 mL of distilled water and absolute methanol. Separate parts of material were extracted with water and methanol. After the filtration, the extracts were concentrated in a rotary evaporator at a reduced pressure (40 ºC) till a constant weight was achieved. By extraction, from 100 g of dry stem, 8.72 g (8.72% of yield) of dry methanol extracts and 2.5 g (2.5% of yield) of dry water extracts were obtained. The extracts were stored at -20 ^{o}C. For experimental purposes the fresh dilutions were prepared on the day of experiments. The dry residues were solubilized in the distilled water in order to obtain 10% solution used for the experiments. All concentrations were presented as final bath concentrations.

Animals

All of the experimental procedures with animals were in accordance with the European Council Directive of September 22nd, Directive 2010/63/EU and were also approved by the Ethic Committee Medical Faculty University of Niš (number 12-2466-3). Fifteen male Wistar albino rats (200–250 g) were used from the Animal Research Center of Faculty of Medicine, University of Niš, Serbia. The animals were maintained at standard environmental laboratory conditions, fed with standard pellet, and had free access to food and water.

Experimental protocol

Animals were anesthetized by ether and the abdominal cavity of rat was opened. The segments of 2 cm long distal parts of the ileum were dissected out and mounted in an organ bath (10 mL) containing the Tyrode's solution (37 ºC, pH 7.4, aerated with mixture of 5% carbon dioxide in oxygen) between two stainless steel hooks with continuous air-bubbling. The change of intestinal activity was recorded using system TSZ-04-E; Spell Iso (Experimetria Ltd). The areas under the curves were evaluated [16]. The aqueous and methanol fennel stem extracts and control chemicals were

added directly to the organ bath. Six segments of isolated ileum were used in each experiment.

The first part of experiments was related to spontaneous rhythmic rat ileum contractions. After the stabilization period the ileum was treated with the aqueous and methanol fennel stem extracts (0.01, 0.03, 0.1, 0.3, 1 and 3 mg/mL). Papaverine (0.01, 0.03, 0.1, 0.3, 1 and 3 μg/mL) was used as a positive control. The relaxant effect of the aqueous and methanol fennel stem extracts was expressed as a percentage of the control contractility without extract.

In the second part of the experiment, the ileum contractions were stimulated by increasing concentrations of acetylcholine (5–1500 nM). Increasing concentrations of acetylcholine were added to the organ bath cumulatively until obtaining the maximum contractile response curve. The contractions were registered in the presence of the aqueous and methanol fennel stem extracts (1–3 mg/mL). Atropine (140 nM) was used as a positive control.

In the third part of the experiment the ileum contractions were induced by KCl solution (80 mM). Then the aqueous and methanol fennel stem extracts were cumulatively added to the tissue bath (0.01–3 mg/mL).

The relaxation of the intestinal preparation in the presence of extracts or antagonist was expressed as a percentage of the maximum contractile response induced by acetylcholine and KCl. After each part of the experiment the tissues were flushed with the fresh Tyrode's solution and left to adapt for 10 minutes.

HPLC analysis

The high performance liquid chromatographic (HPLC) analysis of the aqueous and methanol fennel stem extracts was performed on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, Calif., U.S.A.) with a diode array detector (DAD). The analytical column was Purospher STAR RP-18e (150 × 4.6 mm) with particle size of 5 μm, manufactured by Merck, Darmstadt, Germany. Injected volume was 10 μL and flow rate was 0.7 mL/min. The eluent system consisted of 0.1% water-trifluoroacetic acid solution (A) and acetonitrile (B) with linear gradient: 0–3 min 5%–5% B, 3-32 min 5%–28% B, 32–44 min 25%–50% B, 44–52 min 50%–80% B, 52–54 min 80%–90% B, 54–59 min 90%–5% B, and 59–60 min 5% B. The column temperature was maintained at 30 ºC. The compounds were identified on the basis of UV-VIS signal response in comparison to standards. The extracts were dissolved in methanol, HPLC grade, to a concentration of 10 mg/mL. The measurement was performed on 280, 330 and 360 nm wavelength.

Statistical analysis

All values were expressed as mean ± standard deviation. Statistical analysis of the differences between two means was assessed using Student's *t*-test. Halt maximal effective concentration (EC_{50}) was established by regression analysis. The significance level considered in all tests was $p < 0.05$.

Results

Rat ileum suspended in the Tyrode's solution showed spontaneous contractile activities which were significantly reduced in the presence of the aqueous and methanol fennel stem extracts (Figure 1). The maximal inhibitory effect of the aqueous and methanol fennel stem extracts was achieved with 3 mg/mL concentration. In the presence of the aqueous fennel stem extract at a concentration of 3 mg/mL the spontaneous contractions of isolated rat ileum were reduced. The inhibition was 35.05% ± 3.57% ($p < 0.05$). The EC_{50} value for the aqueous fennel stem extract induced relaxation was 19.53 ± 2.68 mg/mL. In the presence of the methanol fennel stem extract at a concentration of 3 mg/mL the spontaneous contractions of the isolated rat ileum was significantly reduced (48.91% ± 6.31%, $p < 0.01$). The EC_{50} value for the methanol fennel stem extract induced relaxation was 5.14 ± 0.42 mg/mL.

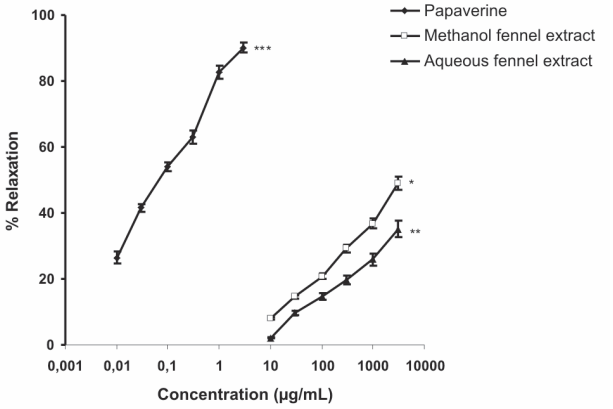

Fig. 1 – Inhibitory effects of the aqueous and methanol stem extracts and papaverine on spontaneous contractions in isolated rat ileum. Each point represents the mean value of at least six experiments and the vertical bars the standard error of the mean (*$p < 0.001$, **$p < 0.01$, *$p < 0.05$).**

It was found that the difference between the relaxant effect of the aqueous and methanol fennel stem extracts was statistically significant ($p < 0.05$). Papaverine was used as a positive control and significantly reduced the intestinal basal tonus in a concentration-dependent manner ($p < 0.001$). The relaxant effects of the aqueous and methanol fennel stem extract on spontaneous rat ileum contractions were terminated after flushing the preparation.

Acetylcholine (5–1500 nM) induced a concentration dependent contraction of the rat ileum. The aqueous fennel stem extract (1–3 mg/mL) caused a significant reduction in contractile response which was registered as a depression of the cumulative concentration response curve for acetylcholine ($p < 0.05$).

The aqueous extract caused a reduction of acetylcholine induced contraction to 80.35% ± 10.22% at a concentration of 3 mg/mL (Figure 2). The methanol fennel stem extract (1–3 mg/mL) significantly and dose-dependently reduced the rat ileum contractions induced by acetylcholine ($p < 0.01$) (Figure 3). The methanol extract caused a reduction of acethyl-

choline induced contraction to 74.89% ± 8.36% at a concentration of 3 mg/mL. Atropine, the non-selective blocker of muscarinic receptors, produced a significant inhibition of contractions caused by acetylcholine ($p < 0.001$). The inhibitory effects of the aqueous and methanol fennel stem extracts on acetylcholine induced rat ileum contractions were terminated after removing the extracts from the organ baths.

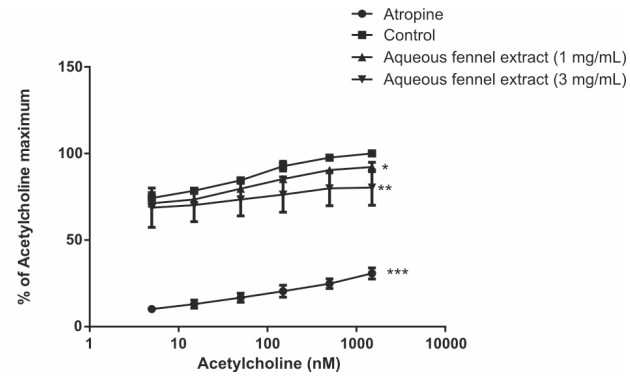

Fig. 2 – Inhibitory effects of the aqueous fennel stem extract and atropine on ileum contraction induced by acetylcholine. Each value is expressed as means ± standard error (n = 6), (*$p < 0.001$, **$p < 0.01$, *$p < 0.05$).**

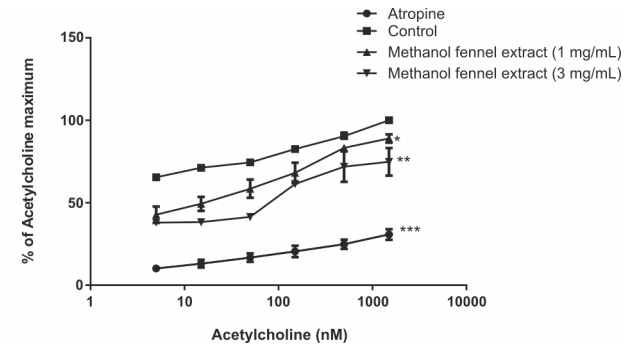

Fig. 3 – Inhibitory effects of the methanol fennel stem extract and atropine on ileum contraction induced by acetylcholine. Each value is expressed as means ± standard error (n = 6) (*p < 0.001, **p < 0.01, *p < 0.05).**

High concentration of potassium ions (80 mM) induced tonic contractions of the rat ileum. The KCl induced contractions of the isolated rat ileum were significantly reduced in the presence of the aqueous and methanol fennel stem extracts (Figure 4). The maximal inhibitory effect of the aqueous and methanol fennel stem extracts was achieved with a concentration of 3 mg/mL. The aqueous fennel stem extract caused a mean contractile response of 88.83% ± 1.52% (at a concentration of 3 mg/mL) ($p < 0.05$). The methanol fennel stem extract caused a mean contractile response of 72.97% ± 3.87% (at a concentration of 3 mg/mL) ($p < 0.01$).

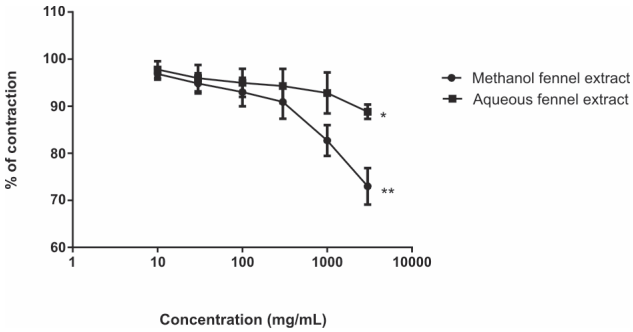

Fig. 4 – Inhibitory effects of the aqueous and methanol fennel stem extracts on ileum contraction induced by potassium chloride (KCl). Each value is expressed as means ± standard error (n = 6), ($p < 0.01$, *$p < 0.05$).**

The HPLC fingerprints of the aqueous and methanol fennel stem extracts were shown in Figure 5. The components of 3-caffeoylquinic acid, chlorogenic acid, 4-caffeoylquinic acid, 1,3-dicaffeoylquinic acid, rutin, miquelianin, quercetin heterosides, 1,5-dicaffeoylquinic acid, 1,4-dicaffeoylquinic acid, apigenin and rosmarinic acid were identified in the fennel methanol stem extract.

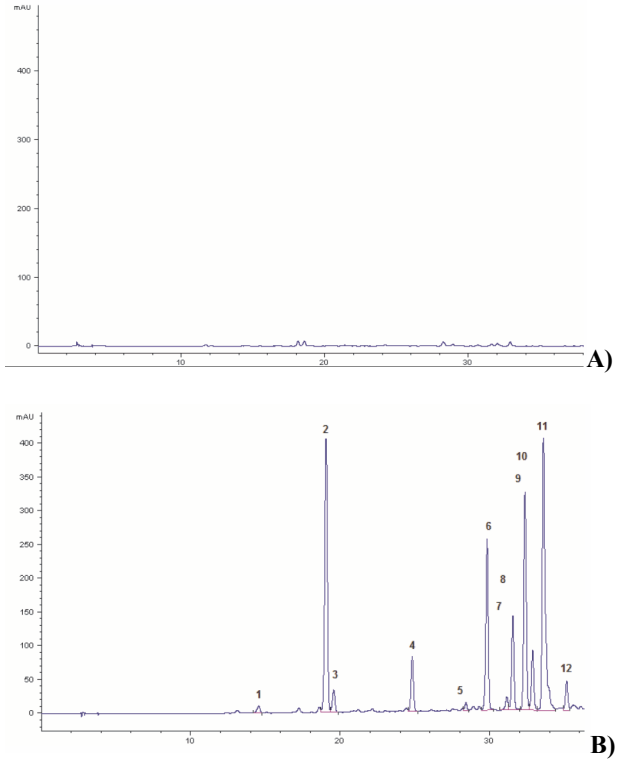

Fig. 5 – Chromatogram from aqueous (A) and methanol (B) fennel stem extracts. Peak identification: (1) 3-caffeoylquinic acid, (2) chlorogenic acid, (3) 4-caffeoylquinic acid, (4) 1.3-dicaffeoylquinic acid, (5) rutin, (6) miquelianin, (7.8) quercetin heterosides, (9) 1.5 dicaffeoylquinic acid, (10) 1.4 dicaffeoylquinic acid, (11) apigenin and (12) rosmarinic acid.

Discussion

Our study demonstrates that the aqueous and methanol fennel stem extracts express spasmolytic effects on rat ileum contractions. The aqueous and methanol extracts induced inhibition of spontaneous contractions of the isolated rat ileum in a concentration-dependent manner. The methanol fennel stem extract is more potent than the aqueous one.

To evaluate possible mechanisms for the spasmolytic activity, isolated rat ileum contractions were induced in two different ways (by application acetylcholine and KCl). Acetylcholine is the main excitatory neurotransmitter in the visceral smooth muscle which induced contractions of the ileum in a concentration-dependent manner. Contractions of the small intestine stimulated by acetylcholine were realized through two different mechanisms related to muscarinic receptors. Activation of non-selective cation channels in the plasma membrane causes membrane depolarization and influx of calcium ions through voltage-dependent calcium channels. Another mechanism of the activation of contraction occurs due to the release of intracellular calcium [17, 18]. The interactions of acetylcholine with muscarinic receptors in the intestinal smooth muscle induce a G protein-mediated signal transduction that increases cytosolic calcium ion concentration, depolarization and the contractions of the intestinal smooth muscle [19, 20].

In our study, it was found that the aqueous and methanol fennel stem extracts caused a significant reduction in contractile response which is registered as a depression of the cumulative concentration response curve for acetylcholine. Moreover, the methanol fennel stem extract had higher effect than the aqueous extract. Inhibitory effects of the aqueous and methanol fennel stem extracts on the acetylcholine induced contraction were less potent than those ones caused by atropine, a muscarinic acetylcholine receptor antagonist. These results indicated that the antispasmodic effect of fennel stem extracts was probably mediated through the blockade of muscarinic receptors. After flushing the contractility of the isolated ileum restored to baseline, meaning that the blockade of muscarinic receptors are reversible.

The contractile activity in the smooth muscle is dependent on the intracellular Ca^{2+}concentration. High concentration of KCl (80 mM) induced tonic contractions of the isolated rat ileum. Activation of the voltage dependent Ca^{2+} channels and influx of extracellular Ca^{2+} [21, 22] was used as a method of depolarization. The aqueous and methanol fennel stem extracts relaxed the tonic contraction of the isolated rat ileum induced by KCl. The methanol fennel stem extracts were more potent than the aqueous extract. According to the literature, plant extracts may cause inhibition of high K^+-induced contractions and act as a blocker of calcium influx [23].

Our results regarding the relaxant activity of the fennel stem extracts are similar to the results obtained by Boskabady et al. [24], who reported that the extracts and essential oil of *F. vulgare* expressed relaxant effect on isolated guinea pig trachea.

Fennel is used in traditional medicine for the treatment of infantile colic. This traditional usage might be related to *in vitro* relaxant activity of fennel. The treatment of infantile colic with *F. vulgare* seed essential oil may improve symptoms in babies [25, 26].

The HPLC analysis of the fennel stem extracts showed that this plant contained 3-caffeoylquinic acid, chlorogenic acid, 4-caffeoylquinic acid, 1,3-dicaffeoylquinic acid, rutin, miquelianin, quercetin heterosides, 1,5-dicaffeoylquinic acid, 1,4-dicaffeoylquinic acid, apigenin and rosmarinic acid. Higher content of these compounds were found in the methanol fennel stem extract than in the aqueous extract.The methanol extract was observed to be more potent than the aqueous extract in inhibiting the rat ileum contraction. The compounds that were analyzed in the aqueous extract were found in traces. It cannot be confirmed that they are responsible for its effects, but sometimes the compounds in small quantities can give effects. It is possible that other unidentified substances may act as smooth muscle relaxants.

The results of this study are in agreement with a study of Salami et al. [27]. They found high variation in composition of methanol extract in 23 fennel samples. The major flavonoid were quercetin, apigenin and rutin and phenolic compounds were chlorogenic, caffeic and 1,5-dicaffeoylquinic acid. It was reported that rutin induced an antispasmodic effect in the isolated ileum of the guinea-pig [28]. The rosmarinic acid was documented to possess an antinociceptive property [29]. Also, a methanol extract of the fennel seeds contains rosmarinic acid, chlorogenic acids, quercetin and apigenin [30]. Our results showed that these compounds are found in the methanol fennel stem extract.

Conclusion

Our research shows that the aqueous and methanol fennel stem extracts (*Foeniculum vulgare* Miller) inhibit spontaneous, acetylcholine and KCl induced ileum contractions. The data we obtained suggest the relaxant activity of the aqueous and methanol fennel stem extracts and may, at least partially, account for the traditional use of fennel for stomach disorders. Further research is needed to clarify whether these extracts exert their effects due to the direct action on smooth muscle or due to the effects on enteric nervous system.

Acknowledgments

The authors are grateful for the financial support of the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grants No. III 46013 and III 41018. The authors are thankful to the assistant professor Bojan Zlatković from the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Serbia for the taxonomical identification of the plant material.

The authors are also grateful for the financial support of Internal project of Faculty of Medicine, University of Niš, named: "Chemical characterization, biological activity and nutritional value of *Ribes nigrum* L, *Salvia sclarea* L. and *Foeniculum vulgare* Miller".

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Received on October 1, 2016.
Revised on November 11, 2016.
Accepted on December 6, 2016.
Online First, December, 2016.