Bronchodilator effect of apigenin and luteolin, two components of *Dracocephalum kotschyi* on isolated rabbit trachea

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A R T I C L E   I N F O

A B S T R A C T

Introduction: *Dracocephalum kotschyi* is a native Iranian plant with antispasmodic activities on smooth muscles such as ileum and uterus. However, so far antispasmodic effect of *D. kotschyi* on tracheal smooth muscle has not been reported. Therefore, the objective of this research was to investigate antispasmodic activity of *D. kotschyi* extract and two of its components luteolin and apigenin on rabbit tracheal contraction *in vitro*.

Methods: Rabbits were euthanized by carbon dioxide and the trachea was dissected and immersed in a Tyrode's solution. Tracheal rings were prepared and mounted vertically in an organ bath at 37°C and gassed continuously with O_2. The tracheal ring preparations were contracted with acetylcholine (ACh) and KCl. The isotonic tension was recorded before and after addition of aminophylline, apigenin, luteolin or flavonoids rich extract of *D. kotschyi*. Flavonoids rich extract were prepared from *D. kotschyi* using solvent-solvent fractionation technique.

Results: Standard drug aminophylline, prevented tracheal ring preparation contracted with ACh. Cumulative addition of aminophylline also attenuated tonic contraction induced by KCl on tracheal smooth muscle. *D. kotschyi* extract at concentration ranges of 32-512 µg/mL in a concentration dependent manner inhibited KCl and ACh induced tracheal contraction. Apigenin and luteolin (range 16–512 µg/mL) relaxed KCl and ACh-induced contraction of tracheal smooth muscle *in vitro* in a concentration-dependent manner.

Conclusion: This study demonstrated that *D. kotschyi* extract is a relaxant of tracheal smooth muscle. The relaxant effect of *D. kotschyi* extract could be due to its flavonoids component such as apigenin and luteolin.

Introduction

Dracocephalum (dragonhead) is a genus of more than 60 species of flowering plants growing with various sizes (1). At least eight species of *Dracocephalum* (family Lamiaceae) have been identified in Iran (2). *Dracocephalum kotschyi* Boiss. is a scientific name of one species in the genus *Dracocephalum* and known as Zaringiah in Iran (3). Zaringiah (*D. kotschyi*) is a medicinal plant native to temperate regions and grows in high altitudes mountainous locations of Iran (4,5). In some part of Iran, local people traditionally use Zaringiah for treatment of arthritis, gastrointestinal disorders as well as for alleviation of asthma (6). Modern pharmacological studies confirm that *D. kotschyi* possesses antispasmodic and anti-inflammatory properties (7-10). Nevertheless, *D. kotschyi* is enriched in many bioactive constituents with wide range of pharmacological properties (11-13). Antispasmodic and anti-inflammatory effects of *D. kotschyi*

extract are attributed to its flavonoids constituents including apigenin and luteolin (14,15).

It has been reported that apigenin has anti-allergic effect in allergic induced airways reaction of mice and has exhibited anti-inflammatory activity in experimental model of asthma (16,17). Furthermore, apigenin prevent proliferation and migration of airways smooth muscle cells by inhibiting transforming growth factor-β_1 (TGF-β_1) a key inflammatory factor in asthma (18,19). Similar effect has been reported for luteolin. For instant, luteolin alleviates airway hyperactivity, airways mucus overproduction and asthmatic features in experimental models (20-22). In addition, apigenin and luteolin ameliorate features of bleomycin induced lung fibrosis (23,24). Although, asthma is an inflammatory disease of airways, however, bronchoconstriction also plays a significant role in breathless of asthmatic patients. As mentioned above, D. kotschyi extract as well as apigenin and luteolin have relatively potent antispasmodic activities on isolated ileum and uterus smooth muscles (9,10,14,15). Therefore, the objective of this research is to investigate bronchodilatory effect of D. kotschyi extract for comparison for luteolin and apigenin.

Materials and Methods
Zaringiah was collected from cultivated garden in Shahankoah at 2650 m altitude from sea level. Shahankoah mountain rang are situated in Meidanak village in Feridonshar (Isfahan, Iran) and is native environment in which Zaringiah growth naturally. The plant was identified as D. kotschyi by a botanist (Mohamad Asfa) from Department of Natural Resource in Isfahan Province. A voucher specimen has been deposited in the School of Pharmacy of Isfahan University of Medical Sciences (No: 1519). Grassy branches of D. kotschyi were cut during flowering season and dried in shade. Dried plant materials were grounded to fine powder by an electronic grainer (Keep, Korea).

Extraction and fractionation procedures
Powdered material of D. kotschyi was extracted with 70% ethanol at room temperature using percolation extraction technique (25). The solvents of hydroalcoholic extract were evaporated under reduced pressure at 60°C on a rotary evaporator. Concentrated hydroalcoholic extract was then subject to liquid-liquid fractionation with chloroform and ethylacetate in the order of their increasing polarity to obtain respective fractions. Initially with sever shaking and stirring the hydroalcoholic extract (100 g) was dissolved in mixtures of distilled water and chloroform (1:1). The chloroform fraction was decanted and the process repeated by addition equal volume of fresh chloroform. The remaining aqueous fraction was fractionated with ethylacetate five times. Combined ethylacetate fraction was concentrated to dryness under reduced pressure (30°C) on a rotary evaporator to give

ethylacetate fraction of D. kotschyi.

Flavonoids contents of separated fractions were assessed by aluminum chloride colorimetric method as described before (15). Ethylacetate fraction possessed highest contents of flavonoids and therefore considered as flavonoids rich fraction for this study.

Tissue preparation and isotonic force recording
Domestic rabbits bred in open filed farm in Esfahan were housed in School of Pharmacy and Pharmaceutical Sciences animal house at room temperature with free access to food and water. Animals were handled according to international guideline for handing laboratory animals (26) approved by the University Ethical Committee (IR. MUI.REC.1396.3.816). For preparation of bladder, a rabbit was placed in a glass chamber with two inlet and outlet slots and euthanized with carbon dioxide gas. The euthanized animal was removed and placed on laboratory bench and longitudinal incisures were made in the frontal section of neck. The trachea was cleaned of adhering tissues and dissected out and placed in oxygenated Tyrode's solution (see drug and solution). Connective and fatty tissues were carefully trimmed off and the trachea was cut into transverse strips of 2-3 adjacent cartilage rings. Four tracheal rings were tied together with cotton thread in such a way that smooth muscle layer was lined up vertically. The prepared tissues were secured in an organ bath for isotonic muscle contraction recording, using a force displacement transducer (Harvard Apparatus) coupled to an oscillograph (Harvard Apparatus, England). The oscillograph was calibrated according to apparatus manual and adjusted to the optimal length for muscle contraction for each tissue. The tissues were placed constantly under 1 g passive tension in the Tyrode's solution at 37°C and continuously gassed with oxygen. The tissues were allowed to settle to their normal basal tension while being washed several times with Tyrode's solution.

Tracheal contraction studies
Tracheal smooth muscles contractions were induced by addition of potassium chloride (80 mM KCl) or acetylcholine (ACh, 100 μg/mL) into the organ bath. ACh remained 1.5 min in contact with the tissue before it was washed out with fresh Tyrode's solution. ACh was added to organ bath at 10-minute intervals. When reproducible tissue responses were established, contractile responses of the trachea to ACh or KCl were recorded in the present of the extract, apigenin, luteolin or aminophylline. Inhibitory effects of the tested compounds were examined in cumulative manner for KCl response while ACh response was assessed 10 min after incubation of test compound in a non-cumulative manner.

Drug and solutions
The Tyrode's composition was 136.9 mM NaCl, 1.8 mM CaCl$_2$, 2.68 mM KCl, 11.9mM NaHCO$_3$, 1.05 mM MgCl$_2$,

0.42 mM NaH_2PO_4 and 5.55 mM glucose. The extract was prepared in dimethylsulphoxide (DMSO) as 20 mM stock solution. Luteolin and apigenin were dissolved in DMSO as 20 mM stock solutions. Further dilutions (1 in 10) were made in 50% DMSO. Aminophylline (25 mg/mL ampoule, Caspian, Iran) was diluted in distilled water. KCl (2M) and ACh (100mM) stock solution were prepared and diluted in distilled water. First stock of ACh was acidified with 1% acetic acid to increase stability. Apigenin and luteolin were purchased from Aktin Chemical Inc (China). Acetylcholine was from Sigma. Other chemicals were from Merck.

Contraction assessments and analysis

Bronchial smooth muscle contraction was measured as amplitude of recorded contraction from baseline and expressed as percentage of pretreated control response. Mean and standard error of mean (SEM) were calculated for each group of data (n=6). Full concentration inhibitory response curves were plotted for assessment of IC_{50} value (inhibitory concentration causing 50% of maximum response). Statistical differences between the groups were analyzed by Student t test or one-way analysis of variance. P values less than 0.05 were considered statistically significant. SigmaPlot computer program was used for statistical analysis and plotting graphs for calculation of IC_{50} values.

Results

Tracheal smooth muscles suspended under 1 g tension in Tyrode's solution gradually relaxed to a stable baseline within 15-30 minutes. Addition of KCl (80mM) produced a sustained contraction. Addition of ACh into the organ bath contracted the tissues within 90 seconds contact time. After washing the tissue with fresh Tyrode's solution, the tissues relaxed to initial baseline position.

After repeatable response to ACh was established effective concentrations of aminophylline, apigenin, luteolin and *D. kotschyi* extract were determined in a series of pilot experiments. Aminophylline reduced tracheal smooth muscle contractile responses to both KCl and ACh. However, with 1.28 mg/mL aminophylline in the bath still 20% and 40% of KCl and ACh responses, respectively were remained (Figure 1). Apigenin also inhibited tissues responses to both KCl and ACh in a concentration dependent manner. The inhibitory effect of apigenin was observed with bath concentration of 16 μg/mL and when apigenin concentration was added up to 512 μg/mL, KCl response was abolished and only 7% of ACh response remained (Figure 2). The inhibitory concentration causing 50% of maximum response (IC_{50}) was calculated as 66±7 μg/mL and 80±14 μg/mL for KCl and ACh, respectively (n=6). Luteolin had similar inhibitory effect as apigenin on tracheal smooth muscle contractions. The first concentration of luteolin (16 μg/mL) had no effect on either KCl or ACh responses. By
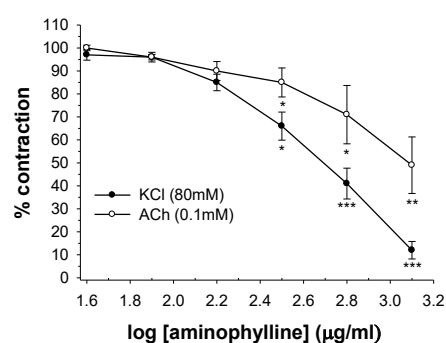

Figure 1. Effect of aminophylline on KCl and acetylcholine (ACh) induced contractions in rabbit isolated trachea. Data are present as mean±SEM (n=6). Stars show statistically significant difference in absence and in presence of increasing aminophylline concentration in the bath (*P<0.05, **P<0.01, ***P<0.001, Student t test).
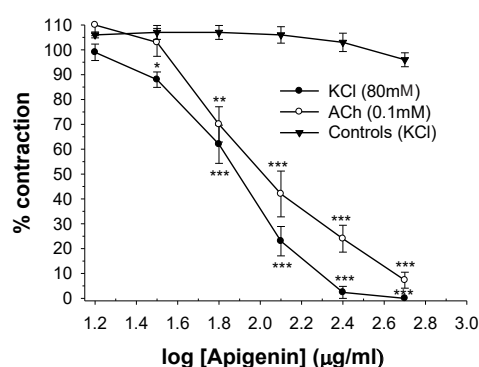

Figure 2. Effect of apigenin on KCl and acetylcholine (ACh) induced contractions in rabbit isolated trachea. Concentration response curve was constructed using two folds increment in bath concentration. Data are present as mean±SEM (n=6). There was no statistically significant inhibition in the vehicle (DMSO) treated control groups (ANOVA). Stars show statistically significant difference in apigenin treated group with their corresponding concentration in the vehicle treated group (*P<0.05, **P<0.01, ***P<0.001, Student's t test).

increasing luteolin concentration to 32 μg/mL response to KCl was attenuated while ACh response was not affected. Higher concentrations of luteolin in a concentration dependent fashion inhibited tracheal contraction induced by both KCl and ACh (Figure 3). The IC_{50} values of luteolin for inhibition of KCl and ACh responses were 65±4 μg/mL and 90±12 μg/mL, respectively. *D. kotschyi* extract at concentration ranges of 32 μg/mL to 1 mg/mL concentration dependently reduced contractile responses to ACh with IC_{50} value of 340±68 μg/mL (Figure 4). After washing the tissues with fresh Tyrode's solution, contractile response to KCl and ACh was gradually restored. Equivalent volume of drugs vehicle (DMSO) did not caused any significant reduction in KCl or ACh responses in the vehicle treated time matched control tissues (Figures 2, 3 and 4).

Discussion

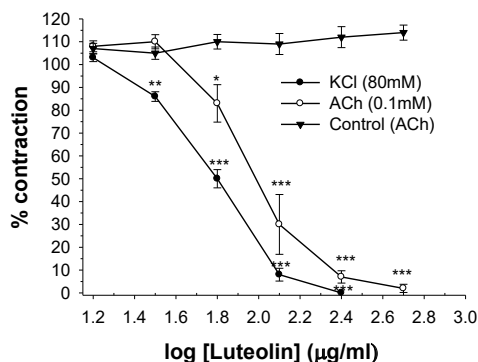Respiratory tract is a compact organ situated in the

Figure 3. Effect of luteolin on KCl and acetylcholine (ACh) induced contractions in rabbit isolated trachea. Concentration response curve was constructed using two folds increment in bath concentration. Data are present as mean±SEM (n=6). There was no statistically significant inhibition in the vehicle (DMSO) treated control groups (ANOVA). Stars show statistically significant difference in luteolin treated group with their corresponding concentration in the vehicle treated group (*$P<0.05$, **$P<0.01$, ***$P<0.001$, Student's t test).

Figure 4. Effect of *Dracocephalum kotschyi* flavonoids rich extract on KCl and acetylcholine (ACh) induced contractions in rabbit isolated trachea. Concentration response curve was constructed using two folds increment in bath concentration. Data are present as mean±SEM (n=6). There was no inhibition in the vehicle (DMSO) treated control groups. Stars show statistically significant difference in the extract treated group with their corresponding concentration in the vehicle treated control group (*$P<0.05$, **$P<0.01$, ***$P<0.001$, Student's t test).

thorax and is responsible for constant gas exchange. Air is drawn into the lungs through trachea and its branches (27). Trachea consists of C-shape rings of cartilage joined by smooth muscle to complete encircles. These rings continuous down the bronchi and bronchioles (28). Bronchial inflammation of respiratory tract induces airway narrowing and decrease in lung function in condition such as bronchitis and asthma (29). These conditions may cause obstruction of airflow from the lungs. Release of inflammation mediators directly or indirectly via activation of vagus nerve, further narrows the bronchial tubes (30). Parasympathetic nerves activation cause bronchoconstriction through muscarinic receptors (30). The primary stage of smooth muscle contraction involves elevation of intracellular calcium ions, activation of myosin light chain kinase and phosphorylation of myosin light chain (27,28). Addition of ACh into organ bath constricted bronchial rings by activating muscarinic M_3 receptors as in the case of vagus nerve activation. These muscarinic receptors are associated with phospholipase-C activation and release of Ca^{2+} from intracellular stores via inositol triphosphate (IP_3) production (27,28). Addition of high concentration of KCl into the organ bath, induced membrane depolarization and activation of voltage gated Ca^{2+} channels and smooth muscle contraction (31).

The standard drug, aminophylline, is a methylxanthine which inhibits phosphodiesterase with resultant increase in intracellular cAMP and/or cGMP concentration (32). These second messengers reverse the phosphorylation of myosin light chain and cause relaxation. Furthermore, they reduce intracellular calcium ion concentration by reducing Ca^{2+} entry and promoting Ca^{2+} storage and Ca^{2+} expulsion from the cell (33). Interfering with final stage of smooth muscle contraction could explain why aminophylline inhibits both ACh and KCl induced cont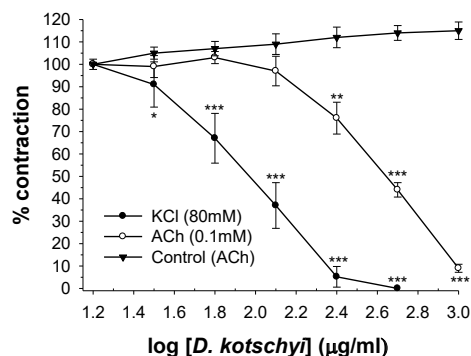ractions. Apigenin and luteolin completely inhibited ACh and KCl induced contractions in isolated bronchial rings. Therefore, like aminophylline they can be regarded as potent bronchodilator agents. It has been reported that luteolin inhibits phosphodiesterase (34) which may contribute to its relaxant effect on smooth muscle. It has been suggested that aminophylline not only relaxes smooth muscle in the respiratory tract but also suppresses airway stimuli involving in inflammatory process of asthma. In fact, asthma is a complicated disease which involves secretion of ranges of inflammatory mediators and airway remodeling (35). Bronchial remodeling in asthma is associated with increase smooth muscle mass, thickening of the bronchial wall and cell migration (35). Activation of inflammatory cells, such as mast cells or T-lymphocytes and secretion of a range of inflammatory mediators, contributes to bronchial inflammation and bronchoconstriction (35). Transforming growth factor-β_1 (TGF-β_1) is a cytokine that is released and is well known inflammatory mediator with significant role in airway remodeling and asthma progression (35). It has been reported that apigenin inhibits TGF-β-induced fibroblast to myofibroblast transition which is known as a key event in asthma progression in human lung fibroblast population (18). This may indicate that apigenin could prevent structural remodeling in asthma. Apigenin also inhibited TGF-β_1 induced proliferation and migration of smooth muscle cells and airway remodeling in animal asthma model (19). Furthermore, apigenin inhibits allergic airway reactions in ovalbumin induced asthma in mice (17) and exhibits anti-inflammatory activity in animal model of asthma (16). Similar anti-nflammatory actions have been reported for luteolin. For instant luteolin alleviates hyperactivity in ovalbumin sensitized mice and prevents mucus overproduction, which is a common symptom in asthmatic patients (21-22).

In this research we demonstrated that both apigenin

and luteolin could relax isolated rabbit tracheal smooth muscles contraction induced by ACh and high extracellular concentration of potassium chloride. These findings further support the idea that apigenin and luteolin are suitable lead compounds to be considered for treatment of asthma and chronic bronchitis. Apigenin and luteolin are two known flavonoids components of *D. kotschyi* extract (36).

In the present study we have demonstrated that flavonoids rich extract of *D. kotschyi* extract concentration-dependently suppressed KCl and ACh-induced contractions of rabbit tracheal ring. *D. kotschyi* extract also reported to have anti-inflammatory and anti-allergic activities and these actions have been attributed to its flavonoids contents (13-15). The flavonoids components in the *D. kotschyi* extract are found in free or glycosides forms (36, 37). Glycosides form of apigenin include apigenin-7-O-apiosylglucoside, apigenin-8-O-glucoside, apigenin-7-O-glucoside, apigenin-6-O-glucoside, apigenin-6-7-diglucoside, 7-methyoapigenin and 7-methoxyapigenin (36,37). Luteolin has close structural similarity to apigenin and shares common glycosides forms. Glycosides forms of flavonoids are polar molecules due to sugar moiety and extractable with hydroalcoholic solution, while aglycones (sugar free from) of apigenin and luteolin are extracted with less polar solvents such as ethylacetate. Our previous researches have revealed that *in vitro* studies mainly the aglycones forms of apigenin and luteolin exhibit antispasmodic activity (38). Nevertheless, when administered orally, the sugar moiety is removed from the glycosides forms and the active forms are released (15). This explain why *D. kotschyi* extract is more effective when is given orally. This experiment supports the idea that apigenin and luteolin contributes to bronchodilatory effect of *D. kotschyi* extract. However, assessment of total flavonoids content of the extract shows that they are accounting for relatively small percentage of the total extract (15). Therefore, it is very likely that apart from apigenin and luteolin, other constituents of the extract have a share in relaxant effect of the extract which needs to be investigated.

Conclusion
Current therapy for treatment of asthma includes bronchodilator drugs which relax bronchial smooth muscles. Corticosteroids are also used for prevention and reduction of inflammation. Previous studies have demonstrated that apigenin and luteolin possess potent anti-inflammatory activities in animal asthmatic models. Our finding clearly shows that apigenin and luteolin are direct airway smooth muscle relaxants and their bronchodilatory actions are comparable to that of aminophylline.

Conflict of interests
The authors report no conflicts of interest. The authors are responsible for the content and writing of this article.

Ethical considerations
Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the Isfahan University of Medicine Sciences. The project was confirmed by the ethical committee of this university (IR.MUI.REC.1396.3.816).

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