The Relaxant Effects of Different Methanolic Fractions of *Nigella sativa* on Guinea Pig Tracheal Chains

Rana Keyhanmanesh¹, Horeyeh Bagban², Hossein Nazemiyeh¹, Fariba Mirzaei Bavil³, Mohammad Reza Alipour⁴*, Mehdy Ahmady²

¹Tuberculosis and Lung Research Centre, Tabriz University of Medical Sciences, Tabriz, Iran
²Department of Physiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
³Research Centre for Pharmaceutical Nanotechnology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran

**ABSTRACT**

**Objective(s):** In regard to the high incidence of asthma and the side-effects of the drugs used, finding novel treatments for this disease is necessary. Our previous studies demonstrated the preventive effect of *Nigella sativa* extract on ovalbumin-induced asthma. In addition, water-soluble substances of *N. sativa* extract and methanol fraction of this plant were responsible for the relaxant effect of this plant on tracheal chains of guinea pigs. Therefore, for the first time, in the present study, in order to identify main constituents of the methanolic extract, the relaxant effects of five different methanolic fractions (20%, 40%, 60%, 80%, and 100%) of *N. sativa* on tracheal chains of guinea pigs were examined.

**Materials and Methods:** The relaxant effects of four cumulative concentrations of each fraction (0.8, 1.2, 1.6, and 2.0 g%) in comparison with saline as negative control and four cumulative concentrations of theophylline (0.2, 0.4, 0.6, and 0.8 mM) were examined by their relaxant effects on precontracted tracheal chains of guinea pig by 60 mM KCl (group 1) and 10 µM methacholine (group 2).

**Results:** In group 1, all concentrations of only theophylline showed significant relaxant effects but all concentrations of these methanolic fractions showed significant contractile effects compared with that of saline (*P*<0.001 to *P*<0.05). However, in group 2, all concentrations of theophylline and these methanolic fractions showed significant relaxant effects compared with that of saline (*P*<0.001 to *P*<0.05).

**Conclusion:** These results showed a potent relaxant effect of 20% methanolic fractions from *N. sativa* on tracheal chains of guinea pigs that were higher than that of theophylline at the used concentrations.

**Please cite this paper as:** Keyhanmanesh R, Bagban H, Nazemiyeh H, Mirzaei Bavil F, Alipour MR, Ahmady M. The Relaxant Effects of Different Methanolic Fractions of *Nigella sativa* on Guinea Pig Tracheal Chains. Iran J Basic Med Sci 2013; 16: 123-28.
Introduction

Asthma is a global health problem affecting 300 million individuals of all ages, ethnic groups, and countries (1). Nowadays, many drugs are used for treating this illness. Although these drugs are effective, there are many side effects. Therefore, physicians try to find new drugs with fewer side effects. One important way for this purpose is studying the therapeutic effect of herbal medicine because some herbs have therapeutic effects without obvious side effects.

Among the promising medicinal plants, Nigella sativa, a dicotyledone of the Ranunculaceae family, is an amazing herb with historical and religious background (2). This plant with green to blue flowers and small black seeds grows in temperate and cold climate areas. The seeds of N. sativa are the source of the active ingredients such as thymoquinone, monotropens-like $\alpha$-cymene and $\alpha$-pinene (3), nigellidine (4), niggellimine (5), and saponin (6).

Several therapeutic effects have been described for the seeds of N. sativa in medical books including anti-asthma and anti-dyspnea (7), hypotensive, anti-nociceptive, anti-fertility, anti-diabetic (8), anti-inflammatory, anti-oxidant, anti-microbial, anti-tumor, and immunomodulatory properties (9). There is evidence for relaxant effects of the volatile oil from this plant on different smooth muscle preparations including rabbit aorta (10), rabbit jejunum (11), and guinea pig isolated tracheal muscle (12).

The results of recent studies also showed different pharmacological effects of N. sativa on guinea pig tracheal chains including relaxant and functional antagonistic effects on muscarinic receptors (13), inhibitory effect on histamine (H1) receptors (14), inhibitory effect on calcium channels (15), opening effect on potassium channels (16), and stimulatory effect on $\beta$-adronceptors (17). The antitussive effect of this plant on guinea pig (18) was also demonstrated.

In addition, the preventive effects of hydro-ethanolic extract of N. sativa on lung pathological changes, tracheal responsiveness to methacholine and ovalbumin, cellular differentiation of bronchoalveolar lavage, and blood cytokines were also demonstrated (19, 20).

Our previous study in which the relaxant effects of hydro-ethanolic, macerated aqueous (MA) and lipid-free macerated aqueous (LFMA) extract of N. sativa were examined on guinea pig tracheal chains demonstrated that mainly water soluble substances of this plant were responsible for its relaxant effect (21).

Moreover, the study of Boskabady et al. in 2008 showed that most concentrations of 3 different fractions of N. sativa (N-hexane, dichlorometane, and methanol fractions) had significant relaxant effects on guinea pig tracheal chains which was more potent for methanol fraction (22).

Therefore in the present study, the relaxant effects of five different methanolic fractions (20%, 40%, 60%, 80% and 100% methanolic extracts) on tracheal chains of guinea pigs were examined to identify main constituents of the methanolic extract of N. sativa.

Materials and Methods

Plant and fractions

Methanolic fractions: N. sativa was collected from north-east Iran and dried at room temperature in the absence of sunlight. The plant was identified by botanists in the herbarium of Ferdowsi University of Mashhad with specimen number of 293-0303-1. Methanolic fraction was prepared like the previous study (22). Briefly, n-hexane was added to 200 grams of the chopped, dried seeds and the solution was kept at room temperature for 10 hr. The solution was then separated and the solvent was dried. Then dichloromethane was added to the remaining powder at room temperature for 10 hr. After that, the solution was...
separated and the solvent was dried. Then methanol was added to the remaining powder at room temperature for 10 hr and the solution was separated and dried. In these manners, 31% lipid remaining of N-hexane fraction, 1% lipid remaining of dichloromethane fraction, and 7% lipid remaining of methanol fraction were obtained.

After that, solid phase extraction (SPE) method was performed for preparing five different methanolic fractions (Figure 1). In this method, Sep-pac cartilages (10 g, ODS, waters, Ireland) were used as solid phase and step gradients of methanol in water (methanol 20%, 40%, 60%, 80%, and 100%) were used as extraction solvents. In each stage, the solution was separated and dried. Then the 40 g% solution was prepared.

**Tissue preparation**

Guinea pigs (400-700 g, both sexes) were killed by a blow on the neck and tracheas were removed. Each trachea was cut into 10 rings (each containing 2-3 cartilaginous rings). All the rings were then cut open opposite the trachealis muscle, and sutured together to form a tracheal chain (21). Tissue was then suspended in a 20 ml organ bath (schuler organ bath type 309, March- Hugstetten, Germany) containing Krebs-Henseliet solution of the following composition (mM): NaCl 120, NaHCO_3 25, MgSO_4 0.5, K_HPO_4 1.2, KCl 4.72, CaCl_2 2.5, and dextrose 11.

The Krebs solution was maintained at 37°C and gassed with 95% O_2 and 5% CO_2. Tissue was suspended under an isotonic tension of 1 g and allowed to equilibrate for at least 1 hr while it was washed with Krebs solution every 15 min.

**Protocols**

The relaxant effects of different solutions were tested with two different experimental designs, (n= 6 for each group) as follows:
1. On tracheal chains contracted by 60 mM KCl (group 1 experiments).
2. On tracheal chains contracted by 10 µM methacholine hydrochloride (Sigma Chemical Ltd, UK), (group 2 experiments).

The relaxant effects of four cumulative concentrations (0.8, 1.2, 1.6, and 2.0 g%) of five different methanolic fractions of *N. sativa* and theophylline anhydrous (Sigma Chemical Ltd, UK) (0.2, 0.4, 0.6, and 0.8 mM) as positive control, and normal saline (1 ml) as negative control were examined. In order to produce the first concentration of each fraction, 0.4 ml of 40 g% was added to a 20 ml organ bath and for other three concentrations; 0.2 ml of 40 g% was added to organ bath, respectively, three times. For theophylline, 0.2 ml of 20 mM theophylline solution was added to organ bath 4 times. The consecutive volumes were added to organ bath at 5 min intervals.

In each experiment, the effect of four cumulative concentrations of each fraction, theophylline or saline on contracted tracheal smooth muscle was measured after exposing tissue to each concentration of the solution for 5 min. A decrease in tone was considered to be a relaxant (bronchodilatory) effect and expressed as positive percentage change in proportion to the maximum contraction. An increase in tone was considered as a contractile (bronchoconstrictory) effect which was expressed as negative percentage change.

The relaxant effects in two groups of experiments were examined in two different series of tracheal chains. All of the experiments were performed randomly with 1 hr resting period of tracheal chains between each two experiments while washing the tissues every 15 min with Krebs solution. In all experiments, responses were amplified with amplifier (ML118 quadrabridge amp, March- Hugstetten, Germany) and recorded on powerlab (ML-750, 4 channel recorder, March-Hugstetten, Germany).

**Statistical analysis**

All data were expressed as mean±SEM. Data of relaxant effects of different concentrations of each fraction were compared with the results of negative and positive control using paired t-test. The data of relaxant effects obtained in two groups of experiments were compared in two groups of experiments were compared with two different experimental designs, (n= 6 for each group) as follows:

1. On tracheal chains contracted by 60 mM KCl (group 1 experiments).
2. On tracheal chains contracted by 10 µM methacholine hydrochloride (Sigma Chemical Ltd, UK), (group 2 experiments).

The relaxant effects of four cumulative concentrations (0.8, 1.2, 1.6, and 2.0 g%) of five different methanolic fractions of *N. sativa* and theophylline anhydrous (Sigma Chemical Ltd, UK) (0.2, 0.4, 0.6, and 0.8 mM) as positive control, and normal saline (1 ml) as negative control were examined. In order to produce the first concentration of each fraction, 0.4 ml of 40 g% was added to a 20 ml organ bath and for other three concentrations; 0.2 ml of 40 g% was added to organ bath, respectively, three times. For theophylline, 0.2 ml of 20 mM theophylline solution was added to organ bath 4 times. The consecutive volumes were added to organ bath at 5 min intervals.

In each experiment, the effect of four cumulative concentrations of each fraction, theophylline or saline on contracted tracheal smooth muscle was measured after exposing tissue to each concentration of the solution for 5 min. A decrease in tone was considered to be a relaxant (bronchodilatory) effect and expressed as positive percentage change in proportion to the maximum contraction. An increase in tone was considered as a contractile (bronchoconstrictory) effect which was expressed as negative percentage change.

The relaxant effects in two groups of experiments were examined in two different series of tracheal chains. All of the experiments were performed randomly with 1 hr resting period of tracheal chains between each two experiments while washing the tissues every 15 min with Krebs solution. In all experiments, responses were amplified with amplifier (ML118 quadrabridge amp, March- Hugstetten, Germany) and recorded on powerlab (ML-750, 4 channel recorder, March-Hugstetten, Germany).

**Statistical analysis**

All data were expressed as mean±SEM. Data of relaxant effects of different concentrations of each fraction were compared with the results of negative and positive control using paired t-test. The data of relaxant effects obtained in two groups of experiments were compared...
using unpaired t-test. The relaxant effects of different concentrations of five different fractions were compared with each other using one-way ANOVA. The relaxant effects of five fractions and theophylline were related to the concentrations using least square regression. Significance was accepted at \( P<0.05 \).

**Results**

**Relaxant (bronchodilatory) effect**

In group 1 experiments, all concentrations of only theophylline showed significant relaxant effects compared to that of saline \( (P<0.001 \text{ for all concentrations}) \). All concentrations of different methanolic extracts showed significant contractile effects compared with that of saline in this group \( (P<0.001 \text{ to } P<0.05) \) (Figure 2).

In group 2 experiments, all concentrations of theophylline and 20% methanolic extract and three last concentrations of other different methanolic extracts \( (40\%, 60\%, 80\%, \text{ and } 100\%) \) showed significant relaxant effects compared with that of saline \( (P<0.001 \text{ to } P<0.05) \). However, the first concentrations of these extracts \( (40\%, 60\%, 80\%, \text{ and } 100\% \text{ methanolic extracts}) \) showed non-significant relaxant effects on tracheal chains (Figure 3).

**Comparison of relaxant effects of theophylline with different extracts**

In group 1 and 2, relaxant effects of all concentrations of all methanolic fractions (except all concentrations of 20% methanolic extract and the third concentration of 40% methanolic extract in group 2) were significantly less than those of theophylline \( (P<0.001 \text{ to } P<0.05) \). However, the relaxant effects of all concentrations of 20% methanolic extract were non-significantly higher than those of theophylline (Figures 1 and 2).

**Comparison of relaxant effects of different extracts**

In group 1, the contractile effects of all concentrations of 20% and 40% methanolic extracts, the first and the last concentrations of 60% methanolic extract and three higher concentrations of 80% methanolic extract were significantly lower than those of 100% methanolic extract \( (P<0.001 \text{ to } P<0.05) \). The most potent contractile effect was seen for 100% methanolic extract and on the contrary, 20% methanolic extract showed the lowest contractile effect in comparison with others.

In group 2, the relaxant effects of all concentrations of 20% methanolic extract were significantly higher than those of others \( (P<0.001 \text{ to } P<0.05) \). There is no significant difference between the relaxant effects of different concentrations of four other extracts \( (40\%, 60\%, 80\%, \text{ and } 100\%) \) (Figures 1 and 2).

![Figure 3. Relaxant effects of five different methanolic extracts from Nigella sativa in comparison with negative control (saline) and positive control (theophylline) in group 2 experiments (contracted tracheal chains by 10 \( \mu M \) methacholin, n=6)](image)

Statistical differences in the relaxant effects of different concentrations of different methanolic fractions and theophylline vs. that of saline. ns: non-significant difference, +: \( P<0.05 \), ++: \( P<0.01 \), +++: \( P<0.001 \). Statistical differences in the relaxant effects of different concentrations of different methanolic extracts vs. those of theophylline. NS: non-significant difference, *: \( P<0.001 \), **: \( P<0.01 \), ***: \( P<0.001 \).

**Comparison of the relaxant effect between two groups of experiments**

The relaxant effects of different concentrations of different methanolic extracts were significantly greater in group 2 compared with group 1 experiments \( (P<0.001) \). However, there was no significant difference in the relaxant effects of different concentrations of theophylline between two groups (Figure 4).

**Correlation between concentrations of solution and their relaxant effects**

There were significant positive correlation between relaxant effects and concentrations for all extracts in group 2 and for theophylline in both groups \( (P<0.001 \text{ to } P<0.01) \). However, the correlation between the relaxant effects and concentrations for all extracts (except 20% methanolic extract) in group 1 were significantly negative \( (P<0.001 \text{ to } P<0.01) \) (Table 1).

**Discussion**

In this study, the relaxant (bronchodilatory) effects of five different methanolic fractions \( (20\%, 40\%, 60\%, 80\%, \text{ and } 100\%) \) from *N. sativa* were compared with saline as negative control and theophylline as positive control. In group 1 experiments (contracted tracheal chains by KCl), all concentrations of only theophylline showed signifi-
In this study was in accordance to the NS

Table 1. Correlation (r) between the relaxant effects of five different methanolic extracts (20%, 40%, 60%, 80%, and 100% methanolic fractions) from Nigella sativa and theophylline with their concentrations in two groups of experiments

| Different solutions | 20% Methanolic extract | 40% Methanolic extract | 60% Methanolic extract | 80% Methanolic extract | 100% Methanolic extract | Theophylline |
|---------------------|------------------------|------------------------|------------------------|------------------------|------------------------|-------------|
|                     | r          | P-value                | r          | P-value                | r          | P-value                | r          | P-value                | r          | P-value                |
| Group 1             | 0.423                  | NS                     | -0.938                   | P<0.001                | -0.602                   | P<0.01                | -0.940                   | P<0.001                | -0.810                   | P<0.001                | 0.978                   | P<0.001                |
| Group 2             | 0.752                   | P<0.001                | 0.708                   | P<0.001                | 0.545                   | P<0.01                | 0.849                   | P<0.001                | 0.793                   | P<0.001                | 0.921                   | P<0.001                |
treatment for cases with increased airway resistance such as asthmatics, it is proposed to assess the relaxant effects of different methanolic fractions on normal guinea pig tracheal muscle. The evaluation of the relaxant effect of this extract on tracheal chains of asthmatic guinea pigs is suggested for future studies and more studies are required for clearing the exact mechanism(s) and the effective substances.

Conclusion
The results of the present study showed a potent relaxant effect of methanolic fractions from N. sativa on tracheal chains of guinea pigs. The most potent relaxant effect was seen for 20% methanolic fraction that was non-significantly higher than that of theophylline at concentrations used.

Acknowledgment
This study was funded by Lung and Tuberculosis Research Centre of Tabriz University of Medical Sciences. This article is derived from MSc dissertation of H. Bagban, titled “Obtaining the effective substance of Nigella sativa methanolic extract on reducing the contraction of airway smooth muscle in guinea pigs”.

References
1. Bouquet J, Mantzouranis E, Alvaro A, Cruz AA, Ait-Khaled N, Baeza-Cagnani CE, Bleecker ER, et al: Uniform definition of asthma severity, control, and exacerbations: Document presented for the World Health Organization Consultation on severe asthma. J Allergy Clin Immunol 2010; 126:926-938.
2. Goreja WG. Black seed: Nature’s miracle remedy. New York, NY: Amazing herbs press; 2003.
3. El-Dakhakhny M. Studies on chemical constitution of Egyptian Nigella sativa L. seeds. II. The essential oil. Planta Med 1963; 11:465-470.
4. Atta UR, Malik SO. Nigellidine, a new indazol alkaloid from seeds of Nigella sativa. J Res Inst 1995; 16:1993-1996.
5. Atta UR, Malik SO. Nigellimine N-oxide, a new isoquinoline alkaloid from the seeds of Nigella sativa. Heterocycles 1985; 23:953-955.
6. Ansari AK, Sadiya HAS. Structural studies on a saponin isolated from the seeds of Nigella sativa. Phyto Chem 1989; 27:377-379.
7. Ave-Sina. Law in Medicine, Interpreter; Sharafkhandy A, Teheran: Ministry of Guidance publication; 1990. p.314.
8. Ali BH, Blunden G. Pharmacological and toxicological properties of Nigella sativa. Phytother Res 2003; 17:299-305.
9. Labib Salem M. Immunomodulatory and therapeutic properties of the Nigella sativa L. seed. Int Immunopharmacol 2005; 5:3749-3770.
10. Aqel MB. The relaxing effect of volatile oil of Nigella sativa seed on vascular smooth muscle. Jordan Ser B 1992; 1:91-100.
11. Aqel MB. Effects of Nigella sativa seeds on intestinal smooth muscle. Int J Pharmacogn 1993; 31:55-60.
12. Reiter M, Brandt W. Relaxant effects on tracheal and ileal smooth muscles of the guinea-pig. Arzneimittelforschung 1985; 35:408-414.
13. Boskabady MH, Shahabi M. Bronchodilatory and anticholinergic effects of Nigella sativa on isolated guinea-pig tracheal chains. Iran J Med Sci 1997; 22:127-133.
14. Boskabady MH, Shiravi, N. Inhibitory effect of Nigella sativa on histamine (H1) receptors of isolated guinea pig tracheal chains. Eur Resp J 2000; 16:461s.
15. Boskabady MH, Shirmohammadi B. Effect of Nigella sativa on isolated guinea pig tracheal chains. Arch Iran Med 2002; 5:103-107.
16. Boskabady MH, Shirzamand, B, Jandaghi P, Kiani S. Possible mechanisms for relaxant effect of aqueous and macerate extracts from Nigella sativa on tracheal chains of guinea pig. BMC Pharmacol 2004; 4:3.
17. Boskabady MH, Kiani S, Jandaghi P. Stimulatory effect of Nigella sativa on β2-adrenoceptors of guinea pig tracheal chains. Med J IR Iran 2004; 18:153-158.
18. Boskabady MH, Kiani S, Jandaghi P, Zarei T, Zarei A. Antitussive of Nigella sativa. Pak J Med Sci 2004; 20:224-228.
19. Boskabady MH, Keyhanmanesh R, Khamseh S, Doostar Y, Khakzad MB. Potential immunomodulatory effect of the extract of Nigella sativa on ovalbumin sensitized guinea pigs. J Zhejiang Univ Sci 2011; 12:201-209.
20. Boskabady MH, Keyhanmanesh R, Khamseh S, Ebrahimi MA. The effect of Nigella sativa extract on tracheal responsiveness and lung inflammation in ovalbumin sensitized guinea pigs. Clinics (Sao Paulo) 2011; 66:879-887.
21. Keyhanmanesh R, Boskabady MH, Ebrahimi Saadatloo MA. The contribution of water and lipid soluble substances in the relaxant effects of Nigella sativa extract on guinea pig tracheal smooth muscle (in vitro). Iran J Basic Med Sci 2007; 10:154-161.
22. Boskabady MH, Keyhanmanesh R, Ebrahimi Saadatloo MA. Relaxant effects of different fractions from Nigella sativa L. on guinea pig tracheal chains and its possible mechanism(s). Indian J Exp Biol 2008; 46:805-810.
23. Miyahara Y, Kizawa Y, Sano M, Murakami H. Effect of organic and inorganic ca+ antagonist on acetylcholine induced contraction in malluscan (myfile edulis) smooth muscle. G Pharmacol 1993; 241:341-1423.
24. Burits M, Bucar F. Antioxidant activity of Nigella sativa essential oil. Phytother Res 2000;14: 323-328.
25. Thippeswamy NB, Naidu KA. Antioxidant potency of cumin varieties-cumin, black cumin and bitter cumin- on antioxidant systems. Eur Food Res Technol 2005; 220:472-476.
26. Yoruk O, Ozabacgil F, Uyanik H, Tasar M, Mutlu V, Atlas E, et al. Antioxidant Effects of Nigella sativa in the Treatment of Experimentally Induced Rhinosinusitis. Macedonian J Med Sci 2010; 31:132-137.