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The Main Relaxant Constituents of *Nigella Sativa* Methanolic Fraction on Guinea Pig Tracheal Chains

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ABSTRACT

Our previous studies demonstrated the preventive effect of *Nigella sativa* extract on asthma and water-soluble substances of this extract, especially its methanol fraction were responsible for this relaxation on contracted tracheal chains of guinea pigs. For the first time, the present study has been conducted to determine the main constituents of its methanolic extract.

Four constituents of 20%-methanolic fraction, consisting of two flavonoids (20-20% and 21-20% fractions) and two polysaccharides (1-20% and 2-20% fractions), were purified by analytical and preparative HPLC. The relaxant effects of four cumulative concentrations of each constituent (50, 100, 150 and 200 mg/lit) in comparison with saline (1 ml) as negative control and four cumulative concentrations of theophylline (0.2, 0.4, 0.6 and 0.8 mM) as positive control were examined on methacholine-precontracted guinea pig tracheal chains (n=6).

All concentrations of theophylline and most concentrations of 20-20, 21-20 fractions showed significant relaxant effects compared to that of saline. 20-20 fraction (Comferol diglucoside) was the most potent bronchodilator. Their relaxant effects were lower than that of theophylline. Polysaccharides (1-20, 2-20 fractions) did not have any relaxant effects compared to that of saline.

These results revealed that two flavonoids of 20%-methanolic fraction of *Nigella sativa* were its main relaxant constituents.

Keywords: Guinea pig; Main constituent; Methanolic fraction; *Nigella sativa*; Relaxant effect

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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, but there are many side effects. So, currently the physicians try to find the new drugs with fewer side effects. Botanical medicine is one important way for this purpose because some of 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 climates. The seeds of *Nigella sativa* are the source of the active ingredients such as thymoquinone, monotropens like *p*-cymene and α -pinene,³ nigellidine,⁴ nigellimine⁵ and a saponin.⁶

Several therapeutic effects have been described for the seeds of *Nigella sativa* in medical books including anti bronchial asthma and dyspnea,⁷ antitussive effect,⁸ hypotensive, anti-nociceptive, anti-fertility, anti-diabetic,⁹ anti-inflammatory, anti-oxidant, anti-microbial, anti-tumor and immunomodulatory properties.¹⁰ There is evidence of relaxant effects of the volatile oil from this plant on different smooth muscle preparations including rabbit aorta,¹¹ rabbit jejunum¹² and guinea pig isolated tracheal muscle.¹³

Our previous studies demonstrated that water soluble substances of *Nigella sativa* extract especially methanol fraction of this plant were responsible for the relaxant effects of this plant on tracheal chains of guinea pigs.^{14,15}

In addition, the preventive effects of hydro-ethanolic extract of *Nigella sativa* on lung pathological changes, tracheal responsiveness to methacholine and ovalbumin, cellular differentiation of bronchoalveolar lavage and blood cytokines were demonstrated.^{16,17}

Although many therapeutic effects of this extract were shown in different studies and in folk medicine, the main constituent of this extract has not been found yet. Therefore, for the first time, in the present study, the constituents of methanolic fractions of *Nigella sativa* were separated and their relaxant effects were assessed on tracheal chains of guinea pigs.

MATERIALS AND METHODS

General Procedures

Ultraviolet spectra were obtained in Methanol using a Shimadzu LC8-A UV-Vis spectrophotometer. NMR spectra were recorded in CD3OD using a Bruker 200 MHz NMR spectrometer (200 MHz for 1hr and 50

MHz for 13sec) with residual solvent peak as internal standard. HPLC separation was performed in a Shimadzu photo-diode-array detector (SPD-10A). A Shim-Pack ODS analytical HPLC column (5 μ m, 25 cm* 4.6 mm) and a Shim-Pack ODS preparative HPLC column (15 μ m, 250 mm* 22 mm) were used. Sep-Pak Vac 35 cc (10 g) C18 cartridge (Waters) was used for pre-HPLC fractionation. MS analyses were performed on a Finnigan MAT95 spectrometer.

Plant Material

Nigella sativa was collected from north eastern of 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; and the specimen number of the plant is 293-0303-1.

Extraction and Isolation of Compounds

The dried and powdered seeds of *Nigella sativa* (200 g) were extracted successively with n-hexane, dichloromethane and methanol, 1.1 liter each, using a Soxhlet apparatus. All these extracts were separately concentrated using a rotary evaporator at a maximum temperature of 45°C. 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.

Then, Solid phase extraction (SPE) method was performed for preparing five different methanolic fractions (Figure 1). In this method, Sep-pac cartridges (10gr- 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.

As the 20%-methanolic fraction had shown the most relaxant effect on guinea pig tracheal chains,¹⁸ the constituents of this fraction were assessed by analytical and preparative HPLC. The analytical HPLC analyses (0-0.01 min, linear gradient of 10% Methanol in water; 0.01-20 min, 30% Methanol in water; 20-25 min, 30% Methanol in water; 25-26 min, 10% Methanol in water; 26-36 min, 10% Methanol in water; flow rate 1 mL/min; detection at 190 and 490 nm) of the 20% methanolic solid-phase extraction (SPE) fraction (Figure 2) and the preparative HPLC analyses (0-0.01 min, linear gradient of 10% Methanol in water; 0.01-50 min, 30% Methanol in water; 50-62 min, 30% Methanol in water; 62-64 min, 10% Methanol in water;

64-74 min, 10% Methanol in water; flow rate 20 ml/min; detection at 190 and 490 nm) of the 20% methanolic SPE fraction afforded 1-20% fraction (499 mg, tR=8.2 min), 2-20% fraction (602 mg, tR = 10.2 min), 20-20% fraction (7 mg, tR =13.1 min), 21-20%

fraction (6 mg, tR = 16.83 min) (Figure 3). 1-20% and 2-20% fractions were polysaccharides and two others were flavonoids. The other constituents of this fraction have not fully known and analysed.

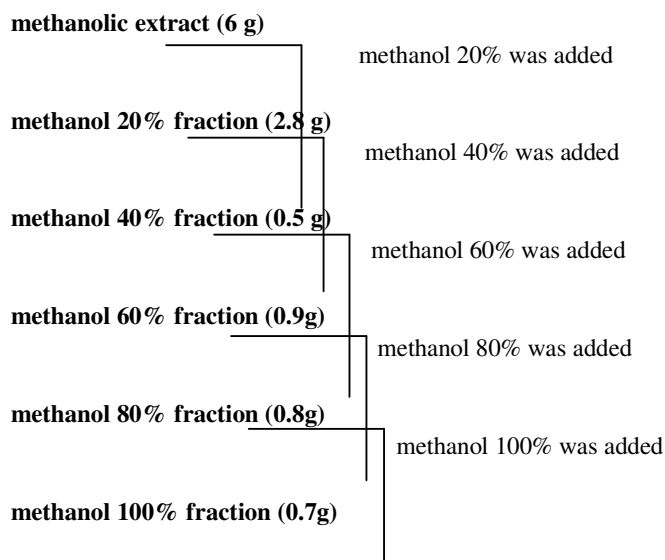


Figure 1. Fractionation of the methanolic extract from *Nigella sativa*

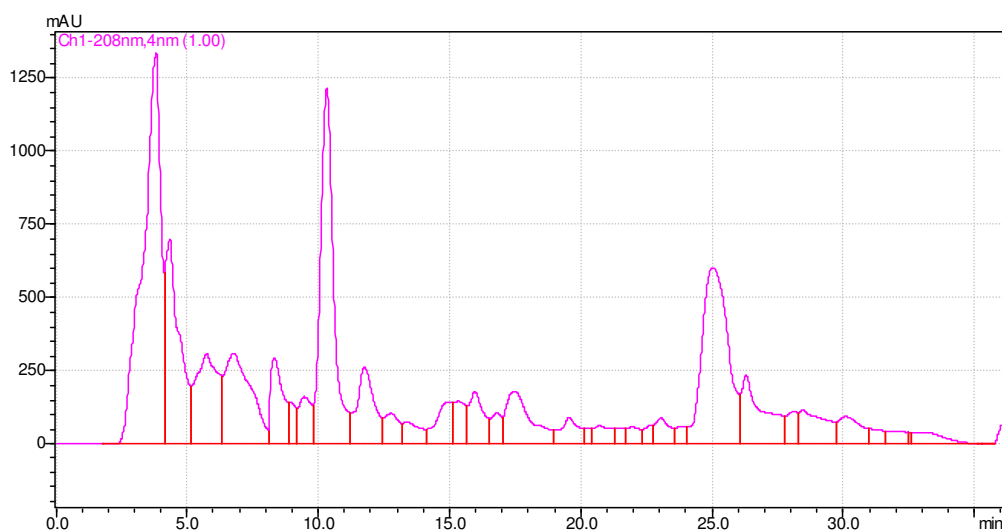


Figure 2. The analytical chromatography

Main Relaxant Constituents of *Nigella Sativa*

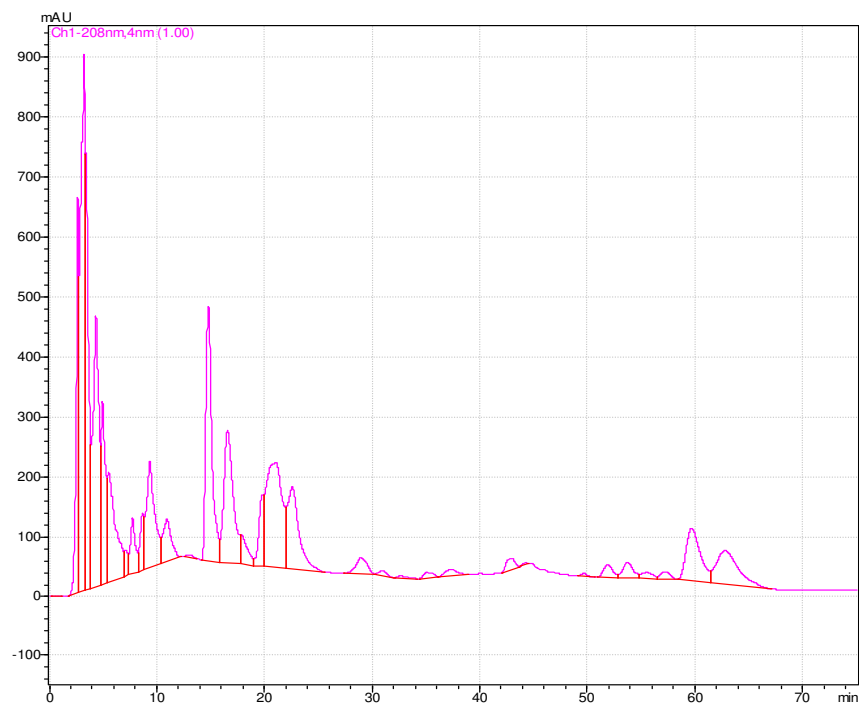


Figure 3. The preparative chromatography

Tissue Preparation

Guinea pigs (400-700 g, of both sexes) were killed by cervical dislocation and tracheas were removed. Each trachea was cut into 10 rings (each containing 2-3 cartilaginous rings). All rings were then cut open opposite the trachealis muscle, and sutured together to form a tracheal chain.¹⁴ Tissue was then suspended in a 20 ml organ bath (schuler organ bath type 809, March-Hugstetten, Germany) containing Krebs-Henseliet solution of the following composition (mM): NaCl 120, NaHCO₃ 25, MgSO₄ 0.5, KH₂PO₄ 1.2, KCl 4.72, CaCl₂ 2.5 and dextrose 11.

The Krebs solution was maintained at 37°C and gassed with 95% O₂ and 5% CO₂. 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.

This study was approved by the ethics committee of Tabriz University of Medical Sciences.

Protocols

The relaxant effects of these different constituents were tested on tracheal chains contracted by 10 μM

methacholine hydrochloride (Sigma Chemical Ltd UK) (n=6).

The relaxant effects of four cumulative concentrations (50, 100, 150, 200 mg/lit) of each constituent 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. The first concentration of each substance was added to organ bath 7-10 minutes after adding methacholine and the consecutive volumes were added to organ bath at five minutes 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 minutes. 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.

All of the experiments were performed randomly with an hour resting period of tracheal chains while

washing the tissues every 15 minutes with Krebs solution. In all experiments, responses were measured using a D-79232 transducer with a sensitivity range of 0–20 mm and resolution of 0.5 mm/turn (Hugo-Sachs Elektronik, Germany); amplified with amplifier (ML/118 quadribridge 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 relaxant effects of different concentrations of different constituents were compared with each other using one way ANOVA. The relaxant effects of four constituents and

theophylline were related to the concentrations using least square regression. Significance was accepted at $p < 0.05$.

RESULTS

Relaxant (Bronchodilator) Effects

The last concentration of 1-20% fraction, two higher concentrations of 21-20% fraction and all concentrations of 20-20% fraction except its first concentration showed significant relaxant effects compared to saline ($p < 0.001$ to $p < 0.05$) but 2-20% fraction did not show any relaxant effect compared to saline.

The relaxant effects of all concentrations of all constituents were significantly lower than those of theophylline ($p < 0.001$) (Figure 4).

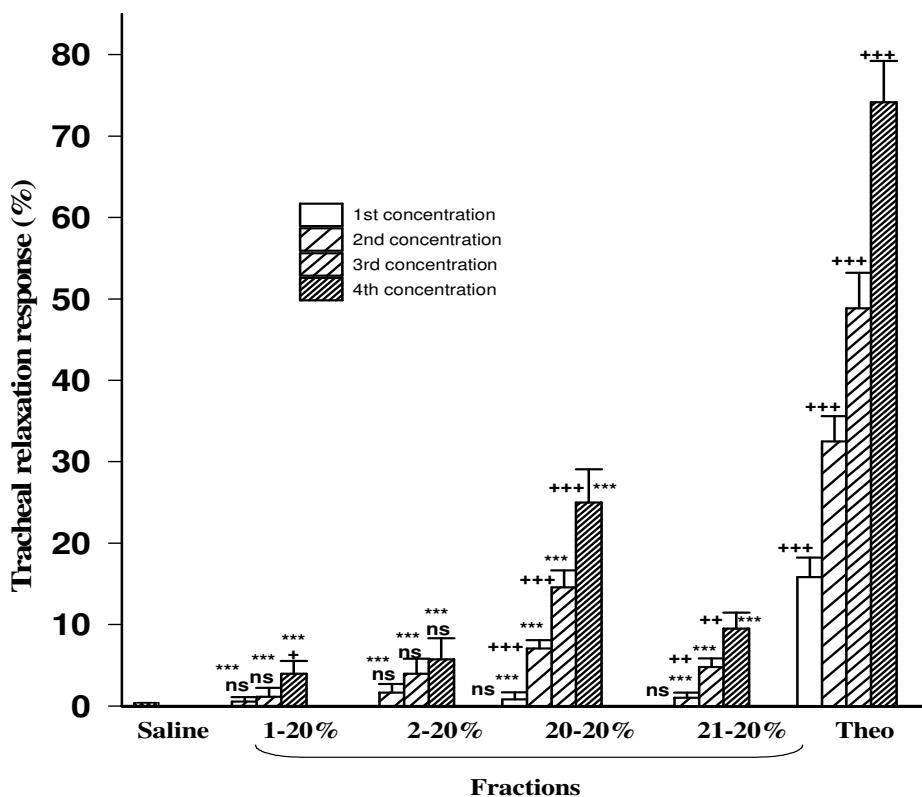


Figure 4. Relaxant effects of four different constituents of 20% -methanolic fraction from *Nigella sativa* in comparison with negative control (saline) and positive control (theophylline) in contracted tracheal chains by 10 μ M methacholin, n=6.

Statistical differences in the relaxant effects of different concentrations of different constituents 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 constituents vs those of theophylline; ***; $p < 0.001$.

Main Relaxant Constituents of *Nigella Sativa*

Table 1. Relaxant effects of four different constituents of 20%-methanolic fraction of *Nigella sativa* in comparison with negative control (saline) and positive control (theophylline) in contracted tracheal chains by 10 μ M methacholine (n=6)

Different Concentrations	Saline	1-20	2-20	20-20	21-20	Theophylline
1		0	0	0.83 \pm 0.83	0	15.83 \pm 2.38
St. Dif. vs Saline				NS p=0.73		p<0.001
St. Dif. vs Theo.				p<0.001		
2		0.55 \pm 0.55	1.62 \pm 1.08	7.08 \pm 1	1 \pm 0.64	32.50 \pm 3.09
St. Dif. vs Saline		NS p=0.97	NS p=0.34	p<0.001	NS p=0.48	p<0.001
St. Dif. vs Theo.		p<0.001	p<0.001	p<0.001	p<0.001	
3		1.11 \pm 1.11	3.96 \pm 1.84	14.58 \pm 2.08	4.79 \pm 1.04	48.83 \pm 4.36
St. Dif. vs Saline		NS p=0.61	NS p=0.09	p<0.001	p<0.01	p<0.001
St. Dif. vs Theo.		p<0.001	p<0.001	p<0.001	p<0.001	
4	0.37 \pm 0.06	3.96 \pm 1.58	5.75 \pm 2.59	25 \pm 4.08	9.05 \pm 1.96	74.16 \pm 5.07
St. Dif. vs Saline		p<0.05	p<0.05	p<0.001	p<0.01	p<0.001
St. Dif. vs Theo.		p<0.001	p<0.001	p<0.001	p<0.001	

Values are presented as mean \pm SEM. St. Dif.: Statistical difference. The four different concentrations for fractions were 50, 100, 150 and 200 mg/lit and for theophylline, 0.2, 0.4, 0.6 and 0.8 mM. Theo: Theophylline.

Table 2. Correlation (r) between the relaxant effects of four different constituents of 20 %- methanolic fraction from *Nigella sativa* and theophylline with their concentrations

	1-20	2-20	20-20	21-20	Theophylline
r	0.512	0.503	0.854	0.795	0.921
p value	p<0.05	p<0.05	p<0.001	p<0.001	p<0.001

Comparison of Relaxant Effects of Different Fractions

The lowest relaxant effect was shown for 1-20% fraction and 20-20% fraction had the highest relaxant effect (Table 1).

Correlation between Concentrations of Solution and Their Relaxant Effects

There were significant positive correlations between relaxant effects and concentrations for all constituents and theophyllin (p<0.001 to p<0.05) (Table 2).

DISCUSSION

In this study, the relaxant (bronchodilator) effects of four different constituents of 20% methanolic fraction from *Nigella sativa* in comparison with saline as negative control and theophylline as positive control were studied. In contracted tracheal chains by

methacholine, all concentrations of theophylline and most concentrations of all constituents except 2-20 fraction showed significant relaxant effects compared to that of saline. The relaxant effects of all concentrations of these constituents were significantly lower than those of theophylline. There were significant positive correlations between the relaxant effects and concentrations for all fractions and theophylline.

As this study was the first investigation in order to declare the main relaxant constituent of *Nigella sativa* on smooth muscle, it was proposed to evaluate their relaxant effects on normal tracheal muscles after adding the contractile substance (methacholine). With analytical and preparative HPLC, four constituents consisting of two flavonoids (20-20% and 21-20% fractions) and two polysaccharides (1-20% and 2-20% fractions) were obtained. Similar to previous studies, the constituents of 20%-methanolic fraction of this plant had relaxant effects on methacholine contracted guinea pig tracheal muscles.^{15,18}

The 20-20% fraction (comferol diglucoside) showed the maximal bronchodilator effects and the 21-20% fraction (comferol digalactoside) was in the second level in tracheal relaxation. In previous studies, the flavonoids showed relaxant effects on smooth muscle such as rat thoracic aorta¹⁹⁻²⁴ although flavonoids differ from one another in the orientation of substituents (hydroxyl and/or methyl etc.), the degree of unsaturation, the type of sugar moiety attached and the position of the benzenoid substitution.

The relaxant effects of flavonoid constituents of 20%- methanolic fraction in this study were lower than that of theophylline. It may be due to the different mechanisms of relaxant effects of theophylline. This drug can inhibit phosphodiesterase competitively and nonselectively²⁵, which raises intracellular cAMP, activates PKA and inhibits leukotriene synthesis²⁶ and reduces inflammation and innate immunity.²⁷ Moreover, this drug can act as a nonselective adenosine receptor antagonist. But the exact mechanism of these constituents evaluated in this investigation is not clear. However, in this study, the exact mechanism of tracheal relaxation by these constituents have not been shown but this effect may be due to their inhibitory effects on muscarinic receptors²⁸, potassium channel opener effect.^{14,15,28}

The relaxant effects of flavonoid constituents of 20%- methanolic fraction in this study were lower than that of 20%-methanolic fraction. Since the effect of each constituent was assessed lonely on contracted tracheal smooth muscle in this experiment design, so there were not any synergistic effects between constituents. The lower relaxant effects of these two constituents may be due to the lack of these synergistic effects. However, two polysaccharides (1-20% and 2-20% fractions) did not show any significant relaxant effect.

As this study is the first investigation which has been done for identification of main constituents, these results are helpful but more studies are required for the exact mechanism(s) and the effective substances causing the relaxant effect of this plant. In addition, the evaluation of the relaxant effect of these constituents on tracheal chains of asthmatic guinea pigs is suggested.

In conclusion, the results of the present study showed that the 20-20% fraction (comferol diglucoside) and 21-20% fraction (comferol digalactoside) had bronchodilatory effect but 1-20%

and 2-10% fractions did not show any relaxant effects on tracheal smooth muscle.

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REFERENCES

1. Bousquet J, Mantzouranis E, Cruz AA, Ait-Khaled N, Baena-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(5):926-38.
2. Goreja WG. In: Black seed: Nature's miracle remedy. New York, NY: Amazing herbs press Ltd, 2003: 5-10.
3. El-Dakhkhny M. Studies on chemical constitution of Egyptian *Nigella sativa* L.seeds. II. The essential oil. *Planta Medica* 1963; 11:465-70.
4. Atta UR, Malik SO. Nigellidine, a new indazol alkaloid from seeds of *Nigella sativa*. *J Res Inst* 1995; 36:1993-6.
5. Atta UR, Malik SO. Nigellimine N-oxide, a new isoquinoline alkaloid from the seeds of *Nigella sativa*. *Heterocycles* 1985; 23:953-5.
6. Ansari AK, Sadiy HAS. Structural studies on a saponin isolated from the seeds of *Nigella sativa*. *Phyto Chem* 1989; 27:377-9.
7. Avicenna: Law in Medicine, Interpreter; Sharafkhandy A, Tehran: Ministry of Guidance publication, 1990: 314.
8. Boskabady MH, Kiani S, Jandaghi P, Ziaei T, Zarei A. Antitussive of *Nigella sativa*. *Pak J Med Sci* 2004; 20(3):224-8.
9. Ali BH, Blunden G. Pharmacological and toxicological properties of *Nigella sativa*. *Phytother Res* 2003; 17(4):299-305.
10. Labib Salem M. Immunomodulatory and therapeutic properties of the *Nigella sativa* L. seed. *Int immunopharmacol* 2005; 5(13-14):1749-70.
11. Aqel MB. The relaxing effect of volatile oil of *Nigella sativa* seed on vascular smooth muscle. *Jordan Ser B* 1992; 1:91-100.
12. Aqel MB. Effects of *Nigella sativa* seeds on intestinal

Main Relaxant Constituents of *Nigella Sativa*

- smooth muscle. *Int J Pharmacogn* 1993; 31:55-60.
13. Reiter M, Brandt W. Relaxant effects on tracheal and ileal smooth muscles of the guinea-pig. *Arzneimittelforschung* 1985; 35(1A):408-14.
 14. 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). *IJBMS* 2007; 10(3):154–61.
 15. 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 of Exp Biol* 2008; 46(12):805-10.
 16. Boskabady MH, keyhanmanesh R, Khamneh S, Doostar Y, Khakzad MR. Potential immunomodulation effect of the extract of *Nigella sativa* on ovalbumin sensitized guinea pigs. *J Zhejiang Univ Sci B* 2011; 12(3):201-9.
 17. Boskabady MH, Keyhanmanesh R, Khamneh 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(5):879-87.
 18. Keyhanmanesh R, Bagban H, Nazemiyeh H, Mirzaei Babil F, Alipour MR, Ahmady M. The Relaxant Effects of Different Methanolic Fractions of *Nigella sativa* on Guinea Pig Tracheal Chains. *IJBMS* 2013; 16:123-8.
 19. Carron R, Sanz E, Puebla P, Martin ML, Roman LS, Guerrero MF. Mechanisms of relaxation induced by flavonoid ayanin in isolated aorta rings from Wistar rats. *Colomb Med* 2010; 41(1):10-6.
 20. Ajaya M, Gilani AH, MR. Effects of flavonoids on vascular smooth muscle of the isolated rat thoracic aorta. *Life Sci* 2003; 74(5):603–12.
 21. Woodman OL, Chan ECH. Vascular and anti-oxidant actions of flavonols and flavones. *Clinic Experiment Pharmacol Physiol* 2004; 31(11):786-90.
 22. Ko WC, Liu PY, Chen JL, Leu IJ, Shih CM. Relaxant Effects of Flavonoids in Isolated Guinea Pig Trachea and Their Structure-Activity Relationships. *Planta Med* 2003; 69(12):1086-90.
 23. Ajay M, Gilani AU, Mustafa MR. Effects of flavonoids on vascular smooth muscle of the isolated rat thoracic aorta. *Life Sci* 2003; 74(5):603-12.
 24. Macedo CL, Vasconcelos LHC, Correia ACC, Martins IRR, Lira DP, Santos BVO, et al. Spasmolytic effect of galeatin 3, 6-dimethyl ether, a flavonoid obtained from *Piptadenia stipulacea* (Beth) Ducke. *J smooth muscle Res* 2011; 47(5):123-34.
 25. Essayan DM. Cyclic nucleotide phosphodiesterases. *J Allergy Clin Immunol* 2001; 108(5):671–80.
 26. Deree J, Martins JO, Melbostad H, Loomis WH, Coimbra R. Insights into the Regulation of TNF- α Production in Human Mononuclear Cells: The Effects of Non-Specific Phosphodiesterase Inhibition. *Clinics (Sao Paulo)* 2008; 63(3):321–8.
 27. Peters-Golden M, Canetti C, Mancuso P, Coffey MJ. Leukotrienes: underappreciated mediators of innate immune responses. *J Immunol* 2005; 174(2):589–94.
 28. Boskabady MH, Shirmohammadi 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.