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The Contribution of Water and Lipid Soluble Substances in the Relaxant Effects of *Nigella sativa* Extract on Guinea Pig Tracheal Smooth Muscle *(in vitro)*

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Abstract

Objective
In previous studies, the relaxant, anticholinergic (functional antagonism), antihistaminic and its stimulatory effects on β-adrenoceptors of *Nigella sativa* have been demonstrated on guinea pig tracheal chains. In the present study, the relaxant effects of hydro-ethanolic, macerated aqueous (MA) and lipid-free macerated aqueous (LFMA) extract of *Nigella sativa* on tracheal chains of guinea pigs were examined.

Materials and Methods
The relaxant effects of four cumulative concentrations of each extract (0.8, 1.2, 1.6 and 2.0 g%) was compared with saline as negative control and four cumulative concentrations of theophylline (0.2, 0.4, 0.6 and 0.8 mM) on precontracted tracheal smooth muscle of guinea pig (60 mM KCl in group 1 and 10 µM methacholine in group 2, n=6 for each group).

Results
In group 1 all concentrations of theophylline, the last two concentrations of MA and the last three concentrations of LFMA extracts showed significant relaxant effects compared to that of saline ($p<0.05$ – $p<0.005$). Two final concentrations of hydro-ethanolic extract caused contraction in comparison with saline in this group. In group 2 all concentrations of theophylline, MA and LFMA and the last three concentrations of hydro-ethanolic extracts showed significant relaxant effects relative to that of saline ($p<0.005$ – $p<0.001$). The relaxant effect of different concentrations of MA, the last three concentrations of hydro-ethanolic and LFMA extract was significantly greater in group 2 as compared with group 1. However, there was no significant difference in the relaxant effect of different concentrations of theophylline, between group 1 and 2. In both groups, the relaxant effect of most concentrations of MA, hydro-ethanolic LFMA extracts was significantly less than those of theophylline ($p<0.05$ – $p<0.001$). In group 1 and 2, the relaxant effect of all concentrations of hydro-ethanolic extract was significantly lower than most concentrations of others ($p<0.05$ – $p<0.01$). There was a significant positive correlations between the relaxant effects and concentrations for theophylline and all extracts (except hydro-ethanolic extract in group1) in both groups ($p<0.05$ – $p<0.001$).

Conclusion
These results showed that mainly water soluble substances of *Nigella sativa* were responsible for the relaxant effect of the plant on tracheal chains of guinea pigs.

Keywords*:* Guinea pig, Hydro-ethanolic extract, Lipid-free macerated aqueous extract, Macerated aqueous extract, *Nigella sativa*, Relaxant effect, Trachea

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Introduction

The seeds of *Nigella sativa* L. (Ranunculaceae) contain thymoquinone, monotropens such as *p*-cymene and α-pinene (1), nigellidine (2), nigellimine (3) and a saponin (4). All chemical composition of the plant was summarized in a recent review (5).

The therapeutic effects described for the seeds of *Nigella sativa* in Iranian ancient medical books are included antiasthma and dyspnea (6). There is evidence of relaxant effects of the volatile oil from this plant on different smooth muscle preparations including rabbit aorta (7), rabbit jejunum (8), and guinea pig isolated tracheal muscle (9). Mahfouz and EL-Dakhakhnsy (10) reported that the volatile oil from *Nigella sativa* protected guinea pigs against histamine-induced bronchospasm, but it did not affect histamine H_1 receptors blockade in isolated tissues. Both systemic and local administrations of essential oil from this plant are showed to have anti-inflammatory activity (11). The therapeutic effect of *Nigella sativa* oil on patients with allergic diseases (allergic rhinitis, bronchial asthma, atopic eczema) also have been demonstrated (12). In addition, Labib Salem in a recent review has summarized the therapeutic properties of the *Nigella sativa* L. seed and emphasized the potent immunomodulatory effects of this plant (5).

The previous studies have shown the different pharmacological effects of *Nigella sativa* on guinea pig tracheal chains including: relaxant and functional antagonistic effects on muscarinic receptors (13), inhibitory effect on histamine (H_1) receptors (14) inhibitory effect on calcium channels (15), opening effect on potassium channels (16) and stimulatory effect on β-adronceptors (17). The antitussive effect of this plant on guinea pig (18) was also demonstrated.

Therefore in the present study, the relaxant effects of hydro-ethanolic, macerated aqueous and lipid-free macerated aqueous extract (lipid and water soluble substances) of *Nigella sativa* on tracheal chains of guinea pigs were examined.

Materials and Methods

Plant and extracts

Nigella sativa was collected from Torbat Heydarieh (north east of Iran) in the spring of 2002, identified by botanists in the herbarium of Ferdowsi University of Mashhad; (specimen number of the plant is 293-0303-1) and dried at room temperature in dark. Macerated aqueous extract was prepared as follows: five hundred grams of *Nigella sativa* seeds were grinded, added to 300 ml distilled water and was kept at room temperature for 48 hours while shaked intermittently. The solution was then filtered and dried. To prepare lipid-free extract, the macerated aqueous extract was shaken with the ether petroleum for 1 hour and the water-phase was separated and dried. The hydro-ethanolic extract was prepared as follows: Two hundred grams of grinded *Nigella sativa* seeds was mixed with 450 ml ethanol 50% for 48 hours at room temperature, while shaking intermittently, and the solution was separated and dried to prepare hydro-ethanolic extract.

Tissue preparation

Adult Dunkin-Hartley guinea pigs (400-700 g, both sexes) were studied. Animals housed in big cages (4 – 5 per cage) with access to food and water *ad libitum*, were maintained at 22º ± 2ºC on a 12 h light/dark cycle (light period 0700 and 1900 hs). Guinea pigs were killed by a blow to their 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 (19). 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, $NaHCO_3$ 25, $MgSO_4$ 0.5, KH_2PO_4 1.2, KCl 4.72, $CaCl_2$ 2.5 and dextrose 11.

The Krebs solution was maintained at 37^oC, pH 7.4 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 h, while it was washed with Krebs solution every 15 mins.

Protocols

The relaxant effects of four cumulative concentrations of hydro-ethanolic, macerated aqueous and lipid-free macerated aqueous extracts of *Nigella sativa* (0.8, 1.2, 1.6 and 2.0 g%) and theophylline anhydrous (Sigma Chemical Ltd UK) (0.2, 0.4, 0.6 and 0.8 mM), and saline (0.5 ml) as negative control were examined. To produce the first concentration of each extract, 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 three times, to organ bath respectively. For theophylline, 0.1 ml of 20 mM concentrated solution was added 4 times, to organ bath. The consecutive volumes were added to organ bath at every five minutes intervals.

The relaxant effect was examined on contracted tracheal smooth muscle, after exposing tissue to each concentration of the solution for 5 mins. 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 (20).

The relaxant effect of different solutions was tested with two different experimental designs, (n=6 for each group) as follows:

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

The relaxant effects in two groups of experiments were examined in two different series of tracheal chains. All the experiments were performed randomly with a 1 h resting period of tracheal chains between each two experiments while washing the tissues every 15 min with Krebs solution. In all experiments, responses were measured using vernier control type 850 N sensor with sensitivity range: 0-20 g and resolution: 0.2 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

Data were expressed as mean±SEM. Data of relaxant effects of different concentrations of each extract 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 matched using unpaired t test. The comparison of relaxant effects of different concentrations of three various extracts with each other accomplished using ANOVA. The relaxant effect of three extracts and theophylline was related to the concentrations, using least square regression. Significance accepted at $p<0.05$.

Results

Relaxant (bronchodilatory) effect

In group 1 experiments all concentrations of theophylline, the last two concentrations of macerated aqueous and the last three concentrations of lipid-free macerated aqueous extracts showed significant relaxant effects compared to that of saline ($p<0.05$ to $p<0.005$). Two final concentration of hydro-ethanolic extract caused contraction relative to saline ($p<0.01$ to 0.005), (Table 1).

Table 1. Relaxant effect (percent decrease in maximum contraction) of hydro-ethanolic, macerated aqueous (MA) and lipid free macerated aqueous extracts (E) from *Nigella sativa* compared to negative (saline) and positive (theophylline = Theo) controls in group 1 experiments (contracted tracheal chains with 60 mM KCl, n=6).

Different Concentration	Saline	hydro-ethanolic E	macerated aqueous E	lipid free MAE	Theo
1	-	0±0	1.00±1.00	3.75±2.39	17.2±2.33
St. Dif. *vs* Saline		-	NS	NS	p<0.001
St. Dif. *vs* Theo.		-	p<0.001	p<0.005	
2	-	-5±2.58	3.33±1.67	7.78±2.97	48.5±4.3
St. Dif. *vs* Saline		NS	NS	p<0.05	p<0.001
St. Dif. *vs* Theo.		p<0.001	p<0.001	p<0.001	
3	-	-10.84±3.51	8.75±2.21	11.67±4.22	66.7±3.42
St. Dif. *vs* Saline		p<0.01	p<0.005	p<0.05	p<0.001
St. Dif. *vs* Theo.		p<0.001	p<0.001	p<0.001	
4	0.37±0.23	-15.84±4.16	15±3.65	15.83±5.69	81.32±2.51
St. Dif. *vs* Saline		p<0.005	p<0.005	p<0.05	p<0.001
St. Dif. *vs* Theo.		p<0.001	p<0.001	p<0.001	

Values are presented as mean±SEM. St. Dif.: Statistical diference. The four different concentrations for extracts were 0.8, 1.2, 1.6 and 2.0 g%, and for theophylline, 0.2, 0.4. 0.6 and 0.8 mM.

Table 2. Relaxant effect (percent decrease in maximum contraction) of hydro-ethanolic, macerated aqueous (MA) and lipid free macerated aqueous extracts (E) from *Nigella sativa* compared to negative (saline) and positive controls (theophylline = Theo) in group 2 experiments (contracted tracheal chains by 10 μM methacholie, n=6).

Different Concentration	Saline	hydro-ethanolic E	macerated aqueous E.	lipid free MAE	Theo
1	-	2.5±1.12	6.67±1.66	15±4.56	15.42±3.56
St. Dif. *vs* Saline		NS	p<0.005	p<0.005	p<0.005
St. Dif. *vs* Theo.		p<0.01	NS	NS	
2	-	6.67±1.67	18.34±4.21	25.38±5.64	43.75±5
St. Dif. *vs* Saline		p<0.005	p<0.005	p<0.005	p<0.001
St. Dif. *vs* Theo.		p<0.001	p<0.005	p<0.05	
3	-	12.5±2.14	25.84±5.23	44.17±8.11	62.5±5.28
St. Dif. *vs* Saline		p<0.001	p<0.001	p<0.001	p<0.001
St. Dif. *vs* Theo.		p<0.001	p<0.001	NS	
4	0.55±0.35	20±3.41	35.84±7.12	50.83±8.80	82.5±6.55
St. Dif. *vs* Saline		p<0.001	p<0.001	p<0.001	p<0.001
St. Dif. *vs* Theo.		p<0.001	p<0.001	p<0.05	

For abbreviations see Table 1.

In group 2 experiments all concentrations of theophylline, macerated aqueous, lipid-free macerated aqueous and the last three concentrations of hydro-ethanolic extracts showed significant relaxant effects as compared with that of saline (p<0.005 to p<0.001), (Table 2).

Comparison of the relaxant effect of theophylline with different extracts

The relaxant effect of all concentrations of three extracts in group 1 and the last three concentrations of hydro-ethanolic extract, all concentrations of macerated aqueous extract and middle (1.2) and high (2.0) concentrations of lipid free macerated aqueous extract in group 2 was significantly less than those of theophylline (p<0.05 to p<0.001), (Table 1 and 2).

Comparison of the relaxant effect of different fractions

In both groups, the relaxant effect of all

concentrations of hydro-ethanolic extract was significantly lower than most concentrations of others ($p<0.05$ to $p<0.01$).

Comparison of the relaxant effect between two groups of experiments

The relaxant effect of different concentrations of macerated aqueous, the last three concentration of hydro-ethanolic and lipid-free macerated aqueous extracts was significantly greater in group 2 than in group 1 experiments ($p<0.05$ to $p<0.001$). However, there was no significant difference between two groups, regarding the relaxant effect of different concentrations of theophylline (Figure 1).

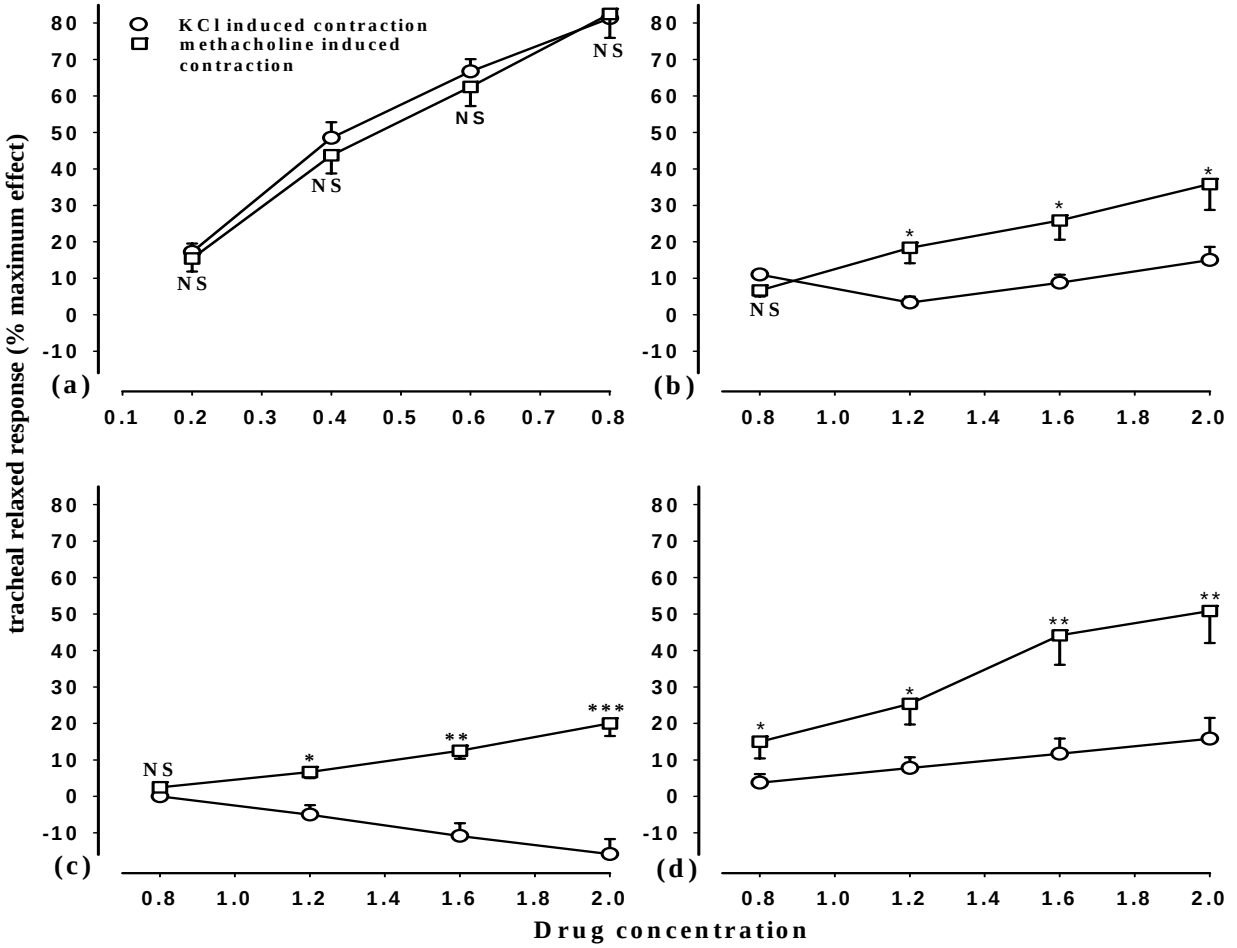

Figure 1. Concentration response curves of the relaxant effect of theophylline (a), macerated aqueous (MA), (b), hydro-ethanolic (c), and lipid free macerated aqueous (LFMA) extracts (d) from *Nigella sativa*, in two groups of experiments. group 1; KCl induced contraction of tracheal chains (o, n=6) and group 2; methacholine induced contraction of tracheal chains (□, n=6). Statistical differences in the relaxant effect of different concentrations of each extract between group 1 with those of group 2; NS: non-significant difference, *; $p<0.05$, **; $p<0.01$. The concentration unite for extracts was g% and for theophylline mM.

Correlation between concentrations of solutions and their relaxant effect

There was significant positive correlation, between the relaxant effect and concentrations of theophylline and all extracts (except hydro-ethanolic extract in group1) in both groups ($p<0.05$ to $p<0.001$, Table 3).

Table 3. Correlation (r) between the relaxant effects of hydro-ethanolic, macerated aqueous (MA) and lipid free macerated aqueous extracts (E) from *Nigella sativa* and theophylline with their concentrations in two groups of experiments

Different solutions	hydro-ethanolic E		macerated aqueous E		lipid free MAE		Theophylline	
	r	p value	r	p value	r	pvalue	r	p value
Group 1	-0.647	p<0.001	0.697	p<0.001	0.424	p<0.05	0.941	p<0.001
Group 2	0.787	p<0.001	0.690	p<0.001	0.644	p<0.005	0.900	p<0.001

Discussion

In this study the relaxant (bronchodilatory) effects of three extracts (hydro-ethanolic, macerated aqueous and lipid-free macerated aqueous) from *Nigella sativa* in comparison with saline as negative control and theophylline as positive control were studied. In group 1 experiments (contracted tracheal chains by KCl) macerated aqueous and lipid-free macerated aqueous extracts showed relatively weak but theophylline a potent relaxant effects on tracheal smooth muscle. However, hydro-ethanolic extract caused contraction effect in this group. In group 2 experiments (contracted tracheal chains by methacholin) in addition to theophylline, lipid-free macerated aqueous extracts also showed potent relaxant effects, macerated aqueous a relatively potent and even hydro-ethanolic extract showed weak but significant relaxant effects on tracheal smooth muscle. Therefore, the relaxant effect of macerated aqueous, hydro-ethanolic and lipid-free macerated aqueous extracts was significantly greater in group 2 compared to group 1 experiments.

The absence of relaxant effect of hydro-ethanolic extract and a weak relaxant effect of MA and LFMA extracts from *Nigella sativa* in group 1 may indicate an opening effect of this plant on potassium channels, because the bronchodilatory effect of potassium channel opening drugs has been demonstrated previously (21). If the extracts from *Nigella sativa* had a potassium channel opening effect, they would not have a relaxant effect on tracheal chains contracted by KCl, while they could show a relaxant effect when the tracheal chain was contracted by metacholine. However, with regard to bronchodilatory effect of calcium channel blockers (21, 22) and because KCl affect calcium channels (23, 24), the result of the present study did not suggest an inhibitory effect of this plant on calcium channels of guinea pig tracheal chain. In fact, these findings are supported by the results of the previous study (16) for aqueous and macerated extracts from *Nigella sativa*.

The greater relaxant effect of the extracts of *Nigella sativa* in group 2 may indicates an inhibitory on muscarinic receptors, which is supported by the results of the previous study (13). The findings of the present study indicated that mainly water soluble ingredients of *Nigella sativa* are responsible for the relaxant effect of this plant on tracheal chains of guinea pigs. However, the lower relaxant effect hydro-ethanolic extract (containing lipid soluble substances) may be due to their low solubility in Kerbs solution of the organ bath which will cause limited access to the muscle of tracheal chains. Therefore, more studies are required for the exact mechanism(s) and the effective substances causing the relaxant effect of this

plant.

In conclusion, the results of the present study showed that mainly water soluble substances of *Nigella sativa* are responsible for the relaxant effect of the plant on tracheal chains of guinea pigs.

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