Vasorelaxant effect of essential oil isolated from *Nigella sativa* L. seeds in rat aorta: Proposed mechanism

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Abstract: The effect of the essential oil extracted from *Nigella sativa* (L.) seeds (Nigella oil) was investigated for its vasorelaxant activity on isolated rat aorta. Nigella oil at concentrations of 10-100µg/mL elicited a dose-dependent relaxation of the aorta, which was pre-contracted with noradrenaline (NA, 10^{-6} M) or KCl (100mM). In the presence of Nigella oil (75µg/mL, the dose response curves to increasing concentrations of NA (10^{-9} M to 10^{-4}M) or KCl (10mM-100mM) were displaced downwards, indicating inhibition of the vasoconstrictive effect. This relaxation effect was independent of the presence of endothelium. In addition, the vasodilatory activity of the Nigella oil was not affected by pre-treatment of the rings with N^G-nitro-L-Arginine (an inhibitor of endothelial nitric oxide synthase; 0.1mM), suggesting that the vasorelaxant effect is not mediated by nitric oxide. Furthermore, pre-treatment of the rings with Nigella oil (75µg/mL suppressed the tension increment produced by increasing external calcium concentration (0.25mM to 1.5mM). Tin conclusion, the essential oil extracted from *Nigella sativa* seeds produces smooth muscle relaxation, which is independent of endothelium and is not mediated by nitric oxide. The results also suggest that the vasorelaxing effect of the oil results from the blockade of both voltage-sensitive and receptor-operated calcium channels, and this may have therapeutic significance, in that Nigella oil may be useful as an antihypertensive agent in humans.

Keywords: Essential Oil, *Nigella sativa*, endothelium, calcium channel, vasodilator, rat.

INTRODUCTION

Nigella sativa Linn (Ranunculaceae) is an annual herb, which commonly grows in Eastern Europe, the Middle East, and Western Asia. Its ripe fruit contains tiny black seeds, known as ''Al-Habba Al-Sauda'' and ''Al-Habba Al-Barakah'' in Arabic and black seed or black cumin in English. It has a very long history of use in traditional medicine, especially for the treatment of respiratory diseases (asthma, bronchitis, cough, chronic obstructive pulmonary disease), rheumatism, gastrointestinal disorders (anorexia, dyspepsia, flatulence, dysentery, diarrhoea), jaundice, skin diseases, gynaecological problems (such as amenorrhea), fever, hair loss, high blood pressure, cancer and diabetes, also as an abortifacient, expectorant, carminative, diaphoretic, digestive, anthelmintic, diuretic, lactagogue and as antiparasitic agent (Ghazanfer, 1994,Bruneton, 1999, Talha *et al*., 2011, Jazieh *et al*., 2012, Ziaee *et al*., 2012, Leong *et al*., 2013,Shabana *et al*., 2013). Its use is very popular in various traditional systems of medicine, such as Unani and Tibb, Ayurveda and Siddha, and Islamic medicine, in which it is considered as one of the greatest forms of healing medicine (Ahmad *et al*., 2013). Several ethnobotanical studies have identified *Nigella sativa* as one of the plant used in traditional medicine to treat high blood pressure (Tahraoui *et al*., 2007, Amel, 2013). *Nigella sativa* seed, its extracts and oil, and isolated constituents have been demonstrated in experimental studies to exhibit analgesic (Al-Ghamdi, 2001, Hajhashemi *et al*., 2004, Islam *et al*., 2013), anti-arthritic (Sajad *et al*., 2010), anti-atherogenic (Al-Naqeep *et al*., 2011), anti-asthmatic (Kalemci *et al*., 2013), anti-malarial (Okeola *et al*., 2011), anti-microbial (Tahir *et al*., 2013), anti-cancer (Randhawa and Alghamdi, 2011), antidiabetic (Mohamed *et al*., 2009), anti-epileptic (Noor *et al*., 2012), anti-fungal (Bita *et al*., 2012), anti-inflammatory (Hajhashemi *et al*., 2004, Sajad *et al*., 2010, Islam *et al*., 2013), anti-oxidant (Woo *et al*., 2012), antipyretic (Al-Ghamdi, 2001), diuretic (Zaoui *et al*., 2000), hypocholesterolemic (Al-Naqeep *et al*., 2011), hypolipidemic (Didi *et al*., 2013), antihypertensive (Zaoui *et al*., 2000,Khattab and Nagi, 2007), immunomodulatory (Hmza *et al*., 2013), cardio- (Ahmad *et al*., 2013), hepato- (Hamed *et al*., 2013), gastro- (Bukhari *et al*., 2011), nephro- (Khattab and Nagi, 2007, Hamed *et al*., 2013), neuro- (Akhtar *et al*., 2013), pneumo- (Tayman *et al*., 2013), and radio- (Assayed, 2010) protective agent, which confirm the reported wide variety of therapeutic effects (Ali and Blunden, 2003, Al-Ghamdi *et al*., 2001, Hajhashemi *et al*., 2004, Khattab and Nagi, 2007, Ahmad *et al*., 2013, Leong *et al*., 2013,Shabana*et al*., 2013).

Oral administration of the seed or its extracts has been shown to reduce blood pressure in patients with mild hypertension (Dehkordi and Kamkhah, 2008,Qidwai*et al*., 2009), or metabolic syndrome (Najmi *et al*., 2013), improve glycemic control in type 2 diabetic patients (Bamosa *et al*., 2010), reduce total cholesterol, triglycerides and LDL-cholesterol in patients with

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dyslipidemia (Qidwai *et al*., 2009) or congestive heart disease (Tasawar *et al*., 2011), improve the oxidative-antioxidative balance in postmenopausal women (Mostafa and Moustafa, 2012), reduce mean frequency of seizures in children with refractory seizures (Akhondian *et al*., 2007) and improve the symptoms in patients with asthma (Boskabady *et al*., 2010) or allergic rhinitis (Nikakhlagh *et al*., 2011).

In clinical trials, Nigella seed oil was found to exhibit anti-allergic (Kalus *et al*., 2003), anti-arthritic (Gheita and Kenawy, 2012), anti-cancer (Jazieh *et al*., 2012),) hypoglycemic (Bamosa *et al*., 2010, Mohtashami *et al*., 2011), hypolipidemic (Amini *et al*., 2011), and hypotensive (Fallah Huseini *et al*., 2013) activities.

The above effects are mediated by some of the compounds identified in the essential oil, including phenols (65% of total oil), such as thymoquinone and its derivatives, thymol, carvacrol and terpenenoids such as carvone, and p-cymene (El-Dakhakhny *et al*., 2002, Nickavar *et al*., 2003, Hajhashemi *et al*., 2004).

The relaxant effect of the volatile oil from *Nigella sativa* seeds has previously been demonstrated in several isolated smooth muscles, including rabbit aorta (Aqel, 1992) and jejunum (Aqel, 1993), guinea pig trachea and ileus (Reiter and Brandt, 1985) and uterus (Aqel and Shaheen, 1996) and rat uterus (Aqel and Shaheen, 1996).

In the present study, we investigated the vasorelaxant activity of the essential oil extracted from *Nigella* seeds on the isolated rat thoracic aorta and to identify possible underlying mechanism(s) involved.

MATERIALS AND METHODS

Plant and extracts

Nigella sativa (L.) seeds were collected in the spring of 2006. Authentic sample was identified by the Botanist, Aïcha Ouyahy. A voucher specimen (No. 10359) was deposited in the Departmental herbarium. The seeds were dried at 50°C for 8hr, ground to a powder and placed in a desiccator prior to use in the absence of sunlight.

Extraction of essential oil

The essential oil used in the experiments was extracted by hydro-distillation of Nigella seed powder using a Clevenger type apparatus. Briefly, 250ml of distilled water was added to 50g of seed powder and the mixture was boiled and the vapors were condensed by using a glass condenser and collected in coated glass bottles. Diethyl ether (10ml) was added to the condensate and after shaking, the mixture was left for 30min to separate the water-ether phases. The supernatant (ether) phase was separated and shaken gently with anhydrous sodium sulfate (as the drying agent) and filtered through 0.22mm filter (Millipore, Bedford, MA, USA). The solvent was removed by using a rotatory evaporator (60^{o}C). The residue (essential oil) stored at 4°C in coated glass bottles; yield =0.37%.

Aortic preparation and mounting

Male Wistar rats (10-12 weeks old, weighing 250-350g), were maintained at a constant temperature (24°±1°C), with a 12-h/12-h dark / light cycle and fed standard chow. All experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care and with institutional guidelines.

Rats were anesthetized with diethyl ether and killed by decapitation using a small animal guillotine. The thoracic aorta was rapidly removed and immersed in cold (4°C) physiological solution (composition in mM: NaCl, 122; KCl, 5.9; $NaHCO_3$, 15; $MgCl_2$, 1.25; $CaCl_2$, 1.25; glucose, 11). Aortic rings (± 2mm) were dissected and mounted in 12.5ml organ bath filled with the physiological solution, gassed with a mixture of 95% O_2-5% CO_2 and maintained at 37°C. The rings were stretched to a resting tension of 20mN. Isometric tension was measured as described (El-Bardai *et al*., 2003). Contraction was evoked either by changing the physiological solution in the bath to a KCl rich solution (composition in mM: NaCl, 27; KCl, 100; $NaHCO_3$, 15; $MgCl_2$, 1.25; $CaCl_2$, 1.25; glucose, 11) or by adding noradrenaline (NA) to the physiological solution in the bath. When required, N-nitro-L-arginine [nitric oxide (NO)-synthase inhibitor, NNA; 0.1mM) was added 30 min before beginning of the contraction. The effect of the Nigella essential oil extract (added at different concentrations) was corrected for time-matched controls.

Drugs and chemicals

NaCl, $NaHCO_3$, KCl, $MgCl_2$, $CaCl_2$, glucose, noradrenaline (NA; norepinephrine), L-NNA (N^G-Nitro-arginine; N^G-nitro-L-Arginine), and acetylcholine were purchased form (Sigma Chemicals).

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Concentrations producing 50% of the maximum effect (IC_{50}) were calculated by non-linear regression of the log concentration-effect curves (Prism, Graph Pad). Nigella oil concentration was expressed as µg/mL.

Student's t-test was used to compare data; mean values were considered significantly different when $P<0.05$.

Effects of Nigella sativa oil on aortic rings

To evaluate the effects of Nigella oil on the contraction of aortic rings, experiments were performed using rings with intact endothelium as well as without endothelium.
Two different experimental designs were used:

Table 1: Inhibitory effect of *Nigella sativa* oil on the NA- and KCl-induced contractions

	Control		*Nigella sativa* oil	
	E_{max} (%)	pD_2	E_{max} (%)	pD_2
NA	2.4±0.2	7.2±0.87	1.02±0.15*	6.35±0.9*
KCl	2.04±0.18	38.24±1.59	1.03±0.14*	29.34±1.43*

E_{max} and pD_2 values were calculated from rat thoracic aorta rings concentration-response curves to NA (10^{-9}-10^{-5}M) and KCl (10-100mM), in the absence (Control) and in the presence of *Nigella sativa* oil (75µg/mL). Results are presented as mean ± S.E.M

Table 2: Effect of endothelial mediators on *Nigella sativa* oil-induced relaxation

	E_{max} (%)	pD_2	N
E+	90±2.9	4.6±1.29	6
E-	85±3.2	4.53±1.47	7
L-NNA	84±2.6	4.52±1.55	8

E_{max} and pD_2 values of the *Nigella sativa* oil concentration-dependent (10-100µg/mL) relaxation in endothelium-intact (E+) aortic rings precontracted with NA (10^{-6} M) compared to L-NNA (0.1mM) treatment and endothelium (E-) aortic rings. Results are presented as mean ± S.E.M

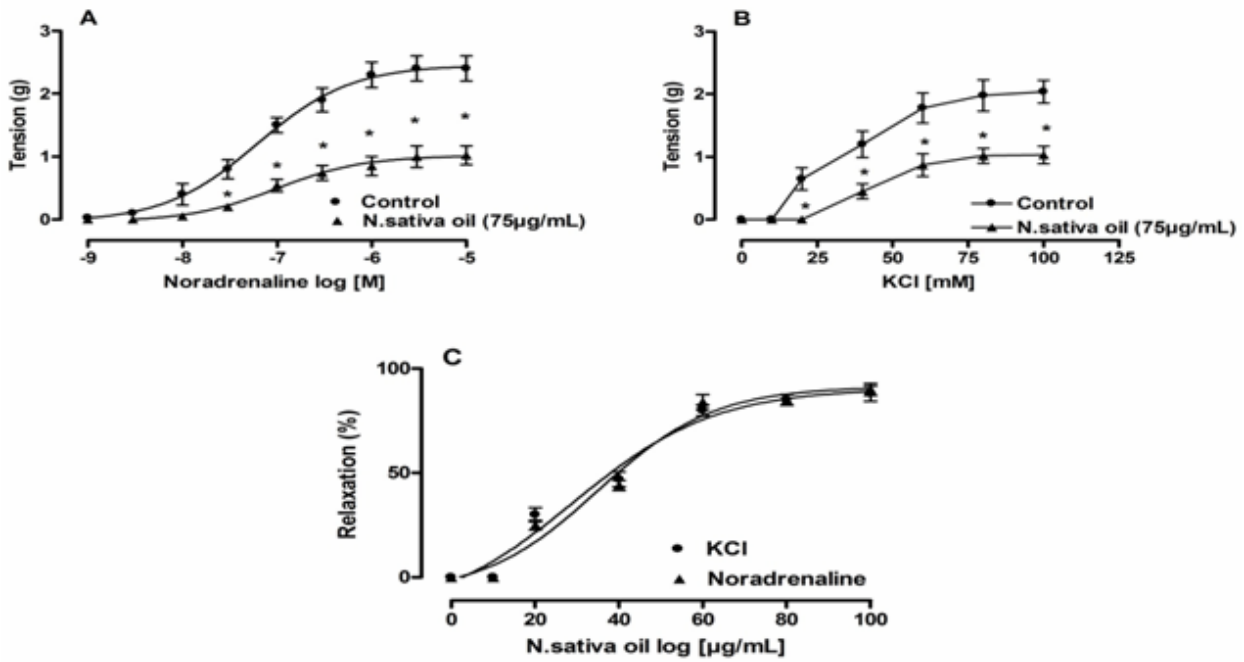

Fig.1: Inhibitory effect of *Nigella sativa* oil (75µg/mL) on the contraction induced by NA (10^{-9}-10^{-5}M) [Panel A] and KCl (10-100mM) [Panel B] in isometric tension in rat thoracic aorta rings. Panel C shows the vasorelaxant effect of *Nigella sativa* oil (10-100µg/mL) on endothelium-intact thoracic aorta rings precontracted with NA (10^{-6}M) or KCl (100mM). Results are presented as mean ± S.E.M. for 4 to 5 experiments. * P<0.05; *Nigella sativa* oil vs. control (A and B): ANOVA followed by Bonferoni test.

a) In one study, after 60 min of equilibration, control concentration-response curves were obtained after adding increasing concentrations of NA (10^{-9}M to10^{-5}M) or KCl (10mM to100mM). In parallel studies, concentration-response curves were obtained 30 min after adding Nigella oil (75µg/mL) to the bath and the addition of NA or KCl at the above concentrations.

b) In the second study, after 60 min equilibration, the aortic rings were pre-contracted with NA (10^{-6}M) or KCl (100mM) and once the plateau was achieved, concentration-dependent curves were obtained for Nigella oil-induced relaxation with increased concentrations of 10-100µg/mL.

Effects of Nigella sativa oil on extracellular Ca^{2+}-induced contraction activated by KCl

Experiments were carried out with aortic rings in calcium-free physiological solution. In control studies, after 60 min of equilibration, KCl (100mM) was added and the effect of addition of cumulative concentrations of $CaCl_2$ (0.25, 0.50, 0.75, 1.00, 1.25 and 1.50mM) on tension of

the rings was determined. The same protocol was repeated in another group of aortic rings 30 min after pre-treatment with Nigella oil (75µg/mL).

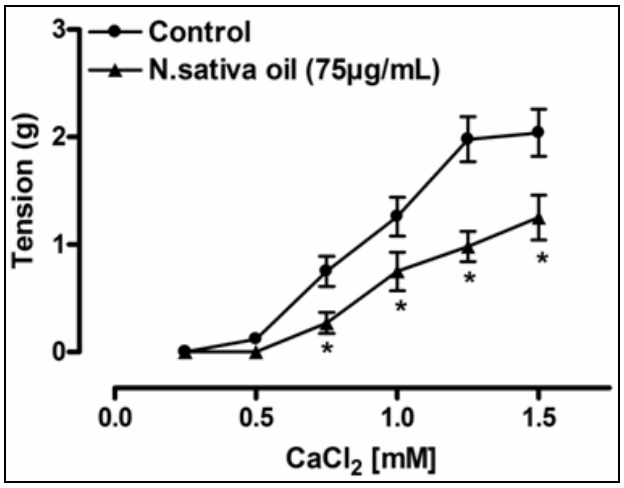

Fig.2: Inhibitory effect of *Nigella sativa* oil (75µg/mL) on the cumulative contraction curve dependent on extracellular Ca^{2+}influx (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mM) induced by KCl (100mM) in Ca^{2+}-free solution of endothelium-intact thoracic aorta rings. Results are presented as mean ± S.E.M. for 4 to 5 experiments. * *P*<0.05; *Nigella sativa* oil vs. control. ANOVA followed by Bonferoni test.

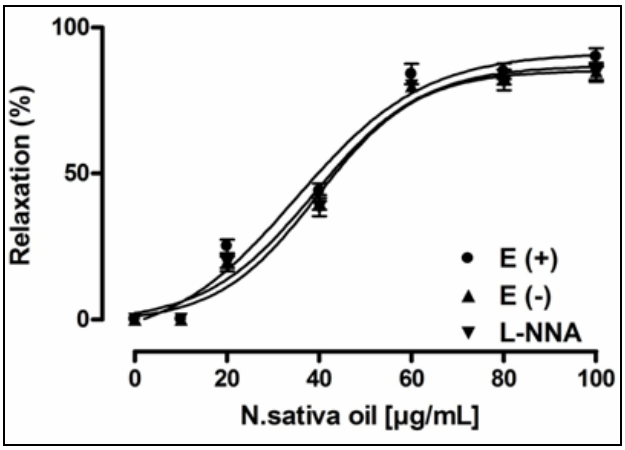

Fig.3. Effect of L-NNA (0.1mM) on relaxation by *Nigella sativa* oil (10-100µg/mL) of endothelium-intact (E+) thoracic aorta rings. For comparison, relaxation by *Nigella sativa* oil (10-100µg/mL) of endothelium-denuded (E-) preparations is also presented. Aortic rings were precontracted with NA (10^{-6}M), and the change in tension is expressed as a percentage of the active tension generated by NA. Results are presented as mean ± S.E.M. for 4 to 5 experiments. * *P*<0.05; L-NNA, E- vs. E+; ANOVA followed by Bonferoni test.

Effects of endothelial mediators on Nigella sativa oil-induced relaxation

To investigate the possible involvement of nitric oxide (NO) in the vaso-relaxing effects of Nigella oil, endothelium intact aortic rings were pre-incubated with the NO synthase inhibitor N-nitro-L-arginine (NNA, 0.1 mM) for 30 min. Cumulative concentrations of Nigella oil (10-100µg/mL) were then applied during the tonic contraction phase induced by NA (10^{-6}M).

In other experiments, endothelium-denuded aortic rings were used, and the same concentration of Nigella oil (10-100µg/mL) was added during the tonic contraction phase induced by NA (10^{-6}M).

Endothelium removal was confirmed by the absence of relaxation induced by acetylcholine (10^{-6}M) in aortic rings pre-contracted with NA (10^{-6}M).

RESULTS

Effect of N. sativa oil in endothelium-intact aortic rings

In endothelium-intact preparations, both the maximal response (E_{max}) and sensitivity (pD_2) of cumulative concentration-effect curves contracted with NA (at concentrations of 10^{-9}M to10^{-5}M) or KCl (at concentrations of 10mM to100mM) were depressed by Nigella oil (75µg/mL) (fig. 1A and B) (table 1). In addition, the vaso-relaxing effect of Nigella oil (at concentrations of 10-100µg/mL) on pre-contracted aortic rings with NA (10^{-7}M) or KCl (100mM) was similar, showing no significant difference in either *Emax* or pD_2 (fig. 1C).

The vasorelaxing effect of Nigella oil at a lower concentration (50µg/mL) was endothelium dependent (data not shown).

Effects of N. sativa oil on extracellular Ca^{2+}-induced contraction activated by KCl

In Ca^{2+}-free solution, aortic rings pre-contracted with KCl (100mM), cumulative addition of $CaCl_2$ (0.25, 0.5, 0.75, 1, 1.25 and 1.5mM) induced a stepwise tension increment (of aortic rings) (fig. 2). The E_{max} attained at the highest concentration of Ca^{2+} (1.5mM) was 2.04±0.22g and the pD_2 was 0.85±0.15. However, when the aortic rings were treated with Nigella oil (75µg/mL), $CaCl_2$-induced contractions were attenuated, suggesting that Ca^{2+} influx was reduced by the oil; E_{max} value attained at 1.5mM Ca^{2+} was 1.25±0.19 g (P<0.05 Nigella oil versus no oil) and pD_2 was 0.37±0.15 (P<0.05, Nigella oil versus no oil) (fig. 2).

Effects of endothelial mediators on Nigella sativa oil-induced relaxation

To investigate whether Nigella oil-induced relaxation was endothelium dependent or independent, experiments were performed in both endothelium-intact and denuded aortic rings pre-contracted with NA (10^{-6}M). The concentration-response curves for cumulative addition of Nigella oil (concentrations of 10-100µg/mL) treatment showed no difference in the maximal response (fig. 3).

To investigate the role of NO in the relaxation caused by Nigella oil treatment (10-100µg/mL) in endothelium-intact aortic rings pre-contracted with NA (10^{-6}M) were performed before and after treatment with L-NNA (0.1mM). The results indicate that the treatment with this nitric oxide (NO)-synthase inhibitor did not affect the maximum relaxation effect (fig. 3 and table 2); the results were very similar to those obtained with endothelium-denuded preparations. Similar results were obtained when the aortic rings were pre-contracted by KCl (100mM) (data not shown).

DISCUSSION

The present study demonstrated that Nigella oil produced a concentration-dependent inhibition of contraction of the aortic rings induced by either NA (10^{-6}M) or KCl (100mM). To determine if the endothelium participates in the vaso-relaxant effect, endothelium-denuded and endothelium-intact aortic rings were used in the experiments, which showed that the inhibitory effect of Nigella oil on NA-induced contraction was not attenuated by the removal of endothelium. Similarly, the role of the vasodilator NO was also investigated by pre-treatment of the aortic rings with L-NNA [a competitive inhibitor of nitric oxide synthase (NOS) with selectivity for the neuronal and endothelial isoforms of the enzyme; Miilsch and Rudi Busse, 1990]; L-NNA had no effect on the vaso-relaxant effect of Nigella oil, suggesting that the effect did not involve the NO-guanylyl cyclase pathway. Thus, the vasorelaxant effect of Nigella oil was independent of endothelium and was not mediated by the release of NO.

In the present study, pre-incubation of the rings with Nigella oil effectively antagonized, Ca^{2+} (concentration-dependent)-induced contractions. Since, smooth muscles contract in response to activation of voltage-dependent and receptor-operated Ca^{2+} channels (Horowitz *et al*., 1996, Karaki*et al*., 1997) and activator Ca^{2+} is released from intracellular stores upon stimulation (Horowitz *et al*., 1996, Savineau and Martahn, 2000), results of the present study suggest that Nigella oil reduced Ca^{2+} influx through voltage-operated Ca^{2+}channel in the isolated aortic smooth muscle, and that the oil may interfere with both voltage and receptor-operated Ca^{2+} channels, thus reducing the Ca^{2+} influx and consequently contraction. These results are somewhat similar to those obtained by previous investigators (Aqel, 1993, Reiter and Brandt, 1985), who used the volatile oil of *N. sativa* seeds but different smooth muscles.

It is likely that the phenolic compounds present in Nigella oil (65% of total oil; see earlier) may act as calcium antagonists (dihydropyridine-type), consistent with reports suggesting that phenolic compounds may act as calcium antagonists (Nishijima *et al*., 1999).

The relaxation of aortic muscle by Nigella oil may be a mechanism by which Nigella oil lowered both systolic and diastolic pressures in healthy volunteers (Fallah Huseini *et al*., 2013) as well as in patients with mild hypertension (Qidwai *et al*., 2009) or the metabolic syndrome (Najmi *et al*., 2013).

Some antihypertensive drugs (such as hydralazine or sodium nitroprusside) act by stimulating the endothelium to release a potent vasorelaxing factor (endothelium-derived relaxing factor; EDRF), now recognized as NO, that modulates the vascular response. This stimulation of NO production by activation of endothelial nitric oxide synthase (eNOS) is mediated by an increase of cGMP content in vascular smooth muscle cells as a result of activation of soluble guanylyl cyclase (Andreopoulos and Papapetropoulos, 2000), and is inhibited by inhibitors of eNOS, such as NNA (Miilsch and Rudi Busse, 1990). Other antihypertensive drugs, such as dihydropyridine calcium channel blockers (for example, amlodipine, felodipine, nifedipine and nitrendipine) act by blocking Ca^{2+} L-channels and the release of Ca^{2+} (which causes vasoconstriction).

In summary, results presented here suggest that the vasorelaxant effect of the volatile oil of *Nigella sativa* seeds is endothelium independent and does not involve NO, but is as a result of inhibition of activation of voltage-and receptor operated Ca^{2+} L-channels. The results may have therapeutic significance, in that Nigella oil or its constituent(s) may be useful as a vasorelaxant and anti-hypertensive agent in humans.

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