**ABSTRACT**

**Background:** It is well known that *Nigella sativa* seeds have been widely used in folk medicine for the treatment of cardiovascular diseases. Little is known, however, about their effect on angiotensin II receptor type I. Studying of such impact will be valuable in producing herbal medicines with much less side effects compared to conventional drugs.

**Objective:** The aim of the current research was to study the blocking effect of hydro-methanolic (NS.HM) and aqueous (NS.Aq) extracts of *Nigella sativa* on angiotensin II (Ang II) receptor type I (AT1) in isolated rat’s aorta.

**Materials and Methods:** Seed's powder was soaked in 50% hydromethanol and distilled water separately for 48 hrs, then filtered through Whatman filter papers. The solvents were evaporated to yield the crude extracts (NS.HM and NS.Aq). The effect of different concentrations (1, 2, 3 & 4 mg/ml) of NS.HM and NS.Aq extracts on isolated rat’s aorta contracted with various doses of Ang II (0.3, 1.0, 3.0, 10, 30 & 100 µM) were evaluated.

**Results:** NS.HM at concentrations 3 and 4 mg/ml, caused a very high significant (*P* < 0.001) inhibitory effect on the dose-response curves (DRCs) in aortic rings at doses 3 and 10 µM of Ang II as compared to the control, and a highly significant (*P* < 0.01) inhibition at doses one µM (for 3 mg/ml), and 1 and 30 µM (for 4 mg/ml). Furthermore, NS.HM at concentrations 1 and 2 mg/ml did not produce any significant right shifting. On the other hand, NS.Aq extract at concentration 4 mg/ml caused a very high significant (*P* < 0.001) right shifting DRC at doses 3 and 10 µM, and highly significant (*P* < 0.01) shifting at 30 µM of Ang II. Besides, significant right shifting (*P* < 0.05) was observed in the DRC in the presence of the extract at dose one µM as compared to the control. Nevertheless, no right shifting in the DRC of Ang II at concentrations 1, 2, and 3 mg/ml of NS.Aq was noticed.

**Conclusions:** We conclude that Both NS.HM and NS.Aq extracts have an anti-hypertensive effect through blocking the AT1 receptors, although NS.HM extract is more potent in blocking effect on AT1R than NS.Aq. In addition, the anti-hypertensive effect of both NS.HM and NS.Aq extracts on the aorta are concentration-dependent.

**Duhok Med J 2020; 14 (2): 73-85**

**Keywords:** Angiotensin II Type 1 Receptor, Anti-Hypertensive, Aqueous Extract, Hydromethanol, *Nigella Sativa.*
Angiotensin receptors have been found in many body organs and systems like the heart, kidneys, pituitary gland, placenta, peripheral vessels, and the central nervous system. In the cardiovascular system, when these receptors (particularly AT1R) are activated by angiotensin II (Ang. II), they lead to vasoconstriction and thence elevation of blood pressure. Therefore, AT1R is known to have an important role in the treatment of cardiovascular disorders. It was shown that blocking of such receptors (e.g., by candesartan, irbesartan, valsartan….etc.) can greatly decrease hypertension and improve the prognosis of cardiovascular diseases such as heart failure. In addition to vasoconstriction and hypertension, AT1R has many other actions like aldosterone synthesis and secretion, increase vasopressin secretion and cardiac hypertrophy.

It was demonstrated that N. sativa causes a pressure-lowering effect by different mechanisms such as calcium channel blockade, inhibition of vasomotor center in the medulla, activations of inositol triphosphate (IP3), ATP-sensitive K+ channel, and Ca2+ activated K+ channel. Furthermore, N. sativa causes a potent inhibition in the contractility and heart rate in isolated hearts of guinea pigs. The later effects may be due to Ca2+ channel blocking or opening of K+ channel of the isolated heart. Despite the aforementioned still, no data are available to date about N. sativa extract’s effect on AT1R. Therefore, the current study was designed to investigate the blocking effect of hydro-methanol (NS.HM) & aqueous (NS.Aq) extracts of N. sativa on AT1R in the isolated rat's aortic rings.

**MATERIALS AND METHODS**

The current study was conducted at the Department of Medical Physiology and Pharmacology, College of Medicine, University of Duhok and Department of Biology, College of Science, University of Zakho, Kurdistan Region–Iraq, from September 2017 to December 2018.

**Experimental Animals**

A total of 40 adult male albino rats (Rattus norvegicus) weighing 200–350g were used in the present study. The animals were bred in PVC cages (46×30×20cm) on wooden chips maintained in the animal house (Department of Biology, College of Science, University of Zakho). Before starting the experiments, 4-6 rats were kept in a cage under standard laboratory conditions of 22 ± 2 oC with free access to dechlorinated water and libitum and a photoperiod of 12L/12D cycle. The animals were fed on standard rat’s pellets obtained from the silage factory in Zakho.

**Chemicals**

All chemicals used in the current study were of analytical grade. Kreb’s physiological solution (composition in mM: NaCl 118, KCl 4.7, Glucose, NaHCO3 25, MgSO4 1.2, KH2PO4 1.2, CaCl2 2.4, EDTA 0.03) was used as a solvent for all drugs. Angiotensin II was purchased from Santa Cruz Biotechnology, USA. n-Hexane 96% and Methanol 99.98% were procured from Scharlau (Spain). Finally, the carbogen (O2 = 95%, CO2 = 5%) was obtained from the Factory of Gas Production – Kirkuk, Iraq.

**Preparation of Nigella Sativa Seed Extract:** Nigella sativa seeds were purchased from a local grocery store in Duhok city and were kindly authenticated by taxonomists (Forestry Department, College of...
Agriculture, University of Duhok). The seeds were ground into a fine powder using an electrical grinder. The powder was first defatted using pure hexane (96%) by maceration method, in which 1000 gm of *N. sativa* powder was soaked in three liters hexane for 48 hours at room temperature with occasional shaking. Afterward, it is filtered through Whatman filter papers (to yield a yellow filtrate). This process was repeated 10 times until a colorless filtrate was obtained. The same procedure was repeated for hydromethanol (light brown) and aqueous (light yellow) filtrates. The filtrates of each fraction were collected alone and then concentrated by evaporation under reduced pressure using a thin layer rotary evaporator (BUCHI, Switzerland) at a temperature of 40 °C (to obtain 145 g coded as NS.Hx extract, 63 g as NS.HM, and 69 g as NS.Aq respectively). The extracts were transferred into plane tubes and stored at –20 °C until use.

Preparation of Isolated Aorta

Rats were anesthetized after inhalation of pure diethyl ether. After thoracotomy, the aorta was removed out and immersed in Kreb's solution aerated with Carbogen (95% O2 and 5% CO2). After removing the excess tissue, the aorta was cut into small rings and mounted in an organ bath chamber containing 10 ml Kreb’s solution, which was continuously aerated with carbogen at 37 °C and pH of 7.4. After setting, the rings were allowed to equilibrate with 2g for at least one hour. Prior to the experiment, the rings were exposed to Potassium chloride (KCl) (60mM) or phenylephrine (PE) (1µM) to verify the functional integrity.

**Experimental Protocol**

To evaluate the effect of NS.HM and NS.Aq (1, 2, 3 and 4mg/ml) on angiotensin II receptors type I (AT1R) on isolated rat's aortic rings, the aortic rings were washed and equilibrated. The contraction was induced by cumulative doses (0.3, 1.0, 3.0, 10, 30 & 100 µM) of angiotensin II alone as a control. After washing and re-equilibration, the aortic rings were pre-exposed to 1, 2, 3, and 4 mg/ml NS.HM separately for 5 minutes, followed by cumulative doses of angiotensin II at 10 minutes intervals between doses. The same protocol was applied for NS.Aq extract.

**STATISTICAL ANALYSIS**

All data were translated into a computerized database structure and expressed as mean ± standard error of the mean (SEM). For multiple comparisons among the data (comparing each cell mean of one group with the cell mean of the other group in the same row), a two-way ANOVA test was used to detect the statistical significance, which was supported by Sidak's multiple comparisons test using Graph Pad Prizm program (version 6). *P*-value of less than 0.05 (*P* < 0.05) was considered statistically significant.

* ≤ 0.05 (Significant), ** ≤ 0.01 (high significant) and *** ≤ 0.001 (very high significant).

**RESULTS**

Effect of Pre-incubation with Different Doses of NS.HM on Angiotensin II Induced Contraction on Isolated Rat's Aorta.
Typical traces representing the experiments for the control and the blocking effect of NS.HM extract (3 mg/ml) on aortic rings precontracted with angiotensin II are shown in (figure 1). Cumulative dose-response curves (DRCs) of Ang. II in the absence (control) and presence of NS.HM extract (1, 2, 3, and 4 mg/ml separately) in aortic rings are shown in (figures 2 and 3). NS.HM extract at concentrations 1, and 2 mg/ml did not cause any significant inhibitory effect on Ang. II-induced contraction at all Ang. II doses were used as compared to the control. In contrast, NS.HM extract at concentrations 3, and 4mg/ml produced a highly significant inhibition of contraction (P<0.01) at a dose 1 µM Ang. II (for 3 and 4 mg/ml of ext.) and 30 µM (for 4 mg/ml ext.).

At the same time, a very highly significant (P<0.001) inhibition of contraction in aortic smooth muscle was observed at concentrations 3 and 4mg/ml NS.HM at doses 3 & 10 µM Ang. II, as compared with the control. The Log EC50 (Log EC50 of CI 95%) and the Emax for the cumulative effect of Ang. II in the absence and presence of NS.HM extract are shown in table 1. Comparing with the control, the dose-response curves of Ang. II at concentrations 3 & 4 mg/ml NS.HM were shifted to the right, with a Log EC50 of -4.895 ± 0.104, (Log EC50 of CI 95% between -5.114 to -4.676) and a Log EC50 of -4.827 ± 0.096, (Log EC50 of CI 95% between -5.028 to -4.626) respectively. In addition, the Log EC50 was -5.744 ± 0.164, (Log EC50 of CI 95% between -6.087 to -5.400) in the absence of the extract.

Moreover, the Emax of NS.HM 4 mg/ml was declined from 29.14 to 25.82%. Whereas, that's of 3 mg/ml has returned back & become 31.34 %. However, the Emax of NS.HM 2 mg/ml has been declined from 11.6 to 8.62%, a Log EC50 of -5.046 ± 0.223, (Log EC50 of CI 95% between -5.513 to -4.579). The Log EC50 of 1mg/ml NS.HM was -5.215 ± 0.167, (Log EC50 of CI 95% between -5.564 to -4.866) & Emax of 11.79%, in comparison to the control that has a Log EC50 of -4.967 ± 0.258, (Log EC50 of CI 95% between -5.505 to -4.428).

Figure 1: LabChart traces showing a dose-dependent contraction of Ang II on isolated rat's aorta in the absence (control) and presence of (3) mg/ml (NS.HM).
Figure 2: Cumulative dose-response curves of Ang. II in the absence (control) and presence of NS.HM extract (1 and 2 mg/ml) in rat's aortic rings.

Figure 3: Cumulative dose-response curves of Ang. II in the absence (control) and presence of NS.HM extract (3 and 4 mg/ml) in rat's aortic rings

Table 1: Log EC50, (Log EC50 of CI 95%) and Emax for the effect of pre-exposure of rat's aorta to NS.HM extract prior to contraction by Ang. II

<table>
<thead>
<tr>
<th>Angiotensin II</th>
<th>Log EC50 ± SEM</th>
<th>LogEC50 of CI 95%</th>
<th>Emax (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-4.967 ± 0.258</td>
<td>-5.505 to -4.428</td>
<td>11.6</td>
</tr>
<tr>
<td>NS.HM 1 mg/ml</td>
<td>-5.215 ± 0.167</td>
<td>-5.564 to -4.866</td>
<td>11.79</td>
</tr>
<tr>
<td>NS.HM 2 mg/ml</td>
<td>-5.046 ± 0.223</td>
<td>-5.513 to -4.579</td>
<td>8.62</td>
</tr>
<tr>
<td>Control</td>
<td>-5.744 ± 0.164</td>
<td>-6.087 to -5.400</td>
<td>29.14</td>
</tr>
<tr>
<td>NS.HM 3 mg/ml</td>
<td>-4.895 ± 0.104</td>
<td>-5.114 to -4.676</td>
<td>31.34</td>
</tr>
<tr>
<td>NS.HM 4 mg/ml</td>
<td>-4.827 ± 0.096</td>
<td>-5.028 to -4.626</td>
<td>25.82</td>
</tr>
</tbody>
</table>
Effect of Pre-incubation with Different Doses of NS.Aq on Angiotensin II Induced Contraction on Isolated Rat’s Aorta

Typical chart traces and cumulative DRCs of Ang II in the absence and presence of NS.Aq extract are shown in figures (4 and 5). The NS.Aq extract at concentrations 1, 2 & 3 mg/ml, did not cause any significant inhibitory effect on the Ang II induced contraction in isolated rat’s aorta. In contrast, the concentration of 4 mg/ml NS.Aq has caused a very high significant (P< 0.001) inhibition in Ang II induced contraction at doses 3 and 10 µM of Ang II, and high significant inhibition (P< 0.01) at a dose 30 µM. In other words, the DRC of Ang II in the presence of 4 mg/ml NS.Aq has shifted to the right. Consequently, the percentage of contraction significantly reduced from (40.69%) in control to (35.68%) in the aorta pre-incubated with 4 mg/ml NS.Aq extract. The Log EC50 was -5.196 ± 0.097M (CI 95% from -5.393 to -4.999) comparing to the Log EC50 of the control which was -5.690 ± 0.071M (CI 95% from -5.835 to -5.545), table 2.

Figure 4: LabChart traces showing dose-dependent contraction of Ang II on isolated rat’s aorta in absence (control) and presence of (4) mg/ml (NS.Aq).

Figure 5: Cumulative dose-response curves of Ang II in absence (control) and presence of NS. Aq extract (1, 2, 3 & 4 mg/ml) in rat’s aortic rings
**DISCUSSION**

Hypertension is a primary risk factor for myocardial infarction (MI), stroke, vascular ailment, and chronic renal disease. It is a serious medical condition with higher intravascular pressure than normal. High blood pressure is, therefore, considered to be a common cause of cardiovascular problems.

The hemodynamic variations seen during hypertension are influenced by different hormonal factors, among which angiotensin II (Ang II) which appears to be a critical one. Ang II receptor type 1 (AT1R) is one of the key sites to which Ang II binds. AT1R promotes many intracellular signaling pathways leading to hypertension, endothelial dysfunction, vascular remodeling, and tissue injury. Different pathways exist for synthesizing Ang II, like Cathepsin G, Chymostatin-sensitive Ang II generating enzyme (CAGE), Chymase, and Angiotensin-converting enzyme (ACE). The inhibitors acting on any of these enzymes (particularly ACE) can only reduce the production of Ang II by about 30–40%.

For this reason, recently, attention was focused on the AT1R blocking. However, the useful impacts of contemporary anti-hypertensive medicines are well reported; nevertheless, the preventive effects of numerous drugs are known to have various side effects. Accordingly, attention now a day is focused on the use of medicinal plants to treat many diseases. One of the most important medicinal plants used in folk medicine is *N. sativa*. It is well known that it exhibits an anti-hypertensive effect, but its exact mechanism is still in debate. Therefore, the current work was carried out for the first time so far to investigate the role of hydromethanolic (NS.HM) and aqueous (NS.Aq) extracts of *N. sativa* in blocking of AT1R in isolated rat's aorta.

The results of the current study demonstrated for the first time that both NS.HM and NS.Aq extracts have significantly shifted Ang II dose-response curve, in Ang II induced contraction in rat's aortic rings, to the right at concentrations 3 and 4mg/ml and 4mg/ml, respectively. Furthermore, various physiological responses to different extracts reflect diverse, active ingredients in each *N. sativa* extract. Therefore, the results of the present work demonstrated that NS.HM (3 and 4mg/ml) has a more potent inhibitory effect than NS.Aq (only 4mg/ml) in Ang II induced contraction on aortic rings. This clearly reflects the presence of different active ingredients in each extract and

<table>
<thead>
<tr>
<th>Angiotensin II</th>
<th>Log EC50 ± SEM</th>
<th>LogEC50 of CI 95%</th>
<th>Emax (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-5.690 ± 0.071</td>
<td>-5.835 to -5.545</td>
<td>40.69</td>
</tr>
<tr>
<td>NS.Aq 1 mg/ml</td>
<td>-5.732 ± 0.089</td>
<td>-5.913 to -5.552</td>
<td>51.05</td>
</tr>
<tr>
<td>NS.Aq 2 mg/ml</td>
<td>-5.557 ± 0.111</td>
<td>-5.783 to -5.332</td>
<td>48.67</td>
</tr>
<tr>
<td>NS.Aq 3 mg/ml</td>
<td>-5.485 ± 0.067</td>
<td>-5.621 to -5.350</td>
<td>43.41</td>
</tr>
<tr>
<td>NS.Aq 4 mg/ml</td>
<td>-5.196 ± 0.097</td>
<td>-5.393 to -4.999</td>
<td>35.68</td>
</tr>
</tbody>
</table>
Nigella Sativa AQUEOUS AND HYDRO-METHANOL EXTRACTS ACT AS A NOVEL BLOCKER

different potencies and different action mechanisms.

Moreover, it was observed that concentrations 1 and 2mg/ml NS.HM were unable to shift the dose-response curve of Ang II to the right. In contrast, highly significant differences were observed in Ang II doses between control and those of 3 and 4mg/ml NS.HM. This indicates that the anti-hypertensive effect of NS.HM is concentration-dependent. Furthermore, NS.Aq also inhibited the Ang II induced contraction in rat aorta in a concentration-dependent manner, but to a lesser extent.

So far, four angiotensin receptors have been described: AT-1, AT-2, AT-4, and Mas receptors (AT1-7)\(^7,20\); among them, AT1R is the most clinically important one, as many drugs competitively block it, thereby reducing blood pressure. Ang II, which is a bioactive peptide, activates both AT1R and AT2R\(^16\). After binding with AT1R, Ang II switches on various intracellular signaling pathways that mediate different physiological responses, including hypertension, atherosclerosis, ventricular hypertrophy, cell proliferation, angiogenesis, matrix synthesis, aldosterone synthesis, and discharge\(^21\). In other words, Ang II induces contraction in smooth muscle cells occurs through Gq/11–PLC–\( \beta \)–PIP2–IP3–PKC\(^22\) and/or G12/13–GEF–ATP–Rho–ROCK–MLC phosphatase\(^23,24\).

The results of the current study revealed that NS.HM extract produced a partial but the more potent effect on Ang II induced contraction than NS.Aq extract. The partial vasorelaxant effect of NS.HM and NS.Aq extracts reflect the differences in the active ingredients present in both extracts. This may be due to the inhibitory effect of their active ingredients on one or more enzymes in the signal transduction pathway. As far as we are aware, at least at the moment, no data concerning the effect of NS.HM and NS.Aq on aortic smooth muscles contracted by Ang II are available. However, a study carried out on the aorta contracted by PMA (a PKC activator) showed that in Ca\(^{2+}\) free solution, PMA activates PKC and induced a slowly developing sustained contraction without changing the [Ca\(^{2+}\)]\(_i\). However, it has been concluded that the flavonoids (pentamethyl quercetin, luteolin, kaempferol, and apigenin) competitively bind ATP binding sites and significantly inhibited the PKC\(^25\). Furthermore, studies on ventricular cardiomyocytes demonstrated that the monoterpene thymol caused a cardio-depression in guinea pigs through inhibition of SERCA, which in turn reduced Ca\(^{2+}\) level in the sarcoplasmic reticulum\(^10,26\), in which the effect was also described for skeletal muscle fibers\(^27\). On the other hand, it has been reported that the flavonoid quercetin has a role in the inhibition of the formation of the calcium-calmodulin complex\(^28\). However, in other observations, the vasodilation effect of quercetin by this mechanism has been rolled out\(^25\). Another expected mechanism is calcium channel blockade. The patch-clamp technique in the whole-cell configuration showed that the monoterpene carvacrol was able to inhibit the calcium ion current through the L-type Ca\(^{2+}\) channel in cardiomyocytes isolated from canine and human ventricles\(^29\). A similar study reported that a phenolic compound in N. sativa oil might act via blockade of Ca\(^{2+}\) channel\(^30\).

From the results of the current study, it was illustrated that NS.HM and NS.Aq extracts
both are able to cause partial blockage in AT1 receptors. Besides, it is concluded that NS.HM has a more potent blocking effect on AT1R than NS.Aq, and the relaxant effect of both NS.HM and NS.Aq extracts on the aorta are concentration-dependent. However, further work is recommended to document the exact pathway and the exact enzyme involved in the blocking effect of the NS.HM and NS.Aq extracts. For future study, NS.HM and NS.Aq extracts may be considered as a substrate for manufacturing drugs for the prevention or even treatment of hypertension.

REFERENCES


Nigella Sativa AQUEOUS AND HYDRO-METHANOL EXTRACTS ACT AS A NOVEL BLOCKER

Research International. 2014; Article ID 247054, 7 pages.


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Pohtah

Nigella Sativa AQUEOUS AND HYDRO-METHANOL EXTRACTS ACT AS A NOVEL BLOCKER FOR ANGIOTENSIN II RECEPTOR TYPE I

Shiwaz va Nahiwax

Pohtah "Nigella Sativa" AQUEOUS AND HYDRO-METHANOL EXTRACTS ACT AS A NOVEL BLOCKER FOR ANGIOTENSIN II RECEPTOR TYPE I

Derenjagam

Nigella Sativa AQUEOUS AND HYDRO-METHANOL EXTRACTS ACT AS A NOVEL BLOCKER FOR ANGIOTENSIN II RECEPTOR TYPE I
الخلاصة

المستخلصات المائية والهيدروميثانولية للحبة السوداء التي تعمل كمناهج جد لمستقبلات نوع I Angiotensin II

خلفية البحث

كان الهدف من البحث الحالي هو دراسة تأثير حجب المستخلصات الهيدروميثانولية (NS.HM) والمستخلصات المائية (NS.Aq) للحبة السوداء على مستقبلات الأنجيوتنسين II (AT1) في الفئران المعزولة الأبهر.

الممرضى وطرق البحث

تم تغذية البذور في 50% من الهيدروميثانول والماء المقطر بشكل منفصل لمدة 48 ساعة، ثم تصفيتها عن طريق أوراق الترشيح Whatman. تم تبخير المذيبات للحصول على المستخلصات الخلية (NS.HM و NS.Aq) على الشريان الأورطي المعزول للجرذان بجرعات مختلفة من الأنجيوتنسين II (0.3، 1، 3 و 10، 30 و 100 µM).

النتائج

تسبب NS.HM بتركيزات 3 و 4 ملغ/مل في إحداث تأثير مثبط كبير جدًا (P≤0.001) على منحنى الاستجابة للجرعة في حالات الشريان الأبهر عند الجرعات 3 و 10 مايكرومول من Ang II بالمقارنة مع السيطرة، وثانيها الكبير (P≤0.01) في جرعات 1 مايكرومول (ل 3 ملغ/مل من المستخلص)، و 1 و 3 مايكرومول (ل 4 ملغ/مل من المستخلص). علاوة على ذلك، أن مستخلصات NS.Aq مع تركيزات 1 و 2 ملغ/مل أي نقاوة معينة إلى البيتين. من ناحية أخرى، تسبب مستخلص NS.HM في تحول معنوي كبير (P≤0.001) في منحنى الاستجابة لجرعة البيتين عند الجرعات 3 من NS.Aq. بالإضافة إلى ذلك، لوحظ تحول معنوي من NS.Aq عند التركيزات 1 و 2 ملغ/مل من Ang II عند الجرعات 3 و 4 مايكرومول و 10 مايكرومول، وتحول كبير (P≤0.01) عند 30 مايكرومول من أنجيوتنسين II. ومع ذلك، لم يلاحظ تأثير معنوي على منحنى الاستجابة لجرعات من NS.Aq عند التركيزات 1 و 2 ملغ/مل من Ang II.

الاستنتاجات

نستنتج أن كل من مستخلصات NS.HM و NS.Aq لها تأثير مضاد لارتفاع ضغط الدم من خلال حجب مستقبلات AT1، وNS.HM تفاعلت هذه الفعالية في تركيزات أعلى من NS.Aq، بالإضافة إلى ذلك، فإن الاستنتاجات تشير إلى أن مستخلصات NS.HM المضادة لارتفاع ضغط الدم لكل من مستخلصات NS.Aq و NS.HM يمكن أن تكون منعولاً على التركيزات العالية من مستخلصات NS.HM.