Effect of *Nigella sativa* seeds extracts on iNOS through antioxidant potential only: Crude/total extract as molecular therapy drug

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There is general belief that only pure phytomolecules may be used as molecular therapeutic agent through one to one action. However, the traditional systems of medicine e.g. Ayurveda, uses the crude extracts, mostly water decoctions and oils, as drug. A comparative study of hexane, ethyl acetate and methanol fractions of *N. Sativa* seeds has been carried out on fresh rat-peritoneal-macrophage culture with reference to their role on various targets of lipopolysaccharide induced release of nitric oxide (NO) and inducible nitric oxide synthase (iNOS) expression. The results indicated significant antioxidant potential with methanolic extract as most effective. Its mechanism of action was proposed primarily through its antioxidant potential and not through direct inhibition of other kinases, involved in its signaling cascade.

**Keywords:** Antioxidant, Anti-inflammatory, Food supplements, *Nigella sativa*

The *Nigella sativa* (NS) seeds are associated with several therapeutic potentials such as radio-protective, hypo-lipidemic, anti-atherosclerosis, anti-obesity, anti-diabetic, anti-arthritis, etc. *Nigella sativa* Linn, Ranunculaceae, (black cumene seeds) grows in the Mediterranean region and in Western Asian countries including India, as herb of 20-30 cm height. It is a common ingredient of Indian kitchen spices. Chemically, the seeds are rich in essential fatty acids and fixed oil as saponins. Thimoquinine (TQ), is one of its bioactive compound, which has been derived from its oil.

The Indian systems of medicine usually use the oil/water decoction of plants as medicine for treatment of various ailments. Although these preparations are clinically effective, their mechanism of action is not known. Since there is a general belief that only pure isolated phytomolecules could be used for exploring the mechanism of action of any herbal preparations, study related to the mechanism of action of the crude preparations remains unattended.

Due to lack of information about these preparations, the physicians of allopathic system of medicine are always hesitant to use these preparations. Several earlier reports have advocated the use of total/crude extract as more effective than the single pure phytochemical. They mention that one of its major components drives the whole biological response and other components act synergistically with this compound. These preparations are, in true sense, behave as a cocktail of several secondary metabolites, with definite therapeutic roles. Many a times, the total extracts have been reported to be more effective than one pure isolated compound. These total extracts are believed to have multi-targeted action with lower side effect and better bioavailability. Thus it is hypothesized that total extract may behave as a single molecule and act through a particular signaling pathway.

To answer this question, effect of 3 different fractions of *Nigella* seeds have been been studied on lipopolysaccharide (LPS) induced inducible nitric oxide synthase (iNOS) expression in macrophage culture. In order to understand the mechanism of action, an experiment has been designed where specific kinase inhibitors have been used to dissect out the specific role of the extracts on various kinases, which are involved in the signaling cascade of LPS induced iNOS expression.

Although, this is a preliminary study, it has provided a definite clue that the total/crude herbal extracts may behave as a single drug at molecular level. The extracts of *Nigella sativa* (NS) seeds were prepared with different solvent systems e.g. hexane,
ethyl acetate and methanol. The solvent free extracts were characterized and then tested on macrophage culture. LPS was used as agonist to induce iNOS. The quercetin, an antioxidant; genistein as tyrosine kinase (PTK) inhibitor; H-7 as protein kinase C (PKC) inhibitor and L-NAME (N\textsuperscript{G}-nitro-l-arginine methyl ester) a nonselective NOS-inhibitor, were used as reference compounds for comparison.

Materials and Methods

Materials—2,2’-azinobis-3-ethyl benzothiazoline-6-sulfonic acid (ABTS\textsuperscript{**}), lipopolysaccharide (LPS), genistein and isoquinolinesulfonamides (H-7) were purchased from Sigma Chemical Co, St Louis, USA. Deoxyribose, nitroblue tetrazolium (NBT), riboflavin, L-methionine thiobarbituric acid, ethylenediamine tetra acetic acid (EDTA), methylene blue, RPMI-1640 and fetal calf serum (FCS) were purchased from HiMedia, Mumbai, India. Ferric chloride anhydrous (FeCl\textsubscript{3}.) ascorbic acid, trichloro-acetic acid, potassium per-sulfate and Vitamin C were purchased from Merck Ltd. Other chemicals were of analytical grade.

Isolation of different fractions of Nigella seeds — Seeds of NS were purchased and their authenticity was verified on pharmacognostical parameters by Prof. K.N. Dwivedi, Department of Dravyaguna, IMS, BHU. The dried seeds were crushed and sequentially extracted with hexane (NSH-hexane extract of NS), ethyl acetate (NSEA-ethyl acetate extract of NS) and methanol (NSME-methanol extract of NS) by continuous Soxhlet extraction method. The extracts were distilled under reduced pressure of NS) and methanol (NSME-methanol extract of NS), ethyl acetate (NSEA-ethyl acetate extract of NS), and desiccated until constant weight was attained, to determine their % yield. Further, thin layer chromatography (TLC) finger print was carried out (Table 1). The voucher specimen of this sample was preserved wide reference no YBT/MC/14/1-2007.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Different fraction of NS seeds</th>
<th>Yield (%)</th>
<th>Solvent systems</th>
<th>No. of spots</th>
<th>R\textsubscript{f} value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hexane</td>
<td>11</td>
<td>B : E.A.+ A.A.</td>
<td>6</td>
<td>0.20, 0.47, 0.53, 0.65, 0.77, 0.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(9.5:0.5+6drops)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Ethyl acetate</td>
<td>5</td>
<td>B : E.A.+ A.A.</td>
<td>4</td>
<td>0.4, 0.74, 0.76, 0.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(9 : 1+3drops)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Methanol</td>
<td>15</td>
<td>B : E.A.</td>
<td>3</td>
<td>0.32, 0.69, 0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1:1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B= benzene; E.A.= ethyl acetate; A.A.= acetic acid

Assessment of antioxidant property in chemical system—For total antioxidant potential, pre-generated 2,2’-azinobis-3-ethyl benzothiazoline-6-sulfonic acid (ABTS\textsuperscript{**}) radicals were mixed with different concentrations of various extracts and change in absorbance was recorded at 734 nm\textsuperscript{9}. The activity of extracts against various species of free radicals was also assessed. For superoxide radical scavenging property, the degree of inhibition of formazone formation, through photochemical reduction of nitroblue tetrazolium (NBT) was assessed\textsuperscript{10,11}. For hydroxyl radical scavenging assay, deoxyribose was used as substrate and the hydroxylated product was monitored at 532nm\textsuperscript{12,13}. Since all free radicals finally induce lipid peroxidation, the protective effect of these extracts was tested against FeSO\textsubscript{4} (70 mM) induced lipid peroxidation, in terms of thiobarbituric acid-reactive species (TBARS) at 535 nm\textsuperscript{14,12}. The egg yolk homogenate was used as substrate and 1,1,3,3-tetraethoxypropane (TEP) was used as standard. The results were compared in terms of their EC\textsubscript{50}.

Effect of Nigella seeds extract pretreatment, on LPS induced nitric oxide (NO) production—The protocol was approved by animal ethical committee of Institute of Medical Sciences, Banaras Hindu University. The normal rats, purchased from central facility of the Institute, were randomly selected for isolation of peritoneal macrophages. About 10 mL sterile ice-cold phosphate buffer saline (PBS; devoid of Calcium and Magnesium ions) was injected to the peritoneal cavity of anaesthetized rats. The abdomen was squeezed for 5 min and then peritoneal fluid was aspirated out, centrifuged and cell-pellets were saved. It was washed 2 times with serum free RPMI-1640 media and suspended in a known volume of complete RPMI-1640 media, supplemented with 5% fetal calf serum (FCS). The viable cells were counted in a hemocytometer by trypan blue exclusion method\textsuperscript{15}. The cells (1 \times 10\textsuperscript{5} cells/well) were taken in a 96 well culture plates and incubated for 2 h at 37 °C in 5% CO\textsubscript{2} atmosphere for their attachment\textsuperscript{16}. Later on, the culture supernatant was replaced with fresh complete media and these attached macrophages were used for various experiments as described in respective tables. Varying concentrations of different fractions (50 µL, dissolved in 10% dimethyl sulfoxide (DMSO) in water) were added to each well and incubated for 30 min. Thereafter, LPS was added with final concentration of 20 ng/mL. For NO estimation and iNOS expression, this culture was further incubated for 17 h.
Effect of seed extracts on post treatment kinetics on LPS induced NO production in macrophages—In order to explore the role of methanolic extract of NS seeds (NSME) on various kinases, involved in the signaling cascade of LPS induced NO production, another experiment was planned. Here, cells were first exposed to LPS and then at different time intervals (2, 4, 6, 8 and 10 min), NSME extract was added to compare its effect on various kinases. The standard kinases inhibitors, described above, had been used in, similar conditions for comparison. The quercetin was used as general antioxidant, genistein as tyrosine kinase inhibitor, and L-NAME as general NOS inhibitor. After 17 h of incubation, culture supernatant was saved and released NO was estimated as accumulated nitrite. Further, the attached cells were saved and subjected to lysis and electrophoresis for determination of degree of iNOS expression, by using western blot analysis.

Estimation of released nitric oxide (NO)—Culture supernatant (50 μl) was incubated with equal volume of Gries reagent (1% sulfanilamide/0.1% naphthalene diamine dihydrochloride 2.5% H₃PO₄) at room temperature for 10 min. Thereafter absorbance was read at 550 nm in an ELISA plate reader (Multiscan).

Western blot analysis for assessment of iNOS expression—The attached macrophages were washed with PBS and then lysed by adding 200 μl lysis buffer (20 mM Tris-Buffer (pH = 7.4), containing 0.25 sucrose, EDTA (1 mM), PMSF (100 μg mL⁻¹), aprotinin (10 μg mL⁻¹), leupeptin (10 μg mL⁻¹). The protein of this cell lysate was estimated by Bradford method and its 20 μg protein was run in each lane on 8% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel electrophoresis. The separated protein bands were transferred to nitrocellulose membrane by electro-blotting, washed with Tris-buffered saline (TBS) containing 0.05% (v/v) Tween 20 and blocked with 5% (w/v) dried non-fat milk in TBS for 2 h. Finally, the blot was incubated with rabbit polyclonal anti-iNOS antibody (SC650, Santa Cruz Biotechnology, 1/1000 in TBS-Tween-20 buffer) at 4 °C overnight and visualized by alkaline phosphataseconjugated anti-rabbit IgG (as the secondary antibody. DAB (diaminobenzidine) was used as substrate. The intensity of bands was analyzed by image analyzer-2254. The equal loading of sample in each lane was confirmed by monitoring the expression of β-actin.

Statistical analysis—Data have been expressed as means±SD. Pearson’s correlation analysis (SPSS 7.5 for Windows, SPSS Inc.) was used to test the significance level for relationship between the concentration and values of P<0.05 was considered as significant.

Results

Phytochemical characterization of each fraction—The hexane fraction (NSH) yielded 11 % of total seed taken for shoxhlet extraction. However, the methanol fraction (NSME) yielded 15 % in similar conditions. Total phenolic content of hexane fraction was 1.782, which was lower than the methanolic fraction (NSME), which had 1.897. Similar pattern was recorded in flavones content of these fractions.

Effect of different fractions of N. sativa seed on different antioxidant parameters—All the 3 fractions were tested in varying concentrations, ranging from 1-5 mg/mL and response was concentration dependent (data not shown). Specific EC₅₀ values for each extract related to various antioxidant parameters (ABTS radicals, superoxides and hydroxyl radicals and FeSO₄ induced lipid peroxidation) are given in Table 2. The NSME (methanol fraction) had lowest EC₅₀ value for all free radicals species, suggesting its better efficacy. Further, the reducing power of hexane fraction was 1.206, whereas for methanol extract, it was 1.78.

Effect of different fractions of N. sativa seed extracts on LPS induced NO production—When macrophages were pretreated with different fractions of NS seeds for 30 min and then exposed to fixed concentration of LPS, there was significant inhibition in NO production with all the extracts. The response was concentration dependent and EC₅₀ value for methanol fraction (NSME) was 626±2.68, which was significantly lower than other 2 fractions (Table 3).

<table>
<thead>
<tr>
<th>Antioxidant parameters</th>
<th>SO₅⁰</th>
<th>Methanol</th>
<th>Ethyl acetate</th>
<th>Hexane</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS⁺⁺ radicals</td>
<td>4.8</td>
<td>5.4</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>SO radicals</td>
<td>5.5</td>
<td>5.9</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>OH radicals</td>
<td>4.8</td>
<td>4.9</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>LPO</td>
<td>4.1</td>
<td>4.5</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>Total phenolic content</td>
<td>1.782</td>
<td>1.568</td>
<td>1.897</td>
<td></td>
</tr>
<tr>
<td>Reducing power</td>
<td>1.206</td>
<td>0.987</td>
<td>1.782</td>
<td></td>
</tr>
</tbody>
</table>

SO= Superoxides radicals; OH = Hydroxyl radicals; LPO= lipid peroxidation.
Effect of different fractions of *N. sativa* seeds on post treatment kinetics on NO production—In the post treatment kinetics, all the fractions and standard kinase inhibitors showed the inhibition for NO release, only when added within 2 min of LPS addition. However, after this time point, there was difference as quercetin showed inhibition only up to 2 min, Genistein, up to 4 min, H-7 up to 6 min and L-NAME more than 6 min (up to 10 min). Thus, it could be suggested that all the fractions of NS seeds behaved as antioxidant only, similar to quercetin (Fig. 1).

Effect on iNOS expression—When NSME was added before or within 2 min of LPS addition, there was significant inhibition in expression of iNOS. However, after 2 min of LPS addition, the NSME did not show any inhibition (Fig. 2). The density of each band is shown in Fig. 3. Further, similar result was observed with quercetin, but genistein and H-7, L-NAME showed different pattern for inhibition. The results pertaining to iNOS expression were similar to those observed with NO release, described above. The genistein, a tyrosine kinase inhibitor, showed inhibition only up to 4 min, H-7 up to 6 min and L-NAME showed inhibition up to 10 min (data not shown).

Discussion

LPS induced NO release is associated with free radical (FR) generation, in its early steps of signaling cascade\(^{20}\). Therefore, this model has been chosen to test the anti-inflammatory property of NS seed extracts, as described earlier also\(^{20, 21}\). Lower activity of hexane fraction, towards FR trapping potential, in

Table 3—Effect of different fractions of *Nigella sativa* seed on LPS induced NO production

<table>
<thead>
<tr>
<th>Concentration of NS seed fraction (µg/mL)</th>
<th>NO production (µM of NO produced by 1×10⁵ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hexane</td>
</tr>
<tr>
<td>200</td>
<td>15.14±1.54</td>
</tr>
<tr>
<td>400</td>
<td>13.51±1.03*</td>
</tr>
<tr>
<td>600</td>
<td>12.77±1.96*</td>
</tr>
<tr>
<td>800</td>
<td>10.57±1.25*</td>
</tr>
<tr>
<td>1000</td>
<td>7.14±1.56*</td>
</tr>
<tr>
<td>EC₅₀ Value</td>
<td>879±2.96</td>
</tr>
<tr>
<td>LPS</td>
<td>16.26±2.45</td>
</tr>
<tr>
<td>Normal</td>
<td>2.26±0.17</td>
</tr>
</tbody>
</table>

\(P\) value : * < 0.01 when compared with Experimental control value

Normal group contain only cell without any treatment.

Comparison was made with respect to control

Fig. 1—Post-treatment effect of different fractions of *N. sativa* (NS) seed on LPS induced NO production: [Q-quercetin, H-hexane fraction, B-EA-ethyl acetate fraction, C-methanol extract, H7- [-1-(5-isoquinolinesulphonyl)-2-methylpiperazine], G-Genistein, L-NAME- (Nω-Nitro-L-arginine methyl ester)]

Fig. 2—Post treatment effect of methanol fraction of *N. sativa* (NS) seeds (NSME) on LPS induced iNOS expression [Lane1=NSME+LPS (2min), Lane2= NSME +LPS (4min), Lane3= NSME +LPS (6min), Lane4= Only LPS, Lane5= Normal cells]
comparison to methanol fraction, is in accordance with the higher phenolic content and higher reducing power of methanolic fraction.

The present yield of methanol fraction was also comparatively higher (15 %), as compared to other fractions. Similarly, the potential for inhibition of LPS induced NO production was also highest in methanol fraction (about 40% better than the hexane fraction). Thus, it could be suggested that the methanol fraction would be a better choice for developing anti-inflammatory food supplement from NS seeds.

The traditional systems of Indian medicine and also of other countries e.g. Traditional Chinese system (TCM) use total-crude extracts of various plants (single or poly-herbal) for clinical use. Many reports indicate that total extract is more effective than single compound. It has also been indicated that one compound of the extract may act as the king and other compounds of the extract support its action. This could be either through enhancing its bio-availability or reducing the side effect etc. The total extract is the natural cocktail of several such phytochemicals and many of the acts synergistically. However, the net therapeutic effect of any crude drug remains one. This prompted us to evaluate the mechanism of action of a crude extract in terms of signaling cascade. In fact, plants make secondary metabolites for their survival. All of them may have different potency or different action, depending on their concentration and structure.

The results of post LPS-treatment experiments related to NO production and iNOS expression indicate that NSME has similar kinetics as that of quercetin, an antioxidant and different than genistein a PTK inhibitor, H-7 a PKC inhibitor or L-NAME a NOS inhibitor. This suggests the antioxidant property of NSME, which is rich in thymoquinone (TQ), a known antioxidant from NS seeds. Pretreatment with these agents, inhibit the LPS induced NO production, because their target kinases are below the free radical action in the signaling cascade. However, genistein is an isoflavone and possesses both antioxidant and tyrosine kinase inhibition property. Its polyphenolic nature might be attributing to its antioxidant potential, but its specific structure makes it PTK inhibitor. These compounds have been selected as reference compounds for specific inhibition of various kinases, based on their earlier reports. These compounds were not selected because of their structural similarity with TQ or other compounds, present in the extract of NS seeds. These agents have inhibition for specific targets in the LPS mediated signaling cascade for NO release.

**Conclusion**

Based on the post treatment kinetics, it appears that all the fractions of *Nigella sativa* (NS) seeds behave as the antioxidant. They have no specific inhibitory role for other kinases, as it has been reported in case of genistein, H-7 or L-NAME. This indicates that its mechanism of action behind the inhibition of LPS induced iNOS expression and NO release is indirectly associated to its antioxidant property only.

**Acknowledgement**

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**References**

8. Proceeding of “Approaches towards Evaluation of Medicinal Plants prior to Clinical Trials”, Organized by The Foundation for Medical Research Mumbai At Yashwantrao Chavan Academy of Development Administration (YASHADA), Pune, India 8 November 2006.