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# Identification of Bioactive Compounds Responsible for the Antimicrobial Activity of Nigella Sativa Seed Extracts Using HPLC and Amino Acid Analyser

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**Abstract:** Nigella sativa medicinal species receiving too much attention in many areas of use, mainly due to its rich source of biologically active constituents in its seeds. Future laboratory research on N. sativa seeds is still required to fully appreciate and support their extensive therapeutic potential in the context of a sound compositional basis. Thus, it was aimed to characterize the main nutrients and active molecules of black cumin seeds and to evaluate, based on phytochemical perspective, the ability of their seeds to inhibit the growth of microorganisms. Proximate analysis revealed good amount of crude lipids (35.70%), carbohydrates (33.34%) and crude proteins (23.2%). The screening performed on free amino acids separated on an amino acid analyzer resolved 16 residues, half of which were the essential amino acids, while the other half were the non-essential ones, and Arginine (44.14 mg/100g) and Glutamic acid (35.65 mg/100g) were the most abundant. The four main phenolic and flavonoids were isolated by HPLC and thymoquinone was the major component (32.5 mg/100 g). Unsaturated fatty acids were the major compounds with linoleic acid (58.57 %) and oleic acid (16.55 %) as the major compounds. The MIC and MBC showed very effective inhibition on all organisms tested, with Alloiococcus otitidis and Staphylococcus aureus being the most susceptible species, followed by Escherichia coli and Klebsiella pneumoniae and finally the fungi, Candida species. All the data together indicate that N. sativa seeds are valuable natural source of therapeutic agents that can fight resistant microbial pathogens.

**Keywords:** Nigella Sativa Seed, Bioactive Compounds, Antimicrobial Activity, High-performance liquid chromatography (HPLC), Amino Acid Analyzer, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC)

## Introduction

Increased rates of bacterial infection have become one of the greatest threats in the world today, along with the proliferation of antibiotic resistance. If antibiotics didn't work, the commonplace practices of organ transplants, chemotherapy, and surgery would be significantly riskier. Recently, there has been a turning of the tide towards herbal remedies, as they are typically readily available without a prescription, inexpensive, natural, and do not result in any harmful side effects when compared to manufactured medicines whose side effects can be quite serious [1][2][3]. Such medicinal plants have high amounts of phytochemicals, flavonoids, tannins, phenols, steroids,

alkaloids and terpenoids which make the biological activity of these plants possible. Phytomedicines are prepared from the fruit, flower, seed, root, leaf and bark material and many active compounds have already been identified in them by standard analytical techniques[4].

Nigella sativa is a medicinal herb which is popularly known as the black seeds due to their color and high concentration of phytochemicals, making the Nigella sativa herb important in different areas of medicine. Studies have revealed that the main chemical constituent of the N. sativa seed is thymoquinone, which provides it with the therapeutic action[5][6]. The extracts of the N. sativa seed have a number of chemical constituents including proteins, carbohydrates, vitamins, minerals (Fe and Zn), crude fiber, alkaloids, saponins, steroids, terpenoids, p-cymene, limonene and fatty acids[7]. It is the chemical composition of the N. sativa seed which makes it exhibit the analgesic, appetite stimulation, antidiabetic, antioxidant, anti-inflammatory, radical scavenging and antimicrobial properties.

Despite extensive research, N. sativa seed oil (NSSO) and some extracts still not completely described chemically[8]. The medicinal value of natural oils and extracts needs to be reassessed, as they are now in great need against the resistant strains[9] and records of their composition should be updated and their therapeutic behaviour confirmed. The antimicrobial activity of plant extracts is of critical importance for preserving food, producing pharmaceuticals, and for natural treatment regimes[10]. If biochemical assays detect phytoconstituents and assess antimicrobial activity in natural materials like extracts from N. sativa or N. sativa oil, novel candidates for antibiotics to combat drug-resistant bacteria could be discovered[11]. For this reason, the objectives of the current research were to find out the chemical composition of the extracts and oil of N. sativa, and to correlate this chemical composition with the antimicrobial effect of the extracts on the selected strains of bacteria and compare this with other published data.

## Materials and Methods

### 1. Sample collection

Nigella sativa seeds purchased from the local market of Al-Muthanna Governorate. The sample was milled and kept under dark and dry condition at 4°C without exposure to oxygen.

### 2. Bacterial strain

Bacterial (Staphylococcus aureus, Klebsiella pneumoniae, and Escherichia coli) and fungal (Candida albicans, Candida krusei and Alloiococcus otitidis) isolates were obtained from the Microbiology Laboratory, Department of Biology, Al-Muthanna University.

### 3. Chemical compositions of Nigella sativa seeds

#### 3.1. Proximate Composition

Protein, Fat, Moisture, and Ash contents were determined using AOAC techniques[12], but with some modification. Crude protein was determined by macro-Kjeldahl method. The mass of the powder was then measured and crude fat was extracted from the milled material using a Soxhlet apparatus in which the powder was extracted in petroleum ether. Moisture was obtained by difference and ash by incineration at 400°C of the sample. Total carbohydrate was calculated.

#### 3.2. Amino acid extraction

About 5mg sample (or 100mg of liquid sample) was weighed accurate to 1 mg. Then, 1 mL of 6 M HCl was introduced and the tube was put aside at 55 °C for 3 hours, acting as the hydrolyzing agent. The hydrolysis reaction was followed by the removal of 100 μL of the hydrolysate which was placed into a vial for complete drying under a flow of N2. Dried amino acid residues were reextracted in 100 μL of acetonitrile and OPA (o-phthalaldehyde) was used to derivatise the amino acids again (100 μL). The sealed vial was sonicated for 1 minute, and the vial was left in a thermoblock at 100 ± 2°C for 30 minutes until the derivatisation was complete. The vial prepared as described above was transferred to the gas chromatograph autosampler and ten injections of 100μL were performed per sample following the procedures described in references [13][14].

### 3.3. Extraction of Phenolic Compounds by Ultrasonic

Nigella sativa seed powder (8 g) was extracted with 60/40 ratio of ethanol–water to obtain the phenolic compounds. Extraction was performed in ultrasonic bath at room temperature for 1 hour. A 5 mL aliquot of the liquid extract was used after the filtration to determine the extraction yield. The solvent was then removed under a reduced pressure on a rotary evaporator and the remaining material was dried at 40°C until the weight did not vary. The individual phenolics were quantified using a reversed-phase high-performance liquid chromatography (RP-HPLC) chromatography system (SYKAMN) and UV detector; dried extracts were kept in glass bottles at 4 °C to avoid any possible oxidative degradation before analysis. The separation was carried out with a Zorbax Eclipse Plus (C18-OSD) column of 25 cm × 4.6 mm, at 30 °C. Samples and the standards were injected in 100 μL volume and the flow rate was kept at 1.2 mL/min. Spectrum of Chromatography was recorded at a wavelength of 280 nm[15].

### 3.4. Fatty Acid Extraction

The 10-gram sample put into a Soxhlet thimble and 250 mL of hexane was poured into the apparatus. Extraction was almost 5 hours long. The solvent was then evaporated and the beaker of fat taken out and put into an electric oven at 60°C for 30 minutes to remove any remaining solvent. The beaker was then cooled to room temperature and weighed to find out how much fat was removed from the vegetable. Next, a 250μl aliquot of 9-fluorenyl chloroformate solution was added to the extracted oil followed by a 25μl aliquot of sodium phosphate buffer (0.05M, pH9.3). The mixture was briefly homogenised and the mixture was then incubated for 10 minutes at 40°C. Then 100 μL of the resulting reaction mixture was injected into the HPLC and separated with the same conditions used for the phenolic compounds in Section 2.3.3.

### 4. Antimicrobial activity of N. sativia seeds

The seed extracts of Nigella sativa were evaluated for their antimicrobial effect against two Gram positive bacteria (S. aureus, A. otitidis), two Gram negative bacteria (E. coli, K. pneumoniae) and two fungi (C. albicans, C. krusei). D. D. Davies[16] standard method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) with some modifications. An overnight culture of bacteria was diluted into an appropriate growth medium and 100μl of the bacterial suspension was added to the microplates. Extracts were serially diluted in order to get 0.33 – 1.64 mg/mL concentrations. After 24 hours of incubation at 37°C the turbidity of each well was measured against the control to determine the inhibition of microbial growth. The lowest concentration of the extract at which there was no visible growth was recorded as MIC. 100 μL of the MIC was removed and also the wells containing higher concentrations (6, 3 and 1.5 mg/mL) was removed from the well and streaked onto the agar plates for the determination of MBC. The MBC was determined after the incubation – the lowest concentration that showed no growth.

### 5. Statistical Analysis

The data obtained were analyzed by SPSS software. One-way analysis of variance (ANOVA) was performed to statistically compare the samples against the controls. Tukey's Post-test was also used to statistically compare the samples and controls. A mean value was deemed significant when $p < 0.001$.

## Results and Discussion:

### 1. Proximate Composition

The results of proximate data analysis showed different physicochemical parameters of N. sativa seed extract. The values thus secured for protein, moisture, ash, lipids and carbohydrates were 23.2%, 4.82%, 3.94%, 35.70% and 33.34% in the above order, as recorded in Table 1.

**Table 1.** Chemical composition of N. sativa seed extract

| Sample | Crude Protein% | Crude Lipid % | Moisture % | Ash% | Carbohydrates % |
|---|---|---|---|---|---|
| N. sativa | **$23.2^{c} \pm 0.01$** | **$35.70^{a} \pm 0.03$** | **$4.82^{d} \pm 0.01$** | **$3.94^{e} \pm 0.02$** | **$33.34^{b} \pm 0.02$** |
| | **SD: standard deviation; P: One-way ANOVA** | | | | |

Protein (23.2%), carbohydrate (33.34%) and lipids (35.70%) were in that order. Moisture and ash content of the seed extract turned out to be 4.82% and 3.94% respectively. The moisture content of the seeds recorded 4.82% while the ash content was 3.94 g/100 g, which are in agreement with the results obtained by Asefa and Gemede [17] for black cumin seeds. The range of crude protein of the seed extract of N. sativa was found to be 23.2 g/100 g to 23.9 g/100 g. This is similar to the average CP content reported by Zanib et al. [18]. The result also demonstrates that Nigella seed is a source of protein and lipid which is suitable for human consumption.

**2. Amino Acid Determination**

Nigella sativa seed extract is rich in protein, containing eight to nine essential amino acids with glutamate, aspartate and arginine being the most abundant, along with amino acids which are important neurotransmitters including GABA and glycine[19]. The amount of free amino acids varied among Nigella sativa species. Assessment on the amino acid analyzer resolved sixteen residues, comprising 8 essential (His, Iso leu, Leu, Lys, Meth, Thre, Val, Phe), 15.98,19.58, 17.88, 19.58, 7.88, 20,14, 22.65, 12.03 and 8 nonessential (Ala, Arg, Glu, Gly, cysl, prol, Ser, Tyr) 23.87, 44.14, 35.65, 16.38, 5.65, 13.25, 17.44, 7.47 mg/100gm. The amino acid that was found at the highest concentration in Nigella sativa seed was Arginine, followed by Glutamic acid, Alanine, Valine and Threonine (Table 2 and figure 1). Methionine and Tryptophan appeared in minor quantity in the essential group while nonessential Glutamic acid, Arginine appeared in leading position in their group. As in our case, Y-Kabir et al [20] found the same.

**Table 2.** Free amino acid contents (mg/100gm.) of N. sativa seed extract

| No | Compound name | Amount (mg/100gm) |
|---|---|---|
| 1 | Glycine | $16.38 \pm 0.02^{d}$ |
| 2 | Lysine | $19.58 \pm 0.03^{d}$ |
| 3 | Serine | $17.44 \pm 0.02^{d}$ |
| 4 | Glutamic acid | $35.65 \pm 0.06^{b}$ |
| 5 | Threonine | $20.14 \pm 0.05^{d}$ |
| 6 | Isoleucine | $19.58 \pm 0.02^{d}$ |
| 7 | Alanine | $23.87 \pm 0.05^{c}$ |
| 8 | Valine | $22.65 \pm 0.07^{c}$ |
| 9 | Tyrosine | $7.47 \pm 0.01^{y}$ |
| 10 | Arginine | $44.14 \pm 0.09^{a}$ |
| 11 | Cysteine | $5.65 \pm 0.01^{y}$ |
| 12 | Methionine | $7.88 \pm 0.03^{y}$ |
| 13 | Proline | $13.25 \pm 0.03^{e}$ |
| 14 | Histidine | $15.98 \pm 0.02^{d}$ |
| 15 | Lucien | $17.88 \pm 0.04^{d}$ |
| 16 | Phenylalanine | $12.03 \pm 0.02^{e}$ |
| | SD: standard deviation; P: One-way ANOVA | |

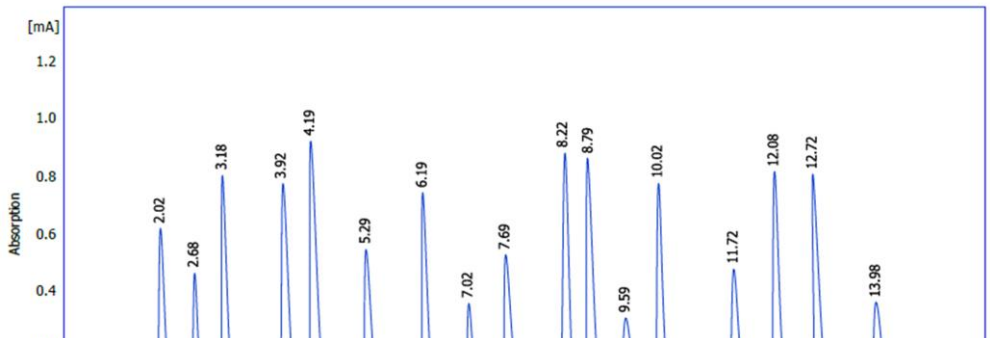


**Figure1: Chromatographic Amino Acid Analyzer of *N. sativa***

As secondary metabolites, phenolic compounds are able to display antimicrobial action[21]. In exciting research, decided to research the phenolic properties of their biological significance. HPLC was used for identification of phenolic compounds (mg/100gm extract). This is shown in Table 3. Thymoquinone, Catechol, Catechine and Qurcetine were the four phenolics detected in that order with their respective concentrations measuring 32.5, 12.6, 2 and 3.6 (mg/100gm). The existing research showed lower levels of phenolics than reported by[22].

**Table 3.** Phenols and Flavonoid compound % (mg/100gm) of *N. sativa* extract

| Phenols and Flavonoid compound % (mg/100gm) | Phenols and Flavonoid compound | Concentration |
|---|---|---|
| | **Thymoquinone** | **32.5+0.09[a]** |
| | **Catechol** | **12.6+0.04[b]** |
| | **Catechine** | **2.0+ 0.01[d]** |
| | **Qurcetine** | **3.6+0.01[c]** |
| | **SD: standard deviation; P: One-way ANOVA** | |

**4. Fatty acid compounds**

The level of fatty acids in Nigella sativa Seed Extracts was determined (Table 4, Figure 2). The data collected lead to the following six fatty acids. The saturated group covered Palmitic acid, stearic acid and Myristic acid with 11.40, 0.80, and 1.44 respectively. The unsaturated group, on the other hand, covered oleic acid, linoleic acid, stearic acid, linolenic acid and Myristic acid at 16.55, 58.57 and 1.05 respectively. On this base the seeds can be used as nutritional sources of essential and non-essential nutrients. The results are similar to the study[23].

**Table 4:** The composition of fatty acids for N.sativa

| Fatty acid % (mg/100gm) | Fatty acids | Concentration |
|---|---|---|
| | **Palmatic** | **11.40 ± 0.02[c]** |
| | **Oleic** | **16.55 ± 0,05[b]** |
| | **Linolic** | **58.57 ± 0.1[a]** |
| | **Stearic** | **0.80 ± 0.00[e]** |
| | **Lenolinic** | **1.05 ± 0.01[d]** |
| | **Myristic** | **1.44 ± 0.02[d]** |
| | **SD: standard deviation; P: One-way Anova; Different letters refer to a significant at p ≤ 0.001** | |

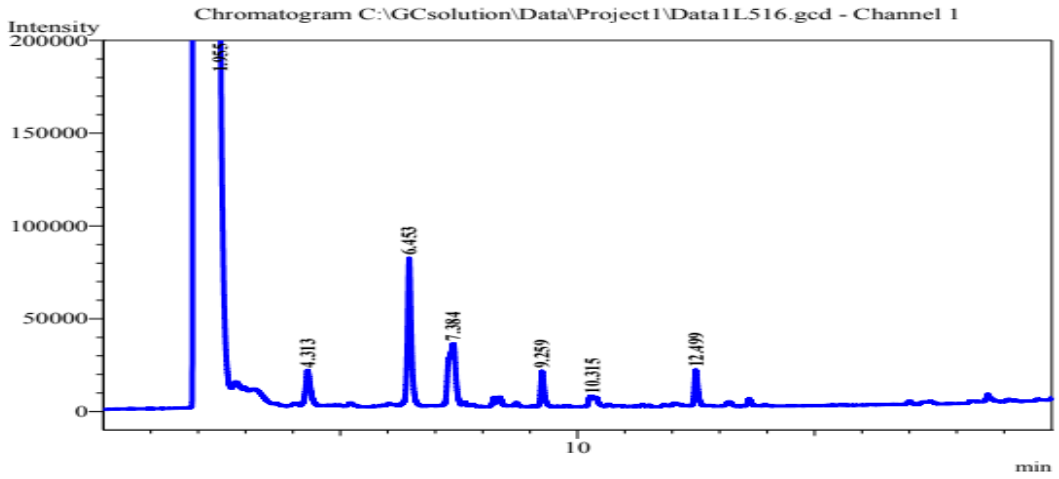


**Figure 2:** Chromatogram for *N. sativa*

**5. Antimicrobial activity of *N. sativia***

The effect of N. sativa seed oils on tested microorganisms was determined using the Minimal inhibitory concentration (MIC) and the Minimal bactericidal concentration (MBC) values as shown in Table 5.

Nigella sativa black seed oil had considerable effects on all microorganisms tested. All strains of alloiococcus otitidis, Staphylococcus aureus, Escherichia coli and Candida krusi showed high sensitivity to the MICs of the Nigella sativa extract. The range of MIC value recorded was from 3.25 to 6.5 mg/ml and the range was not the same as obtained for MBC.

The reason may be attributed to the thymoquinone (TQ) found in black seed, a compound that is known to be very effective against bacteria and fungi, in its action. This substance reduces oxygenation inside microbial cells and thus inhibits growth of these bacteria[24].

The present results corroborate with those of[25] who described thymoquinone as an antibacterial agent of pathogenic bacteria. Nigella sativa oil was observed to have a higher antibacterial activity against Gram-positive bacteria than against Gram-negative bacteria; this may be attributed to the fact that the Gram-positive bacteria has a large amount of peptidoglycan and Gram-negative bacteria has a large amount of lipopolysaccharide which is not resisted by the oil of Nigella sativa seed [26][27].

**Table 5:** The MIC and MBC in mg/ml of Nigella sativa oil against tested microorganisms

| **Bacterial strain** | **MIC** mg/ml | **MBC** mg/ml |
|---|---|---|
| *Alloiococcus otitidis* | 3.25 | 25 |
| *Klebsiella pneumonia* | 6.5 | 50 |
| *Staphylococcus aureus* | 3.25 | 25 |
| *Escherichia Coli* | 3.25 | 50 |
| *Candida albicans* | 6.5 | 25 |
| *Candida krusi* | 3.25 | 12 |

**Conclusions**

The current study reveals that the Nigella sativa seeds are rich source of all essential nutrients and phytochemical substances. Crude lipids, carbohydrates and proteins were found to be significant in their quantity as 35.70%, 33.34% and 23.2% respectively. Sixteen free amino acids were identified in the amino acid profile, and the amino acid arginine was found to be particularly abundant, as well as glutamic acid. The unsaturated fatty acids beneficial to the body were also determined by

chromatographic methods in abundance. In particular, linoleic and oleic acids were found. Thymoquinine is the most abundant phenolic compound found which was 32.5 mg/100 g. All the bacterial and fungal pathogens also exhibited significant antimicrobial activity with the extracts. More susceptibility of the gram positive strains than the gram negative strains may be due to structural and permeability difference of the envelope of gram positive bacteria, particularly, Staphylococcus aureus and Alloiococcus otitidis. Overall, the results clearly suggest the therapeutic potential of extracts from Nigella sativa seeds. Thymoquinone and other bioactive compounds found could be a potential source of natural antimicrobial agents against antibiotic-resistant pathogens.

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