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Original Research Article

Demonstration of antioxidant, antibacterial and antifungal property of *Nigella sativa* seed extract

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Abstract: There is a traditional belief that, *Nigella sativa* seed, commonly known as black seed possess antimicrobial and antioxidant properties. This study was conducted to reveal the antmcrobial and antioxidant property of *Nigella sativa* seed extract to find out the scientific basis of that belief. For that, powder was made from collected *Nigella sativa* seed and seed extract was prepared by Soxhlet Method. Qualitative screening for secondary metabolites was performed with Tannins, Saponins, Flavonoids, Alkaloids, Sterol, Terpenoids, Camcf7io Glycosides and Phenols. Quantitative Assay of antioxidant activity was done by DPPH Method. Antibacterial and antifungal activity were done by disc diffusion and tube dilution method. Results showed that *N. sativa* extracts had highest activity against *B. subtilis* (20 mm zone). MIC values against all the strains were recorded to be < 100 µg/mL. Antimicrobial activity found to be promising from 100-500 µg/mL, whereas moderate from 500-1000 µg/mL and over 1000 µg/mL the extract showed very weak response. *N. sativa* presented a significant activity against *E. coli* and *A. alterneria* with MIC of 5.85 mg/mL and also against *B. subtilis*, *P. multocida* and *A. niger* with MIC of 23.43 mg/mL. Overall, the antimicrobial activity against fungal strains was low versus bacterial strains.
Keywords: *Nigella sativa*, antibacterial, antioxidant, antifungal, antibacterial

INTRODUCTION

Nigella sativa (Black cumin) is a herbaceous plant, used for centuries for the treatment of various ailments, including infectious diseases. The seeds, commonly used in recipes in Asian countries are reported to possess several medicinal properties. Crude extracts and essential oil possess antibacterial activity against several bacteria, fungi and virus also cancer cells. Many active ingredients have been found in the seeds of N. Sativa. The seeds contains both fixed and essential oils, proteins, alkaloids and saponin (Ali and Blunden, 2003). Ghosheh *et al.* (1999) described the quantification of four pharmacologically important components:thymoquinone(TQ),dithymoquinone(DTQ, thymohydroquinone (THQ), and thymol (THY), in the oil of N. Sativa seed by HPLC. The antioxidant action of thymoquinone and its 5-lipoxygenase inhibition may explain the different anti-inflammatory effect of these seeds. *Nigella sativa* seeds caused concentration-dependent inhibition of Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* and *E.coli* and a pathogenic yeast *Candida albicans*. The extract showed antibacterial synergism with streptomycin and gentamicin and showed additive antibacterial action with spectinomycin, erythromycin, tobramycin, doxycycline, chloramphenicol, nalidixic acid, ampicillin, lincomycin and sulfamethoxyzole–trimethoprim combination. Interestingly, the extract successfully eradicated a non-fatal subcutaneous *staphylococcal* infection in mice when injected at the site of infection. (Hanafy and Hatem, 1991). Today, black seed is used for treating digestive tract conditions including gas, colic, diarrhoea, dysentery, constipation, and haemorrhoids. It is also used for respiratory conditions including asthma, allergies, cough, bronchitis, emphysema, flu, swine flu and congestion. In this research work, we aimed to demonstrate the antioxidant, antibacterial and antifungal property of *Nigella sativa* seed extract.

MATERIAL AND METHOD

Collection of Seeds

The Seeds of *Nigella sativa* were bought from a local herbal market in Velachery, Chennai, and Tamil Nadu, India.

Preparation Of Powder

The dried seeds of *Nigella sativa* were collected. These dried were washed with distilled water thrice and dried on a blotting paper in the laboratory at 37^{o}C for 24 hrs. After the seeds were powdered by using a kitchen blender and stored in an airtight container. These powdered materials were used for further experimentation.

Preparation Of Extract

Seed extract was prepared by SOXHLET METHOD (Franz von soxhlet). 25 g of dried seeds powder of *Nigella sativa* were taken and the crushed seeds were taken into the filter paper. Filter paper were kept carefully into the condenser and take 150ml of methanol/chloroform in a clean sterile rounded flask. Fix the condenser carefully and kept the cooling apparatus inside the condenser. The cooling apparatus will maintain the heat temperature. The temperature was maintained up to 55-60^{O}C and keep it for 4-6 hrs. After 4-6hrs the two cycles were completed and the extracts were collected. The extract /distillate were transferred in a clean sterile beaker. It was incubated in a room temperature for evaporation. The weight was measured and the yield was calculated. This extract was used for the phytochemical analysis, Antioxidant, Antimicrobial assay.

Phytochemical Analysis (Trease And Evans, 1986)
Qualitative Screening For Secondary Metabolites:

Nigella sativa seed extracts (Methanol/ Chloroform) were subjected to phytochemical screening for secondary metabolites. Qualitative phytochemical analysis was carried out by standard protocols (;Trease and Evans, 1989; Sofowra,1993**).**

Tannins :1ml of sample was taken where few drops of 0.1% Ferric chloride was added and observed for color changes to blue /brownish green

Saponins : 1ml of sample was taken where 2ml of H_2O was added and shake vigorously and observed for foaming appearance.

Flavonoids: 1ml of sample was taken where few drop of conc. Hcl and Magnesium chloride was added and observed for pink tomato red color.

Alkaloids: 1ml of sample was taken where few drops of Iodine and Potassium Iodide was added and It was observed for orange red colour/brownish red.

Sterol: 1ml of sample was taken where 10% of conc.H_2SO_4 added and observed for green color development.

Terpenoids: 1ml of sample was taken where 200µl of chloroform and conc.H_2SO_4 was added and observed for development of reddish brown colour.

Camcf7io Glycosides: 1ml of sample was taken where 0.4 ml of Glacial acetic acid, Ferric chloride and conc.H_2SO_4 was added. Then observed for development of brown ring colours.

Phenols: Methanol Extract To 2mL of extract, 5% ferric chloride solution was added. Deep blue black colour indicates the presence of phenol

Quantitative Assay Of Antioxidant Activity By DPPH (2, 2-diphenyl- l-picrylhydrazyl) Method (Sasidharan *et al.,* 2007])

Chemicals: 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Butylated hydroxyl toluene (BHT)
Methanol

Reagents: DPPH – 1mg/ml in methanol**,** BHT (standaMCF7) – 1.6mg/ml in methanol

Procedure:

Aliquot 3.7 ml of absolute methanol was taken in all test tubes along with blank.. Then, 100µl of absolute methanol was added to blank. 100 µl of BHT was added to tube marked as standaMCF7 and 100 µl of respective samples to all other tubes marked as tests. Then, finally 200 µl of DPPH reagent was added to all the test tubes including blank, Incubate all test tubes at room temperature and dark condition for minimum of 30 minutes. Then, absorbance of all samples at 517nm were checked.

Calculation

% Antioxidant activity={(absorbance at blank)– (absorbance at test) / (absorbance at blank)} X 100.

Microbial Strains Used
Collection Of Sample

The specimen for culture which was previously identified by 16S rRNA, obtained from Louis Pauster clinic in Velachery, Chennai. Below strain were used to evaluate the antimicrobial activity of solvent extract.

Bacteria: *Escherichia. Coli. Staphylococcus aureus. Bacillus subtilis. Klebseilla pneumonia. Streptococcus pyogenes. Pasturella multocida. Shigella flexneri. Salmonella typhimurium*

Fungi *Aspergillus niger, Aspergillus flavus, Alternaria alternate, Rhizopus solani*

Maintanance Of Culture

Bacterial isolates were subcultured on Nutrient Agar slant. Fungal isolates were subcultured on Potato Dextrose Agar (PDA). Cell lines were subcultured on serum added Minimal Essential Medium (MEM) media.

Determination of Antimicrobial Activity

Antimicrobial activity was performed by standaMCF7 method, disc diffusion on agar and tube dilution method in broth for MIC .

Antimicrobial Activity of *Nigella sativa* Seeds Extract By Disc Diffusion Method

The 200mg of the methanolic extract of *Nigella sativa* was diluted with 800µl DMSO. It was serially diluted with 500µl of DMSO. (1000, 500, 250, 125, 62.5 µg/disc). The filter paper discs (6mm diameter) were cut and sterilized. The 20µl of sample loaded on every disc. The discs were placed on the Muller Hinton Agar plate swabbed with bacterial strains. The plates were incubated at 37^{o} c for 24 hours. The diameter of the inhibition zones were measured in mm and the result were recoMCF7ed. The zones of inhibition were compared with zone formed by Streptomycin antibiotic as standard control.

Antifungal Activity of *Nigella Sativa* Seeds Extract By Disc Diffusion Method

The 200µg of methanolic extract was added with 800µl of the DMSO. It was serially diluted with 500µl of DMSO. (1000, 500, 250, 125, 62.5 µg/disc). The each disc was loaded with 20µl of each dilution. The PDA plates were swabbed with fungal culture. The loaded discs were placed on swabbed plates and incubated. The zones of inhibition were observed after the incubation. The Amphotericin B antibiotic was used as the standaMCF7 control.

MIC

Overnight Mueller Hinton broth cultures of *Staphylococcus aureus, E. coli, Bacillus pumillus, Shigella* spp. *and Pseudomonus* spp. at 37^{0}C were prepared .The culture was adjusted to obtain turbidity comparable to that of the turbidity of McFarland 0.5 standaMCF7. Different concentration of black seed extract was added to the tube and incubated at 37^{0}C for 24h.The inhibition of growth was determined and recoMCF7ed.

RESULT

The phytochemical analysis carried out on the dry sample of different germination phases of N. sativa seed extracts showed the significant change in some bioactive compounds. In eleven extracts of samples of germination phases, eight bioactive constituents were tested. The results of effect of seed germination on alkaloid composition of *N. sativa* seeds.

Table I: **Preliminary screening of secondary metabolites from N. sativa**

Day	Alkaloids	Sterols	Phenols	Flavonoids	Tannins	Saponins	Terpenoids	CaMCF7ia glycosides
0	+++	+++	+	+	-	++	+	++
1	++	+++	++	+	-	++	+	++
2	++	++	++	+	-	++	+	++
3	++	+++	++	+	-	+++	+	+
4	++	++	+++	++	+	+++	+	+
5	++	++	++	+++	+	+++	+	+
6	++	++	++	+++	+	++	+	+
7	++	++	++	++	++	+	+	+
8	+	+	++	++	++	-	+	-
9	+	+	++	+	++	-	-	-
10	-	+	++	+	++	-	-	-
11	-	+	++	-	++	-	-	-

* '-' symbolizes absence of the metabolite; *'+' symbolizes presence of the metabolite; *'++' symbolizes moderate presence; *+++ symbolizes good presence.

Table II: Absorbance of methanol, test extracts (DPPH Method)

Extracts	*Absorbance*	*% inhibition*
Methanol	***0.51***	***3.77***
Chloroform	***0.04***	***92.45***

The antimicrobial activity of *N. sativa* seed crude extracts was tested against different bacterial and fungal strains. Results showed that *N. sativa* extracts had highest activity against *B. subtilis* (20 mm zone). MIC values against all the strains were recoMCF7ed to be < 100 µg/mL. Antimicrobial activity recorded to be promising from 100-500 µg/mL, whereas moderate from 500-1000 µg/mL and over 1000 µg/mL the extract showed very weak response. *N. sativa* presented a significant activity against *E. coli* and *A. alterneria* with

MIC of 5.85 mg/mL and also against *B. subtilis*, *P. multocida* and *A. niger* with MIC of 23.43 mg/mL. Overall, the antimicrobial activity against fungal strains was low versus bacterial strains. Activity-guided fractionation was carried out to isolate the proteins from extracts.

Table III: Antibacterial and antifungal activity and MIC values of N. sativa crude extract

Microorganism	**Disc diffusion method Zone of inhibition in mm**	**Minimum inhibitory concentration (MIC) Conc. (µg/mL)**
Bacterial strains		
Escherichia coli	16	23.43 ± 0.234
Bacillus subtilis	20	14.85 ± 0.015
Pasturella multocida	14	46.87 ± 0.0468
Staphylococcus aureus	7	123.43 ± 0.023
Fungal strains		
Alterneria alterneria	19	15.75 ± 0.058
Aspergillus flavis	17	16.71 ± 0.011
Aspergillus niger	15	34.43 ± 0.023
Rhizopus solani	21	14.64 ± 0.014
Terbinafine	23	
Rifampcin	27	5.56 ±0.055
Negative Control (Autoclaved water) -		

Table IV. Correlation between concentration of *Nigella sativa* extract and inhibition zones against different types of bacteria

Test bacteria	**Concentration / disc (5 ìL/disc) iameter of inhibition zones (mm)**							
	Concentration (mg/m)						**Methanol**	**Sperman's correlation**
	1	5	10	20	50	100		
Klebseilla pneumonia	Nil	Nil	Nil	0	11.0 ±1.8	15.0 ± 4.5	Nil	0.845
Pseudomonas aeruginosa	Nil	Nil	Nil	0	12.0 ± 2.7	15.0 ± 4.0	Nil	0.845
Streptococcus pyogenes	Nil	Nil	Nil	10.0± 0.0	16.0 ± 9.9	19.3 ± 1.9	Nil	0.88
Proteus vulgaris	Nil	Nil	Nil	0	10.7 ± 1.0	15.0 ± 4.0	Nil	0.845
P				0.012	0.012	0.341		

Table V. Correlation between concentration of *Nigella sativa* extract and inhibition zones against different types of bacteria

Test Bacteria	Concentration / disc (5 µL/disc) Diameter of inhibition zones (mm)							
	Concentration (mg/mL)						Methanol	Spearman correlation
	1	5	10	20	50	100		
Klebseilla pneumonia	Nil	Nil	Nil	0	10.0 ± 0.5	10.0 ± 0.5	Nil	0.778
Pseudomonas aeruginosa	Nil	Nil	Nil	11.0 ± 1.4	12.0 ± 1.5	15.0 ± 0.0	Nil	0.941
Streptococcus pyogenes	Nil	Nil	Nil	11.0 ± 0.5	12.0 ± 1.0	20.0 ± 0.9	Nil	0.941
Proteus vulgaris	Nil	Nil	Nil	0	11.0 ± 0.5	12.0 ± 1.0	Nil	0.845
P				0.021	0.205	0.016		

Correlation between log dose and size of zones is significant it 0.01 (2-tailed)

(Mean diameter of inhibition zones in mm around 6 mm disc impregnated with *Nigella sativa* extract) P-Positive correlation

DISCUSSION

This study reports the antimicrobial activity of *Nigella sativa* against *Streptococcus pyogenes*, *Klebseilla pneumonia*, *Pseudomonas aeruginosa* and *Proteus vulgaris*. The results of the antimicrobial activity of the investigated extract are shown in tables 1 and 2. in this study, both methanol and aqueous extracts showed no inhibition against all the bacteria tested at lower concentrations (<50 mg/ml). Generally, the methanol extract of *Nigella sativa* exhibited higher antibacterial effect compared with aqueous extracts extraction of the biologically active compounds from the plant material depends on the type of solvents used in the extraction procedure. According to Parekh *et al*. (2006), methanol, ethanol and water are the most commonly used solvents for determining the antimicrobial activity in plants. The diameter of inhibition zone in methanol extract are higher (Mdn=11.5) than aqueous extract (Mdn=11.0). on the contrary, statistical analysis using Mann-whitney analysis showed that there are no significant differences between methanol extract and aqueous extract used, U=282.0, z=-0.142 with p-value≥0.01. This is because different sources of the extracts, agro-climate factor, handling of experiment and phytochemical ingredients in the extract also contribute to the differences of results obtained (Erdman *et al*. 2007).

In this study, positive result was only observed in methanol extracts of *Nigella sativa* at 20 mg/mLagainst *Streptococcus pyogenes* measured at 10 mm; (p≤0.01) while all other bacteria were resistant in aqueous extract. At concentration of 100 mg/ml, the highest antibacterial activity of 19 mm was recorded in *Streptococcus pyogenes* and similar activity was recorded in *Klebseilla pneumonia*, *Pseudomonas aeruginosa* and *Proteus vulgaris* with an inhibition zone measuring around 15 mm. Aqueous extract of *Nigella sativa* had a remarkable sensitivity towards *Pseudomonas aeruginosa* and *Streptococcus pyogenes* with inhibition zones of 20 mm and 15 mm at concentration of 100 mg/ml, respectively. At concentration of 50 mg/mL, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Klebseilla pneumonia* exhibited modest inhibition at 10, 12, 12 and 11 mm, respectively. there was no difference in inhibition zone showed by *Klebseilla pneumoniae* when the concentration was increased from 50 mg/ml to 100 mg/ml.

On the basis of the above results, it showed that methanol extract of *N. sativa* exhibited a greater inhibition compared with aqueous extract. Parekh *et al*. (2006) reported that most of the antimicrobial active compounds were soluble in polar solvent such as methanol instead of water. this result is comparable to the study by de Souza *et al*. (2004) using methanol extract of *L. sibiricus* that showed effective antibacterial activity on *Bacillus subtilis.* Bajwa and Shafique (2008) showed that methanol fraction of *A. rabiei* exhibited more promising results in suppressing the fungal growth rather than aqueous extract. was also reported by Zafar *et al*. (2002), where chloroform extract of *Melia azedarch* leaves was active against *Fusarium chamdosporum* while water extract of the leaves did not show any positive results.

In this study the extracts were found to be more effective on Gram positive than Gram negative bacteria, which is in conformity with a number of earlier studies where compounds derived from plants often show considerable activity against Gram positive bacteria but not against Gram negative species (Nagi *et al*. 2008). Gram negative bacteria have effective permeability barrier, comprised of the outer membrane, which restricts the penetration of amphiphatic compounds and multidrug resistance pumps that extrude toxins across this barrier (Nagi *et al*. 2008). it is possible that the apparent ineffectiveness of the plant antimicrobial activity is largely due to this permeability barrier.

Results of the study indicate that black seed extract showed a dose of dependent inhibition against concentration. Statistical analysis using Spearman's Rho, indicates that there is a significant correlation between zone of inhibition and concentration's used. both extracts showed that all the bacteria tested (n=4) showed strong and positive correlation value. Positive correlation value indicates that increasing the concentration will increase the diameter of inhibition zone formed by the bacteria. This finding is in agreement with results reported by Hannan *et al*. (2008) using the same genus of plant tested.

The antimicrobial activity of *N. sativa* seed crude extracts was tested against different bacterial and fungal strains. Results showed that *N. sativa* extracts had highest activity against *B. subtilis* (20 mm zone). MIC values against all the strains were recorded to be < 100 µg/mL. Antimicrobial activity recorded to be promising from 100-500 µg/mL, whereas moderate from 500-1000 µg/mL and over 1000 µg/mL the extract showed very weak response and this trend is in line with previous investigation (Holetz *et al*., 2002). *N. sativa* presented a significant activity against *E. coli* and *A. alterneria* with MIC of 5.85 mg/mL and also against *B. subtilis*, *P. multocida* and *A. niger* with MIC of 23.43 mg/mL. Overall, the antimicrobial activity against fungal strains was low versus bacterial strains which were enhanced by increasing the working concentration of extracts (Banso and Adeyemo, 2007).

Activity-guided fractionation was carried out to isolate the proteins from extracts. The supernatant did not show any antibacterial activity except a weak effect against *E. coli*. Strong antibacterial activities were observed for the residual material and might be correlated to peptides/proteins present in the extract because the activity was diminished by treating the residue with proteinase K which confirmed that the antimicrobial activity was due to proteins/peptides in the extracts (Jamil *et al.,* 2007).

CONCLUSION:

All findings discussed above indicate that *N. sativa* seeds have antimicrobial effects against different pathogens, including bacteria, and fungus.

Black seed in traditional medicine and in recent years for the treatment of microbial diseases has been used without any reported side effects. Therefore, this plant can provide a valuable agent for microbial diseases. However, additional studies are required to evaluate and explore the specific cellular and molecular mechanisms of the antimicrobial effects of *N. sativa*, alone or in combination with other drugs.

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