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Full Length Research Paper

Antibacterial activity of *Nigella sativa* seed in various germination phases on clinical bacterial strains isolated from human patients

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***Nigella sativa* is an important spice and flavoring agent which is widely used in various European and Asian cuisines. It harbors an array of medicinal properties as shown by various researches. Germination is a phenomenon during which rapid changes in metabolic activities and the interconversions of metabolites take place. The objective of present study was to evaluate the antibacterial activity of *N. sativa* seed that are on various germination phases against clinical bacterial strains isolated from pus, urine, ascitic fluid and cerebrospinal fluid of various patients. The minimum inhibitory concentration (MIC) values were determined by using a modified macro-broth dilution technique. The agar well diffusion method was used to test the antimicrobial effects of *N. sativa* extracts. Some broad spectrum antibiotics were used as positive control. The phytochemical constituents of *N. sativa* seed were also studied in germination phases. The distilled methanolic extracts of *N. sativa* showed significant antimicrobial activity against tested clinical strains of Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*, Klebsiella *pneumoniae, Pseudomonas aeruginosa* and *Proteus mirabilis* bacteria. Results showed day-dependent and dose-dependent activity and a significant antimicrobial effect was observed as germination proceeded.**

Keywords: *Nigella sativa* seed, germination phases, phytochemicals, clinical isolates, antibacterial activity.

INTRODUCTION

Nigella sativa Linn. is commonly known as black seed which belongs to the botanical family of Ranunculaceae. *N. sativa* seeds have been used for nutritional and medicinal purposes in many Middle Eastern countries and other parts of the world (El-Dakhakhny et al., 2000; Al-Ghamdi, 2001). *N. sativa* plant is commonly referred as "Love in a Mist" in English. Seeds of *N. sativa* are frequently used in folk medicine in the Middle East and some Asian countries for acquiring good health and treatment of many ailments including fever, common cold, headache, asthma, rheumatic diseases and various microbial infections and to expel worms from the intestine (Akhtar and Riffat, 1991; Al-Jassir, 1992; Al-Ghamdi, 2001).

The present study was conducted on the evaluation of antibacterial properties of *Nigella sativa* during different phases of germination on some clinical pathogenic bacteria. Germination of seeds has been used for centuries to soften the kernel structure, to increase the nutritional value and to decrease the Anti-nutritive compounds. During the recent years interest has arisen also in the secondary metabolites produced during germination which can have valuable health promoting properties and act as bioactive or functional compounds in foods. All this requires knowledge and know-how of the germination process and the biochemistry behind it (Kaukovirta-Norja et al., 2004). The seeds of *N. sativa* in different germination stages have revealed the presence

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Table 1. Human clinical isolates used in the present study and their sources.

S/N	Organism	Patient code	Date	Disease	Age/Gender	Source
1	*E. coli*	U/397	22-02-2012	Intestine infection	17 yr/M	Urine
2	*S. aureus*	P/312	13-02-2012	Stomach ulcer/ infection	26yr/M	Ascitic Fluid
3	*K. pneumoniae*	P/299	11-02-2012	wound at surgical sites	50yr/F	Pus
4	*P. aeruginosa*	P/322	14-02-2012	Jaundice	1day/F	CBF
5	*P. mirabilis*	U/401	25-02-2012	Cystitis	51yr/M	Urine

of alkaloids, tannins and flavonoids (Kamal et al., 2010).

Bacterial infection is responsible for upto a quarter of the deaths of patients with liver disease (Sloth et al*.,* 1970; Correia et al*.,* 1971; Powell et al*.,* 1971; Rimola et al*.,* 1984). 58% of Gram-positive and 42% of Gram-negative bacteria responsible for chronic liver diseases (Jones et al*.,* 1967; Wyke et al*.,* 1982). Few prospective surveys of the incidence of bacteraemia among patients with liver disease are present. Patients with chronic active hepatitis or primary biliary cirrhosis are rarely affected by bacteraemia (Mistilis and Blackburnm*,* 1970; Crowe et al*.,* 1980; Kirk et al*.,* 1980). In immunocompromised patients *P. aeruginosa* frequently causes septicemia and a high incidence of *P. aeruginosa* bacteremia was observed in patients with impaired barrier function of the liver (Pollack, 1990; Korvik et al*.,* 1991).

Clinical isolates of multi-drug resistant Gram-positive *Staylococcus aureus* and Gram-negative *Escherichia coli*, *Klebsiella pneumonia, Pseudomonas aeruginosa* and *Proteus mirabilis* bacteria were used in present study. They may be responsible for many types of infections including bacteraemia and liver infections or diseases.

The production of new and potent antibacterial agent is urgently needed, especially for hospitals and health centers, keeping in mind that study on strains of clinical microbes are scarce. So, the present study was taken to investigate the antimicrobial effects of *N. sativa* crude methanolic extracts of successive germination phases against five clinical bacterial strains.

MATERIALS AND METHODS

Collection of *Nigella sativa*

Seeds of *N. sativa* were procured in the month of March, 2012 from a grocery shop in Lucknow and authenticated by Dr. Shanthy Sundaram, Centre for Biotechnology, University of Allahabad, Allahabad (U.P.) India.

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Germination of seeds

Seeds of *Nigella sativa* were grown in glass petri plates. Seeds were placed on four folds of damp filter paper at 25°C and incubated in the dark till the initiation of sprouting 3rd day after which they were placed at a light intensity of 100 µmol m^{-2} s^{-1} measured by LI-190SA quantumSensor (Li-COR Co., USA) and a 14/10 h (day/night) photoperiod till the complete plantlet with two leaves was obtained.

Preparation of distilled extracts

The samples of seed and germinated phases 3rd, 5th, 7th, 9th and 11th day were shade-dried and ground to a fine powder. The powder (20gm) was extracted by using soxhlet apparatus with 200 ml methanol solvent for 48 h in order to extract bioactive compounds (AOAC method 1980). The extracts were filtered using Whatman filter paper and evaporated using rotary distillation apparatus. Oily fraction of extracts (12g) stored at 4^0C until use.

Qualitative study of phytochemicals of *Nigella sativa* during germination

The phytochemical properties (saponin, tannin, alkaloids, polyphenols, sterols, flavonoids) were determined by the methods of Sofowora (1993), Trease and Evans (1983) and Evans and Brightman (1980).

Clinical bacterial strains used for the study

The clinical bacterial isolates used in study are listed in Table 1. These isolates were collected from Era's Lucknow Medical College and Hospital U.P. (India) from various patients. They were authenticated by Dr. Vineeta Mittal, MD, Department of Microbiology, Era's Lucknow Medical College, Lucknow.

Inoculum preparation

The test microorganisms were maintained at 4°C on nutrient agar slants. Active cultures for each bacterial species were prepared by transferring a loopful of cells from the stock cultures to test tubes of nutrient broth. The inoculated tubes were incubated without agitation for 24 h at 37°C. The cultures were diluted with fresh nutrient broth to achieve optical densities corresponding to 10^6 cfu mL^{-1} (Duraipandiyan et al., 2006).

Determination of *in vitro* anti-microbial effect

Broth dilution assay

The minimum inhibitory concentration (MIC) values were

Table 2. Screening of phytochemicals in methanol extracts of *N. sativa* during germination.

Days	Sterols	Alkaloids	Tannins	Saponins	Phenols	Flavonoids	Terpenoids	Cardiac glycosides
0	+++	++	+++	+++	+++	+++	+++	+++
3	+++	+++	+++	+++	+	+++	+++	+++
5	+++	+++	++	+++	++	+++	+++	+++
7	+++	+++	++	++	++	+++	+++	+++
9	+++	++	+++	+	+++	+++	++	+++
11	+++	++	+++	+	+++	+++	++	+++

*Data is a mean of three replications.

determined by using a modified macro-broth dilution technique (Ibrahim et al., 1997). Overnight culture of bacteria grown in nutrient broth cultures were diluted 100 folds in NB (100µl bacterial cultures in 10ml NB which contained 10^5 cfu of bacteria). Gradually increasing volumes of the extracts were added to test tubes containing the bacterial cultures to know the inhibitory concentration in a particular tube inhabiting the bacterial growth. The tubes were incubated at 37°C for 18-24 hours. The tubes were examined for visible turbidity and optical density of cultures was determined at 620nm using NB as control. The lowest concentration that inhabited visible growth of the tested organisms was recorded as the MIC.

Agar well diffusion assay

The agar well diffusion method was used to test the antimicrobial effect of *N. sativa* methanolic extracts in different stages of germination. (Perez et al., 1990; Okeke et al., 2001). All media plates (9 cm in diameter) were prepared with nutrient agar. After agar solidification, the well (7 mm in diameter) was cut from the agar to produce a total of four wells per agar plate. For test, three doses of extract (25, 50, 75 µg/well) were use. Standard antibiotics (HIMEDIA) streptomycin (30 µg), ciprofloxacin (10 µg), doxycycline (30 µg), ampicillin (10 µg) and ofloxacin (5 µg) were used as positive control. 100 µl (10^5 cfu) of each diluted microbial suspension were inoculated on nutrient agar plates using sterile cotton swab. The extracts and positive control (streptomycin, ciprofloxacin, doxycycline, ampicillin and ofloxacin) were added separately to each well of agar plate and allowed to diffuse at room temperature for 15-20 min. After incubation at 37°C for 24h, all plates were examined for zones of growth inhibition and the diameter of these zones was measured. The assay was repeated three times for each extract. The antimicrobial effects were recorded as the mean diameter of the resulting inhibition zones of growth in millimeter.

RESULTS

The qualitative analyses of phytochemicals present in the methanolic extract of *N. sativa* seed showed the presence of sterols, alkaloids, saponins, phenols, flavonoids, terpenoids and cardiac glycosides. No effect of germination was observed on the presence of sterols, phenols and cardiac glycosides. There was a slight increase in the alkaloid content from 3rd to 7th day of germination. A slight decrease was observed in tannins, saponins, phenols and terpenoids from 5th to 7th day, from 7th day, from 3rd to 7th day and from 9th to 11th day respectively (Table 2).

A clear increase in sterol, tannins, phenols, and in cardiac glycosides was observed as the germination proceed. The presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols *etc*. remedial properties in medicinal plants. Thus the preliminary screening tests for bioactive compounds may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development.

In this study, we investigated the antibacterial effects of methanol extracts of successive stages of the germinating seed on Gram-positive and Gram-negative clinical bacterial isolates collected from different pathological samples. The results of antibacterial test is presented in Table 3 and 4 which indicated that different germination extracts of *N. sativa* showed different degrees of growth inhibition depending on the day of germination and bacterial strains.

The general trend of the inhibition of bacterial strains by the extracts was the increase in the activity from 0 day reaching maximum on 5th day and then showed decline again. On 11th day, the inhibitory activity was equal to that of 5th day extract in case of *S. aureus, P. aeruginosa and P. mirabilis* (Figure 1). The 9th and 11th day extracts of germinating seeds did not show the inhibition of *E. coli*. Maximum sensitivity was shown by *S. aureus* (5th and 11th day inhibition zone, 34mm) followed by *P. aeruginosa* (5th and 11th day inhibition zone, 30mm) and *P. mirabilis* (5th and 11th day inhibition zone, 30mm). *K. Pneumonia* showed moderate sensitivity (5th day inhibition zone, 28mm) and *E. coli* was the least sensitive organism towards these extracts (5th day inhibition zone, 13mm). From the above results, it could be said that the extracts showed day-dependent activity and the 5th day extract of germinating seed was most effective in inhibiting the growth of bacterial pathogens isolated from human patients (Table 4).

The minimum inhibitory concentration of the germinating seed extract was moderate (0.80 µg ml^{-1}) for *E. coli*, *S. aureus* and *K. pneumoniae* whereas it was

Table 3. Minimum Inhibitory Concentration of methanol extracts of *N. sativa* seed in different germination phases on clinical bacterial isolates.

S/N	Organism	Minimum Inhibitory Concentration (µg ml-1)					
		Day of germination					
		Day 0	Day 3	Day 5	Day 7	Day 9	Day 11
1	***E. coli***	1.6±0.09	1.0±0.11	0.80±0.06	3.0±0.11	-	-
2	***S. aureus***	1.5±0.11	0.80±0.03	0.80±0.09	2.0±0.10	0.80±0.02	3.0±0.15
3	***K. pneumoniae***	1.3±0.10	0.80±0.07	0.80±0.03	3.1±0.14	1.0±0.12	3.3±0.24
4	***P. aeruginosa***	1.3±0.12	1.0±0.041	1.0±0.02	3.0±0.31	3.0±0.11	3.0±0.26
5	***P. mirabilis***	1.0±0.05	0.80±0.04	0.60±0.03	2.0±0.12	0.60±0.02	2.2±0.11

*Data is a mean ± SD of three replications.
** Technique used was broth dilution assay.

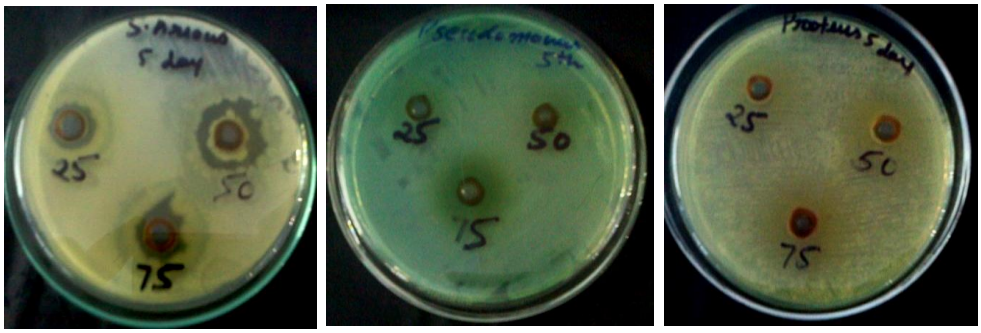

Figure 1. The zones of inhibition shown by 5th day germinating seed extract of *N. sativa on S. aureus, P. aeruginosa* and *P. mirabilis*.

maximum for *P. aeruginosa* (1.0 µg ml^{-1}) and least for *P. mirabilis* (0.60 µg ml^{-1}). From these data, it could be said that the extracts of *N. sativa* during various germination phases were effective in inhibiting *P. mirabilis* at very low concentration (MIC, 0.60 µg ml^{-1}) (Table 3) but they were most effective against *S. aureus* (5^{th} and 11^{th} day inhibition zone, 34mm) as it showed maximum zone of inhibition (Table 4).

All the pathogenic organisms were resistant towards ciprofloxacin (10 µg), doxycycline (30 µg), ampicillin (10 µg) and ofloxacin (5 µg) and sensitive towards streptomycin (30 µg). The discs of these standard antibiotics (HIMEDIA) served as positive controls in the experiment. The extracts were more effective on Gram-positive *S. aureus* (5^{th} and 11^{th} day inhibition zone, 34mm) as compared to Gram-negative *E. coli* (5^{th} day inhibition zone, 13mm), *K. Pneumonia* (5^{th} day inhibition zone, 28mm), *P. aeruginosa* (5^{th} and 11^{th} day inhibition zone, 30mm) and *P. mirabilis* (5^{th} and 11^{th} day inhibition zone, 30mm) (Table 4).

DISCUSSION

Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties. The extracts of seeds of *N. sativa* in different germination stages have exposed the presence of sterols, alkaloids, tannins, saponins, phenols, flavonoids, terpenoids and cardiac glycosides in most of the samples (Table 2). An interesting consequence is that these compounds are potent bioactive compounds that could be used for therapeutic purpose or which are precursors for the synthetic of useful drugs (Sofowora, 1982).

Level of antimicrobial activities of the methanolic extracts on clinical bacterial strains was compared with the chemical composition of extract to determine the chemical composition-activity relationship of extract. The alkaloid and saponins content showed a decrease while the phenol, tannin and flavonoid contents have showed an increase with germination. High tannin and flavonoid contents might also be responsible for the antibacterial activity in later stages of germination (Table 2).

Recent studies have shown that the secondary metabolite content varies during germination of seeds. A significant antibacterial effect of *Allium roseum* L (bulb, leaf, seed and flower) extracts on *S. aureus*, *B. subtilis*, *B. cereus*, *E. faecalis, E. coli*, *P. aeroginosa, S. typhimurium* and *C. albicans* strains has been s hown

Table 4. Zone of Inhibition shown by methanol extracts of *N. sativa* seed in different germination phases.

S/N	Organism	Zone of inhibition (mm)										
		Days						Antibiotics				
		0	3	5	7	9	11	ST	CF	DO	AM	OF
1	***E.coli***	12±0.24	12±0.1	13±0.31	12±0.24	-	-	16±0.21	-	-	-	-
2	***S. aureus***	20±0.16	32±0.24	34±0.22	20±0.31	24±0.61	34±0.45	20±0.12	16±0.21	14±0.18	18±0.21	24±0.32
3	***K. pneumoniae***	28±0.32	25±0.41	28±0.18	21±0.12	20±0.11	24±0.51	12±0.12	-	-	-	-
4	***P. aeruginosa***	22±0.42	18±0.35	30±0.56	24±0.91	28±0.51	30±0.25	16±0.12	-	-	-	-
5	***P. mirabilis***	22±0.51	15±0.51	30±0.55	28±0.32	28±0.33	30±0.35	18±0.31	-	-	-	-

*Data is a mean of three replications. **"-" No inhibition observed. ***ST: Streptomycin (30 µg), CF: Ciprofloxacin (10 µg), DO: Doxycycline (30 µg), AM: Ampicillin (10 µg), OF: Ofloxacin (5 µg), **** Technique used was agar well diffusion assay. ***** Extracts (75µg/well).

(Najjaa et al., 2009).

Several investigations have been directed towards *N. sativa* antibacterial properties (Voravuthikunchai *et al*., 2005; Salman *et al*., 2008; Hannan *et al*., 2008; Suresh *et al*., 2010). The preliminary assessment of the *in vitro* antimicrobial effects of different germinating stages of *N. sativa* extracts revealed some basic outcomes in the present study. First, the methanol extracts of *N. sativa* showed good inhibitory effect against Gram-positive and Gram-negative clinical bacterial strains during germination phases as compared to seed extract, the extracts showed highest activity from 5^{th} day to 11^{th} day of germination (Table 4).

The activity exerted by the methanol extract may be due to the presence of some active phytoconstituents such as thymol and thymoquinone. Thymol is a phenolic alcohol present in the essential oil of *N. sativa* (Randhawa and Al- Ghamdi, 2002) that has been reported to possess antibacterial activity (Karapinar and Aktug, 1987). Thymol and thymoquinone is present in the methanol soluble portion of *N. sativa* oil (Abou Basha et al*.,* 1995). In a study by Kahsai (2002), thymoquinone present in volatile oil obtained from the crude extract exhibited remarkable inhibition of the growth of various strains of bacteria. Thymoquinone is present in the methanol soluble portion of *N. sativa* oil (Abou Basha et al*.,* 1995) and thus will be extracted in methanolic extract of seed also.

Second, the extracts of germination phase revealed good inhibitory effect when compared with the standard antibiotics (Table 4). These antibiotics are the inhibitors of cell wall synthesis, the cross-linking of different peptidoglycan strands etc. The extracts of *N. sativa* showed the activity against standard antibiotics resistant bacteria from 5^{th} to 11^{th} day of germination. This might be due to the presence of metabolites in methanolic extract on these days of germination which could act by cell wall synthesis inhibition, by inducing changes in membrane structure, by inhibiting bacterial protein synthesis or by binding to ribosomal 50S subunit and interfering with the peptidyl transferase activity. The illustration of the exact mechanism of inhibition by the extracts needs further investigation.

Conclusion

This is the first study on the alterations in the antimicrobial properties of *Nigella sativa* seed in germination phases against clinical bacterial isolates from human patients. *N. sativa* seed extracts during various germination phases possesses potential antimicrobial activity against several multidrug resistant clinical bacterial isolates. High metabolic activity and higher contents of secondary metabolites during germination might be responsible for the antibacterial activity.

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