

Antimicrobial activity of Black Cumin seeds (*Nigella sativa*) against multidrug resistant strains of Coagulase negative Staphylococci.

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Summary

Nigella sativa (black cumin) seed oil and methanolic extract were tested in varying dilutions against strains of *Staphylococcus epidermidis* and other coagulase-negative staphylococci resistant to a number of clinically used antibiotics isolated from patients attending JN Medical College Hospital, Aligarh, using disc agar diffusion technique on inoculated Mueller Hinton agar plates under standard laboratory conditions. Both the oil and extract showed remarkable dose dependant antibacterial activity against the tested strains upto a dilution of 1:50 as evident from the zones of inhibition. No cross resistance was noticed with any of the tested antibiotics. To the best of our knowledge, the activity of *N. sativa* against multidrug resistant clinical strains of Coagulase negative staphylococci is being reported for the first time.

Keywords: *Nigella sativa*, *Staphylococcus epidermidis*, Coagulase negative staphylococci, Antimicrobial activity, Antibiotic resistance,

Introduction

Staphylococcus epidermidis and other coagulase-negative staphylococci (CONS) represent the major components of the microflora of human skin and mucosae and have been acquiring increasing importance in the etiology of hospital infections (Kloss and

Bannerman, 1994). This fact probably results from the large number of debilitated and immunosuppressed hospitalized patients, and also from the large-scale employment of invasive procedures leading to spread of patient's endogenous strain to normally sterile sites (Von Eiff *et al.* 2001). An additional problem is the resistance of CONS to multiple antimicrobial agents due to person-to-person spread of staphylococci, particularly those that have acquired antimicrobial resistance in the hospital (Patrick, 1990).

Nigella sativa L. (Black cumin) is a herbaceous plant that has been used for centuries for treatment of various ailments, including infectious diseases. It is one of the important medicines of Tibbe Nabawi (Prophetic Medicine) according to the following Hadiths:

- 1) Narrated Khalid bin Sa'd, "Ibn Abi 'Atiq said to us, "Treat him with black cumin.....for Aisha has narrated to me that she heard the Prophet saying, 'This black cumin is healing for all diseases except As-Sam.' 'Aisha said, 'What is As-Sam?' He said, 'Death.' "(Sahih al Bukhari a)
- 2) Narrated Abu Huraira, "I heard Allah's Apostle saying, 'There is healing in black cumin for all diseases except death'." (Sahih al Bukhari b)

The seeds have been thoroughly studied scientifically in the last 3-4 decades and have been reported to possess a number of medicinal properties (Ali and Blunden, 2003; Randhawa and Al- Ghamdi, 2002). Their crude extracts (Ali *et al.* 2001; Mouhajir and Pedersen, 1999) and essential oil (Halwani *et al.* 1999) have been shown to possess antibacterial activity against several bacteria. However, their effect against multidrug resistant CONS isolated from patients had not been studied. Hence this study was undertaken.

Methodology

Acquisition of seeds and oil of Nigella sativa: Seeds of *N. sativa* (locally known as Kalonji) were procured from a local dealer at Aligarh and were authenticated by a botanist at Department of Botany, Aligarh Muslim University, Aligarh. A voucher specimen of the same was deposited in the Department of Pharmacology, Aligarh Muslim University, Aligarh. *N. sativa* oil (Kalonji oil) was procured from Mohammedia products, Red Hills, Nampally, Hyderabad, Andhra Pradesh, India. As per manufacturer's information, it was prepared by steam distillation at Hyderabad, India.

Preparation of extract: The seeds were washed thoroughly with water, to remove dust and impurities, and dried in the air. These were grounded into fine powder and 150 grams of grounded seeds were soaked in 150 ml of HPLC grade methanol in a sterile bottle and kept for 7 days at room temperature with stirring with a sterile rod twice daily. It was then filtered using sterile filter paper under UV lamp. The filtrate was kept in a petri dish at room temperature for 3 days to allow the solvent to evaporate. The extract thus prepared was transferred as aliquots of 1 ml each into sterile vials and stored at - 20 °C till further use.

Preparation of Drug impregnated filter paper discs: The extract and oil were diluted using methanol and Ethylene glycol respectively upto dilution of 1:100. During sensitivity testing, 4µl of methanolic extract or oil in pure or diluted form was kept on filter paper disc of 6 mm diameter, placed on Mueller Hinton Agar plate inoculated with bacteria.

Inoculation of plates: This was done using flood-inoculation technique (Acar and Goldstein 1996). Bacterial suspension in Nutrient Broth having turbidity equivalent to 0.5

McFarland was freshly prepared and 2 ml of this was transferred onto the Mueller Hinton Agar plate and distributed gently over the surface of medium with gentle rocking. The excess fluid was removed from the plate and the plate was kept in incubator at 37 °C for 30 mins for drying before application of discs.

Disc susceptibility testing: This was carried out by placing discs impregnated with test material on surface of inoculated agar plates (Bauer *et al.* 1966). For sensitivity testing with standard antibiotics, commercial antimicrobial susceptibility testing discs obtained from HiMedia Laboratories Limited, Bombay were used. The plates were then kept in incubator at 37 °C for 18 hours and diameters of zones of inhibition were measured.

Strains of Coagulase negative staphylococci were isolated from pus, blood, conjunctival swab, and urine (Table 1) of various patients attending Jawaharlal Nehru Medical College Hospital, Aligarh, classified as *Staphylococcus epidermidis* or other Coagulase negative staphylococci by standard microbiological techniques, and tested for their sensitivity to extract, oil as well as a number of other clinically used antibiotics. The concentrations of Antimicrobial sensitivity testing discs used and interpretation of sizes of zones of inhibition were in accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests, NCCLS, 2002 (Wayne 2002). The antibiotics tested and their concentrations were: Ampicillin (10µg/disc), Amikacin (30 µg/disc), Cotrimoxazole (trimethoprim-1.25, sulphamethoxazole-23.75 µg/disc), Cefaclor (30 µg/disc), Chloramphenicol (30 µg/disc), Ciprofloxacin (5 µg/disc), Ceftriaxone (30 µg/disc), Cephalexin (30 µg/disc), Ceftazidime (30 µg/disc), Erythromycin (15 µg/disc), Gentamicin (10 µg/disc), Gatifloxacin (5 µg/disc), Methicillin (5 µg/disc), Ofloxacin (5

µg/disc), Roxithromicin (5 µg/disc), Sparfloxacin (5 µg/disc), Tetracycline (30 µg/disc) and Tobramycin (10 µg/disc).

Statistical analysis: The relationship between dose of extract or oil and zone sizes was evaluated using Spearman's rho test. Zone sizes produced by equal doses of oil and extract were compared by Wilcoxon signed-rank test. Effect of oil and extract on *S. epidermidis* and other Coagulase negative staphylococci was compared using Mann Whitney U test. Further, relationship between sensitivity to various antibiotics and to oil or extract was explored using Chi square test. All statistical analyses were done using SPSS for windows 12.0 version. P value < 0.05 was considered as significant.

Observations

Staphylococcus epidermidis: Thirteen strains were tested which showed variable resistance pattern to the tested antibacterials. Four were resistant to 0-1 antibiotics, 8 to 5-7 antibiotics and 1 was resistant to 10 antibiotics. Resistance was highest to second generation Cephalosporins and Aminoglycosides. Five of these were Methicillin resistant (Figure 1). Twelve out of 13 strains were inhibited by oil which showed activity upto a dilution of 1:100, 1:50, 1:10 and 1:1 against 1, 5, 2 and 2 strains respectively (Tables 1 and 2) in a dose dependant manner ($r = 0.526$, $P < 0.05$). The Methanolic extract produced zones of inhibition significantly larger than oil at concentrations of 1:1 and 1:10. It inhibited 6 strains upto 1:50 dilution, the least concentration tested, and 6 upto 1:10 dilution (Tables 1 and 2) in a dose dependant manner ($r = 0.701$, $P < 0.05$). One strain, which was resistant to 10 antibiotics, was also found resistant to oil as well as extract. Zone sizes produced by equivalent doses of oil and extract were found to be strongly correlated ($r = 0.803$, $P < 0.05$).

Other Coagulase negative Staphylococci: Out of 18 strains tested, 4 were resistant to 1-3 antibiotics, 8 to 4-6 antibiotics, 3 to 8 antibiotics and 1 each to 13 and 15 antibiotics. Resistance was highest for Cotrimoxazole, followed by Tetracycline, Amikacin, Ampicillin, Ciprofloxacin and Tobramycin (Figure 2). The oil inhibited 17 of the above strains dose dependently ($r = 0.581$, $P < 0.05$). It was active against 11 strains upto a dilution of 1:50, against 2 strains upto 1:10 dilution and only in undiluted state against 4 strains (Tables 1 and 2). The Methanolic extract was active against all 18 strains. It showed zones of inhibition significantly larger than oil at concentrations of 1:1 and 1:10 and was active in dilution of 1:50, 1:10 and undiluted state against 9, 4 and 5 strains respectively (Tables 1 and 2) in a dose dependant manner ($r = 0.697$, $P < 0.05$). The zone sizes produced by equivalent doses of oil and extract showed good correlation ($r = 0.647$, $P < 0.05$). Both the oil and extract showed better activity against other coagulase negative staphylococci than *S. epidermidis* but the difference was not significant. No significant relationship was found between sensitivity of bacteria to any of the tested antibiotics and to oil or extract of *N. sativa*.

Results and Discussion

An alarming increase in bacterial strains resistant to a number of antimicrobial agents demands that a renewed effort be made to seek antibacterial agents effective against pathogenic bacteria resistant to current antibiotics. One of the possible strategies towards this objective is the rational localization of bioactive phytochemicals. Traditional healers have long used plants to prevent or cure infectious conditions. Many of these plants have been investigated scientifically for antimicrobial activity and a large number of plant products have been shown to inhibit growth of pathogenic bacteria. A number of these

agents appear to have structures and modes of action that are distinct from those of the antibiotics in current use, suggesting that cross-resistance with agents already in use may be minimal (Zahner and Fiedler 1995). This hypothesis is further supported by our study which failed to find any relationship between the sensitivity of tested strains to *Nigella sativa* and to other commonly used antibiotics.

Nigella sativa oil as well as methanolic extract have been found to possess remarkable antibacterial activity against multidrug resistant Coagulase negative staphylococci. Topozada (1965) reported antibacterial activity of the phenolic fraction of *N. sativa* seeds. Thymol is a phenolic alcohol present in the essential oil (Randhawa and Al- Ghamdi, 2002) that has been reported to possess antibacterial activity (Karapinar and Aktug, 1987). Since Thymol is present in the methanol soluble portion of oil (Enomoto, 2001), it will also be extracted in the methanolic extract. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason and Wasserman 1987).

Toama *et al* (1974) and El-Fatratry (1975) isolated Thymohydroquinone from volatile oil of *N. sativa* seeds and found it to have high antimicrobial effect against gram-positive bacteria. 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 (Basha *et al.* 1995) and thus will be extracted in methanolic extract of seeds also. In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins which may lead to inactivation of

the protein and loss of function (Stern *et al* 1996). Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism (Mason and Wasserman, 1987).

The seeds also contain tannins, which can be extracted by methanol (Eloff, 1998). A number of studies have reported antimicrobial properties of tannins (Scalbert, 1991). One of their molecular actions is to complex with proteins through forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Hashem and El-Kiey 1982). Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, etc.

It may be concluded that *Nigella sativa* oil as well as extract are active against multidrug resistant strains of Coagulase negative staphylococci and may be used, at least topically, in susceptible cases. Further research is needed to advocate its use in systemic infections and to elicit its mechanisms of action.

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Table 1: Sensitivity of Coagulase negative Staphylococci to *Nigella sativa* (Number of strains sensitive to varying dilutions of Extract and oil)

Bacteria	Source	Total	Extract			Oil		
			1:1	1:10	1:50	1:1	1:10	1:50
<i>S. epidermidis</i>	Blood	9	9	9	5	9	7	4
	Pus	4	3	3	1	3	1	1
	Total	13	12	12	6	12	8	5
Other Coagulase negative Staphylococci	Pus	13	13	9	5	12	8	6
	Urine	3	3	2	2	3	3	3
	Conjunctiva	2	2	2	2	2	2	2
	Total	18	18	13	9	17	13	11

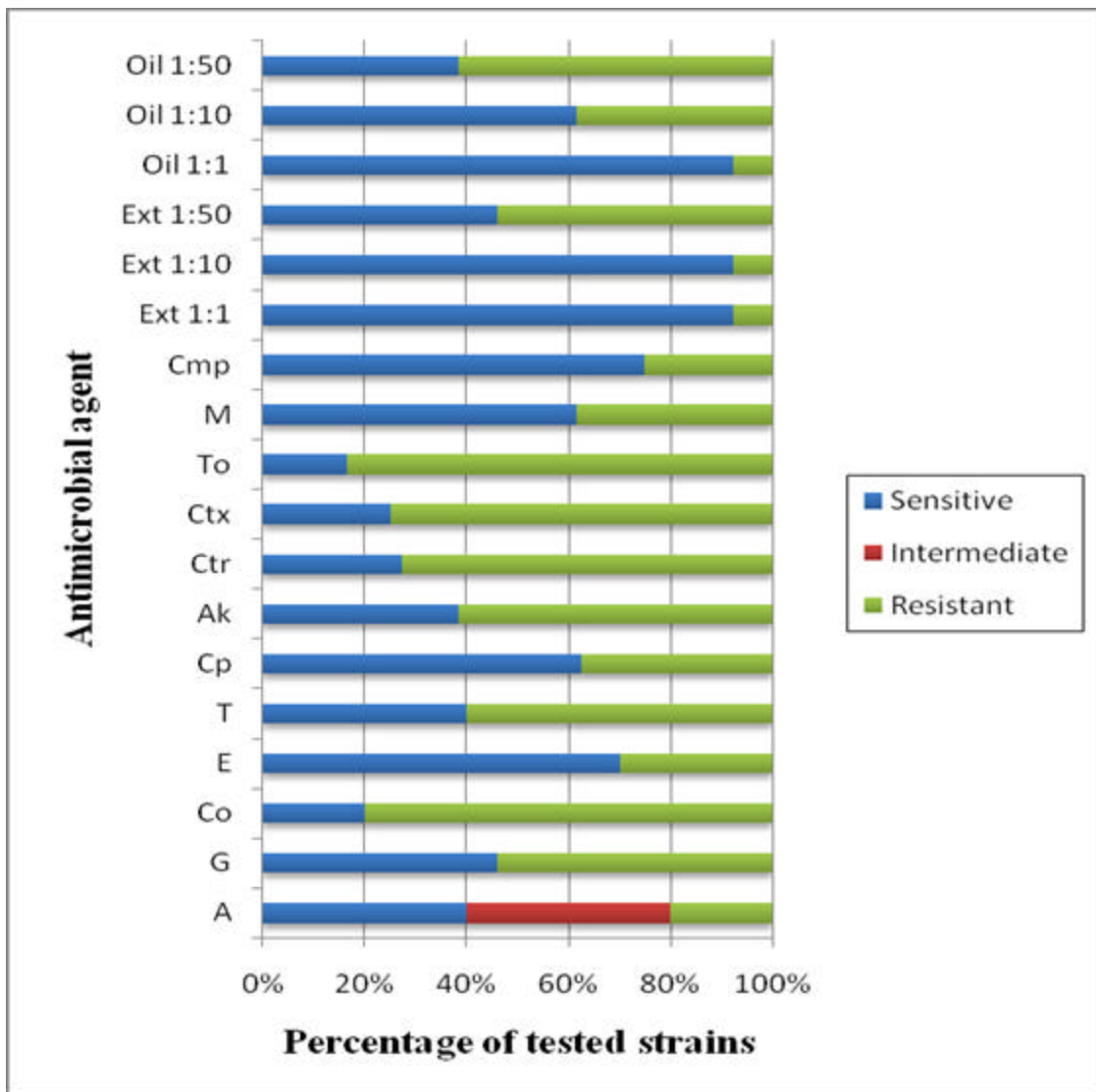
Table 2: Sensitivity of Coagulase negative Staphylococci to *Nigella sativa* (Average zones of inhibition around 6 mm filter paper discs impregnated with varying dilutions of Extract or oil)

Bacteria	Source	Total	Extract			Oil		
			1:1	1:10	1:50	1:1	1:10	1:50
<i>S. epidermidis</i>	Blood	9	38.4	23.9	12.8	26.2	22.3	16.0
	Pus	4	15.3	10.7	10.0	11.3	8.0	7.0
	Average	13	32.7 ^a	20.6 ^b	12.3	22.5	20.5	14.2
Other Coagulase negative Staphylococci	Pus	13	30.7	20.9	15.0	21.1	22.8	14.5
	Urine	3	46.7	28.0	9.0	26.0	13.3	11.0
	Conjunctiva	2	24.0	22.0	12.0	22.0	11.0	10.0
	Average	18	33.0 ^a	22.3 ^b	12.8	22.3	17.6	12.3

a- $P < 0.005$ when compared to oil

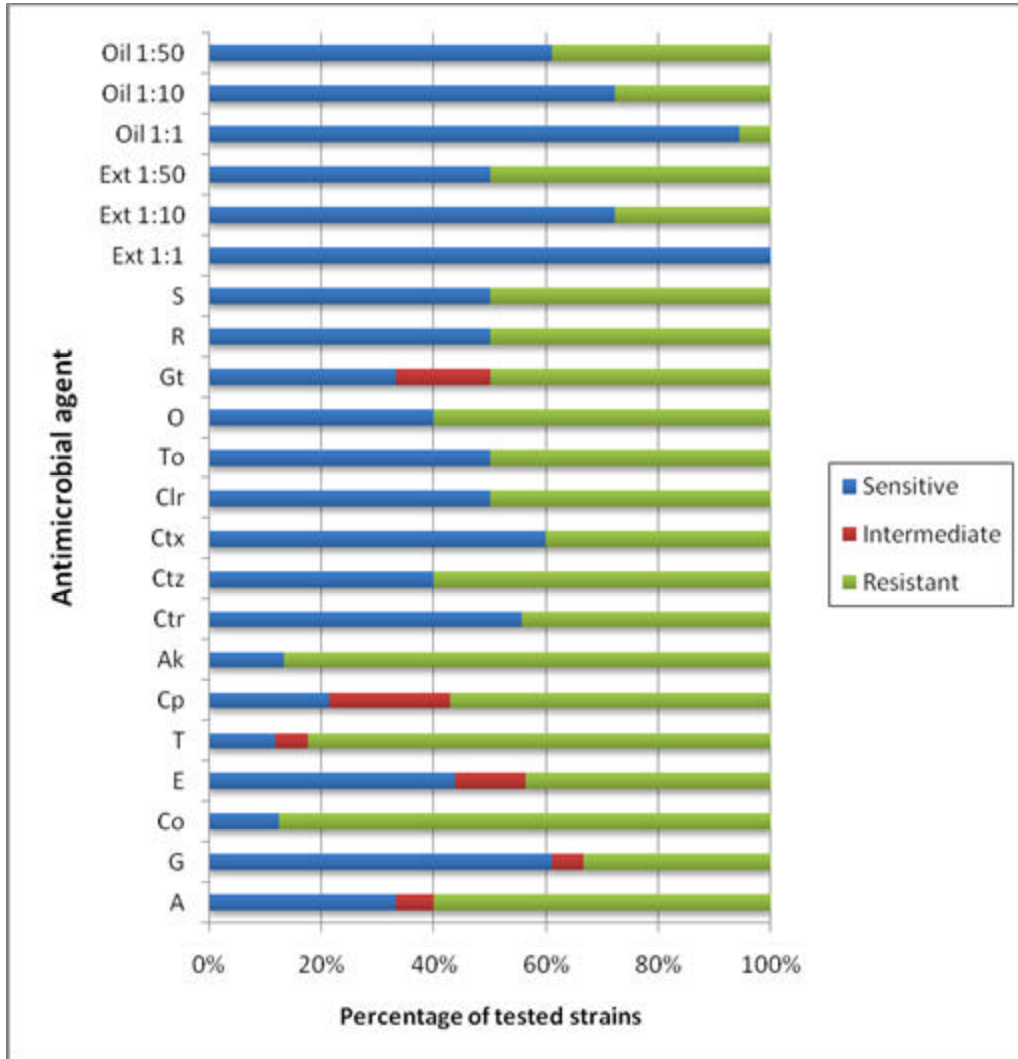
b- $P < 0.01$ when compared to oil

Figure 1



A- Ampicillin, Co- Cotrimoxazole, Cmp- Chloramphenicol, Cp- Ciprofloxacin, Ctr- Ceftriaxone, Ctx- Cefotaxime, E-Erythromycin, G- Gentamicin, M- Methicillin, T- Tetracycline, To- Tobramycin, Ext- *N. sativa* methanolic extract, Oil- *N. sativa* oil

Figure 2



A-Ampicillin, Ak- Amikacin, Co- Cotrimoxazole, Clr- Cefaclor, Cp- Ciprofloxacin, Ctr- Ceftriaxone, Ctx- Cefotaxime, Ctz- Ceftazidime, E Erythromycin, G Gentamicin, Gt- Gatifloxacin, O- Ofloxacin, R- Roxithromicin, S- Sparfloxacin, T- Tetracycline, To- Tobramycin, Ext- *N. sativa* methanolic extract, Oil- *N. sativa* oil

Legend of Figures:

Figure 1 : Sensitivity pattern of *Staphylococcus epidermidis*

Figure 2 : Sensitivity pattern of Other Coagulase negative staphylococci