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Research Article

# Synergism Between *Nigella sativa* Seeds Extract and Synthetic Antibiotics Against Mec A Gene Positive Human Strains of *Staphylococcus aureus*

[1]Bushra Uzair, [2]Abdul Hameed, [3]Sidrah Nazir, [4]Barkat Ali Khan, [5]Fehmida Fasim, [6]Samiullah Khan and [7]Farid Menaa

[1]Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad-44000, Pakistan
[2]Institute of Biomedical and Genetic Engineering (IBGE), 24-Mauve Area, G-9/1, KRL, Islamabad, Pakistan
[3]Department of Biochemistry, PMAS Arid Agriculture University, Rawalpindi, Pakistan
[4]Faculty of Pharmacy, Gomal University, Dera Ismail Khan, Pakistan
[5]Department of Biological and Environmental Sciences, Qatar University, Doha, Qatar
[6]Gomal Center of Biochemistry and Biotechnology, Gomal University, Dera Ismail Khan-29050, KPK, Pakistan
[7]Fluorotronics USA, San Diego, California, United State of America

## Abstract

**Background and Objective:** Synergistic combinations of various antimicrobial agents have been introduced as extra successful strategies to combat multidrug resistant (MDR) infections. This study was undertaken to evaluate MDR profiling of *Staphylococcus aureus* strains isolated from clinical specimens of two public hospitals of twin Pakistani cities as well as to explore *in vitro* antibacterial potential of *Nigella sativa* L. (black cumin or black seeds) extract against Mec A gene positive *Staphylococcus aureus* strains. **Materials and Methods:** The MDR strains were then screened for positive Mec A gene by PCR and sequencing. A total of 500 bacterial strains were subjected to antimicrobial and *Nigella sativa* seeds susceptibility and minimal inhibitory concentration assays. The data were evaluated as Mean±Standard deviation of five independent experiments. The results were analyzed using t-test with SPSS version 16.0. **Results:** Methanolic extracts of *Nigella sativa* seeds showed maximum activity against Mec A gene positive *Staphylococcus aureus* alone and in combination with the penicillin antibiotic Augmentin® plus the second-generation Cephamycin, Cephalosporins, Mefoxin® (Cefoxitin). The anti-MRSA activity was reduced when methanolic extracts of *Nigella sativa* seeds were used in combination with the pain reliever and the fever reducer paracetamol (acetaminophen). Eventually, it has been observed that the anti-staphylococcal activity of *Nigella sativa* led to changes in bacterial cell morphology indicating that the cell wall of Gram-positive bacteria as likely a target of action. **Conclusion:** This study provides new insights about synergistic antimicrobial and *Nigella sativa* crude extract activities against MDR *Staphylococcus aureus* strains.

Key words: *Nigella sativa* synergism, β-lactam antibiotics, strategies against MDR, methicillin resistant *S. aureus*

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Corresponding Author: Samiullah Khan, Gomal Center of Biochemistry and Biotechnology, Gomal University, Dera Ismail Khan-29050, KPK, Pakistan
Tel: 0092-966-750131/0092-3367228768



Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The third significant cause of mortality around the world is infectious diseases as estimated by World Health Organization[1]. Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections have become a global health problem particularly in hospital setup causing simple skin infections to life-threatening infections[2,3]. In many developing countries, including Pakistan, the situation appears depressing due to inadequate implementation of policy on infection control, lack of political will, too little resources, poor motivation of health care workers and researchers[4-6]. *Staphylococcus aureus* (*S. aureus*) is a Gram positive bacterium and is known to develop quick resistance to antimicrobial agents. *Staphylococcus aureus* usually cause community acquired post-operative infections, endocarditis, toxic shock syndrome, food poisoning and osteomyelitis[7-9].

Genetic mutation was involved in the development of resistance of antibiotic. The attainment of Mec A gene by horizontal transmission by conjugation was the main cause of antibiotic resistance in *S. aureus*[10]. In bacterial genome mobile hereditary component called *Staphylococcal* chromosome cassette (SCC*mec*) carried Mec A gene[11,12]. The altered penicillin binding protein abbreviated as PBP2a is coded by Mec A gene that has a low affinity for antibiotics belongs to beta lactam, the active site of altered PBP2a cannot bind to methicillin or other β-lactam antibiotics[13,14].

The increasing incidence of drug-resistant pathogens has drawn the attention of the pharmaceutical and scientific communities towards studies on the potential antimicrobial activity of plant-derived substances, which are used in traditional medicine in different countries[15-17]. The use of and search for drugs derived from plants have accelerated in recent years. Ethnopharmacologists, botanists, microbiologists and natural-products chemists are working in collaboration to find new plant based antibiotics for treatment of infectious diseases[18,19]. 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[20,21]. Scientists from divergent fields are investigating plants with an eye to their antimicrobial usefulness. A sense of urgency accompanies the search as the pace of species extinction continues[22]. Laboratories of the world have found literally thousands of phytochemicals which have inhibitory effects on all types of microorganisms *in vitro*[19]. More of these compounds should be subjected to animal and human studies to establish their efficacy in whole-organism systems, including in particular toxicity studies as well as an examination of their effects on advantageous normal microbiota. It would be useful to standardize methods of extraction and *in vitro* testing so that the search could be more systematic and interpretation of results would be facilitated[23].

*Nigella sativa* (*N. sativa*) (Family Ranunculaceae) is a broadly used medicinal plant throughout the world. It is very popular in a variety of traditional systems of medicine like Unani and Tibb, Ayurveda and Siddha. Seeds and oil have a long history of folklore usage in various systems of medicines and food. The seeds of *N. sativa* have been broadly used in the treatment of different diseases and ailments[24]. In Islamic literature, it is well thought-out as one of the best forms of healing medicine. It has been suggested for using on regular basis in Tibb-e-Nabwi (Prophetic medicine). It has been widely used as antihypertensive, liver tonics, diuretics, digestive, anti-diarrheal, appetite stimulant, analgesics, anti-bacterial and in skin disorders. Due to its miraculous power of healing, *N. sativa* has got the place among the top ranked evidence based herbal medicines. It has a potent bioactive known as thymoquinone which shows promise in treating epilepsy, allergies and boosting the immune system[25]. The ethnobotanical studies showed it is a medicinal plant essential in the treatment of microbial infections[26]. Bakathir and Abbas indicated that the aqueous extract of *N. sativa* had a significant antibacterial activity[27]. However, as a further study in cognizance of challenges of multidrug resistant bacteria, its antibacterial effect in combination with different first-line antibiotics commonly used against infectious agents needs to be investigated. The synergistic antimicrobial effects of crude methanolic extract in combination with β-lactam antibiotics will advance new knowledge because it has not yet done.

The aim of this study was to screen the antibacterial activity of *N. sativa* (black seeds), which is commonly used in the Pakistani population. Aqueous and methanolic preparations of plant extracts were tested for antibacterial activity against a *S. aureus* strains isolated from diverse clinical specimens. *In vitro* synergistic potential of *N. sativa* seeds extract with second generation cephalosporins and augmentin, a β-lactamase inhibitor was determined. Their effects on bacterial morphology were also determined.

## MATERIALS AND METHODS

**Reagents:** All solvents (ethanol, ethyl acetate, methanol, dimethylsulfoxide (DMSO) were purchased from Merck, Germany. Nutrient media: Mueller-Hinton broth and Mueller-Hinton agar were purchased from Oxoid, Hamshire, UK. Standard discs of antibiotics (amoxicillin, chloramphenicol, streptomycin, tetracycline, rifamycine) were from Oxoid, Hamshire, UK.

**Plant material:** *Nigella sativa* (seeds) were obtained commercially from the market. Dried, ground seeds (50 g for each extract) were extracted with water, ethanol, methanol and ethyl acetate by maceration. After being concentrated, stock solutions of extracts (80 mg $mL^{-1}$) were obtained by dissolving a certain amount of crude extract in solvent (5% DMSO). The yields of the extracts were 11.6 g for water extract, 5 g for ethanol extract and 5.3 g for ethyl acetate extract and 15 g for methanol.

**Isolation of *S. aureus* from clinical specimens:** Total 500 different strains of *S. aureus* were collected from Pakistan Institute of Medical Sciences hospital Islamabad and Holy Family hospital Rawalpindi, Pakistan over a period of one year (Dec., 2014-Jan., 2016). Samples collected were of different nature such as blood, pus, wounds, trachea and ear discharge. Blood agar and Mueller Hinton agar media were used to inoculate the samples. *Staphylococcus aureus* was identified on the basis of colonial, cellular morphology and biochemical test like catalase test, coagulase test, DNase test. Identification was also confirmed by 16S rRNA gene sequencing by using GAGTTTGATCCTGGCTCAG and AGAAAGGAGGTATCCAGCC forward and reverse primer sequence, respectively.

**Inoculum preparation:** Bacterial suspension was prepared from overnight cultures by the direct colony method. Colonies were taken directly from the plate and suspended into 5 mL of sterile 0.85% saline. The turbidity of initial suspension was adjusted comparing with 0.5 McFarland standard. When adjusted to the turbidity of a 0.5 McFarland standard, a suspension of bacteria contains about $10^8$ Colony Forming Units (CFU) $mL^{-1}$. Ten-fold dilutions of initial suspension were additionally prepared into sterile 0.85% saline to achieve $10^6$ CFU $mL^{-1}$.

**Antibiotic sensitivity profiling:** Antibiotic susceptibility testing was done for *S. aureus* strains under the standard CLSI guidelines[28]. *Staphylococcus aureus* was cultured on Mueller-Hinton agar. Different antibiotic drugs (chloramphenicol, gentamicin, clindamycin, ceftoxitin, sulfamethoxazole) discs were used to check their sensitivity. Antibiotic discs were placed on the surface of a Mueller-Hinton agar that has been inoculated with *S. aureus*. After 18-24 h the diameter of zone of inhibition were measured according to reference tables[29].

**Molecular characterization of *S. aureus*:** The MRSA isolates were subjected to 16S rRNA sequencing. The PCR was done on following conditions; 94°C for 4 min followed by 94°C for 1 min then 57°C for 1 min and 72°C for 1 min and at the end 72°C for 7 min. Reaction volume was 30 µL with (16S) forward (GAGTTTGATCCTGGCTCAG) and reverse (AGAAAGGAGGTATCCAGCC) primer sequence.

**Detection of Mec A gene by PCR and sequencing of Mec A gene:** Out of 500 isolated *S. aureus* strains from diverse clinical specimen 278 strains showed resistance to more than one antibiotic. Multiple antibiotic resistant strains were selected for screening Mec A gene positive strains. Cells were suspended in a lysis buffer containing 1 M tris HCl, 5 M NaCl and 0.1 M EDTA, which was incubated at 95°C for 10 min. After incubation, the suspension was centrifuged at 23 000×g for 5 min. The supernatant was used as a template in PCR. Three step PCR method was carried out using XP thermal cycler[29]. Published primer Mec A were used for amplification[30,31]. The final PCR products were visualized using UV-transilluminator after electrophoresis on 1.5% agarose gel containing 50 mg $mL^{-1}$ EtBr.

**Primer for Mec A gene:** For amplification of 163 bp region forward primer sequence (CTGGTGAAGTTGTAATCTGG) and reverse primer sequence (ACTGCTATCCACCCTCAAAC) were used.

**DNA sequencing on ABI3130 genetic analyzer:** After electrophoresis the amplified PCR product was purified to be further used for sequencing reaction. For sequencing reaction 4 µL of big dye, 5 µL purified PCR product, reverse and forward primers 1 µL were added to make total reaction volume of 10 µL. Sequencing conditions were 96°C for 1 min for one cycle, then 96°C for 15 sec and 55°C for 4 min for 25 cycles. Product of sequencing reaction was purified. Sequencing was run using ABI 3130 genetic analyzer and results were analyzed.

**Antimicrobial activity of crude extract of seeds of *N. sativa*:** A total of 500 bacterial strains were subjected to antimicrobial susceptibility and MIC assays. In addition, reference strains such as *S. aureus* ATCC 25923, was run each time as control for susceptibility and MIC assays. Plant extracts were screened for antimicrobial activity using the agar well diffusion method. Bacterial cultures were inoculated in LB broth for 3 h at 37°C and turbidity was adjusted in Phosphate Buffered Saline (PBS) to 0.5 McFarland's index. Using a sterile cotton swab, a bacterial lawn was spread on 90 mm MHA plates containing 6 mm wells. Twenty microliters of plant extracts were poured into each well and plates were incubated at 37°C aerobically for 18 h. Plates were incubated in microaerophilic conditions

using a Campygen kit (Oxoid, Hamshire, UK) at 37°C for 48 h. The diameter of the zone of bacterial inhibition around each well was measured[32].

**Determination of MIC and MBC:** Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentration (MBC) were determined by agar dilution and micro broth dilution assays using MH agar and MH broth, respectively[33]. The concentrations of the extracts tested ranged from 5000-50 μg $mL^{-1}$. In the case of agar dilution, experiments were performed on MH agar plates containing various concentrations of extracts (1:20 v/v). Plates were inoculated with 10 μL of the test organism containing $10^5$ CFU log phase bacteria per spot and a total of 20 spots were tested on one plate. In the case of the micro broth dilution assay, sterile flat-bottom 96-well plates were loaded with 100 μL of two-fold dilutions of extracts into each well. The starting bacterial inoculums were $1.5\times10^5$ CFU $mL^{-1}$. Plates without any plant extracts were served as growth control. The assay plates were incubated as described in this study. The highest dilution of extract that showed no visible bacterial growth per spot and no turbidity in agar dilution and micro broth dilution assay respectively was considered as MIC.

To determine Minimum Bactericidal Concentration (MBC) of extracts, 100 μL of MH broth from each well of micro broth assay was sub-cultured on MH agar plates after 24 h of initial incubation. The MH plates were incubated for another 24 h. The lowest concentration of extract that resulted in no bacterial growth was considered as MBC. Experiments were performed in quadruplicate on five different occasions.

**Synergistic antimicrobial assays:** Synergistic activity of *N. sativa* extract with second-generation cephalosporins and augmentin against Mec A gene positive human strains of *S. aureus* was determined. *Nigella sativa* seeds methanolic and aqueous extracts were tested against representative strains of *S. aureus* ATCC 25923 and isolated clinical strains. Each combination was first tested by the checkerboard titration method using MH broth in microtiter plates as described previously and by disk diffusion method[34]. Concentrations of extracts ranged from 5000-5 μg $mL^{-1}$. The antimicrobial activity of the extract and antibiotic combination was interpreted as one of the following categories: Synergy, additive effect or antagonism. The Fractional Inhibitory Concentration (FIC) of each agent was calculated as the MIC of the agent in combination, divided by the MIC of the agent alone. The interpretation was made on the basis of the fractional inhibitory concentration index (ΣFIC), which is the sum of the FICs of both agents. The FICI results were interpreted as follows: <0.5 synergy, 0.5-1 additive effect, 1-2 indifferent or no effect and >2 antagonism[35].

**Time kill kinetic assay:** Time kill kinetic assays were performed on successful synergistic combinations obtained by checkerboard titration methods and disc diffusion assay. Flasks containing 100 mL MH broth and the drug-plant extract combination were inoculated with a log phase culture of the test organism to a density of $1\times10^5$ CFU $mL^{-1}$. Test strains included *S. aureus*. Individual components of each combination, either extract or antibiotic, were added to the control flask to compare the effects of the synergistic combinations to their individual effects on the bacterial growth curve, while no drug was added to the growth control flask. Flasks were incubated for 24 h at 37°C. Aliquots (100 μL) were collected at different time intervals from each flask, serially diluted in PBS and cultured on MHA plates to obtain colony counts[35,36]. Curves were constructed by plotting the $\log_{10}$ of CFU $mL^{-1}$ versus time. Synergy was defined as $\geq$2 $\log_{10}$ decreases in CFU of organisms treated with the drug combination compared to the most active component of the alone as described previously[37].

**Phase contrast microscopy:** On the basis of biological activity, bacterial strains treated with sub-inhibitory concentrations (0.5×MIC) of *N. sativa* seeds methanolic extract was subjected to phase contrast microscopy to see their effects on bacterial cell morphology. Exponential phase cultures ($A_{600}$, 0.05) of *S. aureus* grown in Luria broth were treated with Minimal Inhibitory Concentration (MIC) and sub lethal concentration (1/2 MIC) of *N. sativa* methanolic extract. Slides were immediately made after treatment in 1% agarose using 4 μL of antibacterial compound (methanolic extract of *N. sativa*) treated cultures, time lapse images were obtained under phase contrast by OMAX 40X-2500X Phase Contrast LED Trinocular Compound Microscope, USA. Particular care was taken to minimize the sample exposure to UV light. Image grabbing were performed essentially as described by Edwards and Errington[38].

**Statistical analysis:** It is a retrospective study, therefore, both descriptive and influential statistical analysis was considered. The data were evaluated as Mean±Standard deviation of five independent experiments. The results were analyzed using t-test with SPSS version 16.0. The $p<0.001$ were considered significant.

## RESULTS

**Antimicrobial resistance profiling of isolated *S. aureus* strains:** The antibiotic resistance pattern of all the isolated *S. aureus* strains from diverse clinical samples are presented in Fig. 1. Distribution of *S. aureus* in clinical isolates is presented in Fig. 2. *In vitro* susceptibility test in Fig. 1 shows that, out of the 5 antibiotics tested, cefoxitin worked best on all *S. aureus* isolates including MRSA while clindamycin was observed to be the least effective.

**Molecular characterization of MRSA (*S. aureus*):** The confirmed MRSA both with Mec A positive and cefoxitin resistant codes SA 13 and SA 14 were subject to molecular characterization for genetic confirmation of *S. aureus* using standard 16S r RNA standard protocol. Results revealed 97% identity to reference strain *S. aureus* subsp. *aureus* ATCC 35844 (Fig. 3). Accession no D83355 when analyzed by genome database on ezbiocloud. Accession no given to the new sequence by gene bank was SUB927972 SA_13 KR336552.

**Mec A gene detection in multiple antibiotic resistant *S. aureus*:** Out of 500 samples 200 confirmed MRSA were selected on the basis of antibiotic resistance and analyzed for Mec A gene detection by PCR. Amplified product of 163 bp was observed in 104 samples when checked on 1.5% agarose gel by electrophoresis (Fig. 4).

**Sequencing of Mec A gene:** The Mec A positive MRSA confirmed by PCR codes SA_02 and SA_03 were further processed for gene sequencing by ABI3130 Genetic Analyzer. The genetic analysis data revealed 100% identity to reference strain *S. aureus* subsp., *aureus* MSHR 1132 and M0480 when blast to genome database on ezbiocloud. All samples were resistant to drug cefoxitin but only 52% were Mec A gene positive.

**Black seed extract antimicrobial activity assay:** Methanolic extract of *N. sativa* extract showed excellent anti MRSA activity, the zone of inhibition was in range of 18-25 mm in diameter as compare to aqueous extract exhibiting zone of inhibition in the range of 11-14 mm (Table 1).

**Antimicrobial activity of seeds of *N. sativa* extracts:** The most potent inhibitory effect of *N. sativa* seed extract was observed in methanolic extract against MRSA strains with MICs values in the range of 0.39-1.5 mg $mL^{-1}$ ($p<0.001$) and zone of inhibition of more than 18 mm whereas, aqueous extract exhibited zone of inhibition in range of 11-14 mm with MICs in range of 0.22-1.5 mg $mL^{-1}$ ($p<0.001$) for clinical *S. aureus* strains (Table 1). Interestingly, 2-4-fold increases in MIC were found when drug-sensitive clinical strains of *S. aureus* were tested, but the MIC/MBC ratio was 0.5 in both cases. The solvent control (5% DMSO) did not inhibit growth of the tested bacteria. Our data clearly indicate strong antimicrobial activity of crude extract of seeds of *N. sativa* against Gram-positive organism, particularly methicillin resistant S. *aureus* and multiple drug resistant *S. aureus*.

**Synergistic activity of plant extracts with antimicrobial agents:** Two different antibiotics (augmentin and cefoxitin) were used for evaluation of synergistic activity of crude extract and antibiotic. When *N. sativa* methanolic extract was combined with ceftoxitin, it was able to inhibit methicillin resistant *S. aureus* and reduction in MICs was observed against *S. aureus*. In this combination, cefoxitin was able to inhibit MRSA strains at 0.312 μg $mL^{-1}$, which was 64 times lower than the MIC of ceftoxitin alone. When *N. sativa* seed extract was combined with augmentin, it was able to inhibit *S. aureus* at sub-MIC levels. A significant reduction was also observed in MICs which explains the strong synergy (FICI 0.37) in this combination. Synergism was also studied by disc diffusion method, enlargement in sizes of zone of inhibition was observed in both the antibiotica and extract combination.

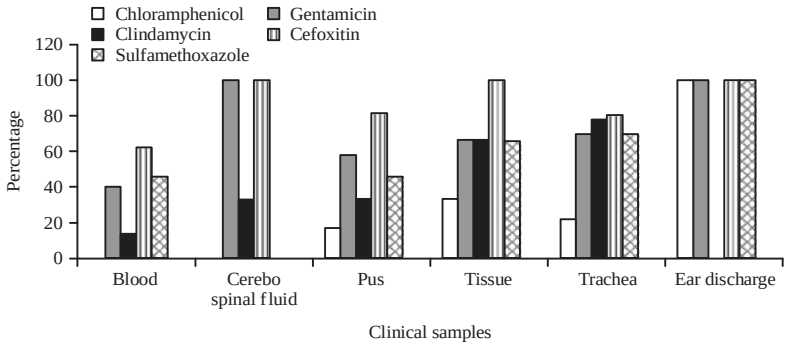


Fig. 1: Antibiotic resistance profiling of MRSA strains isolated from clinical samples

Table 1: Anti MRSA activity of *Nigella sativa* seeds extract in different solvent system

*Staphylococcus aureus*

| | MIC (mg mL$^{-1}$) | | | | MBC (mg mL$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| Clinical samples | Aqueous extract | Zone (mm) | Methanolic extract | Zone (mm) | Aqueous extract | Methanolic extract | R-Type | Significant p-value |
| Blood | 0.75±0.03 | 12.0 | 0.39±0.03* | 25 | 1.5±0.12 | 0.50±0.05* | MRSA | (p-value<0.001) |
| CSF | 0.22±0.12* | 11.0 | 0.39±0.04 | 25 | 1.5±0.20 | 0.50±0.03* | MDR | (p-value<0.001) |
| Pus | 3.50±0.50 | 13.0 | 1.25±0.09* | 22 | 3.3±0.40 | 1.25±0.12* | MSSA | (p-value<0.001) |
| Tissue | 0.22±0.05* | 12.5 | 1.25±0.11 | 21 | 0.4±0.08* | 1.50±0.08 | MRSA | (p-value<0.001) |
| Tracheae | 0.75±0.22* | 12.0 | 1.50±0.22 | 18 | 1.5±0.25* | 2.50±0.15 | MRSA | (p-value<0.001) |
| Ear discharge | 3.70±0.20 | 14.0 | 0.78±0.08* | 20 | 3.4±0.30 | 1.25±0.11* | MDR | (p-value<0.001) |
| Reference strain | 3.12 | 13.0 | 1.50±0.15 | 18 | 3.7 | 1.5 | ATCC | - |

*Statistically significant value (p<0.001), MRSA: Methicillin resistant *Staphylococcus aureus*, MDR: Multidrug resistant, n: number of isolates, R: Type resistant phenotype, MIC: Minimal inhibitory concentration, MBC: Minimal bactericidal concentration

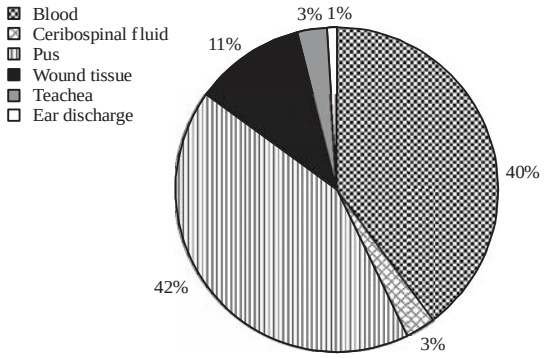


Fig. 2: Distribution of *Staphylococcus aureus* in clinical isolates

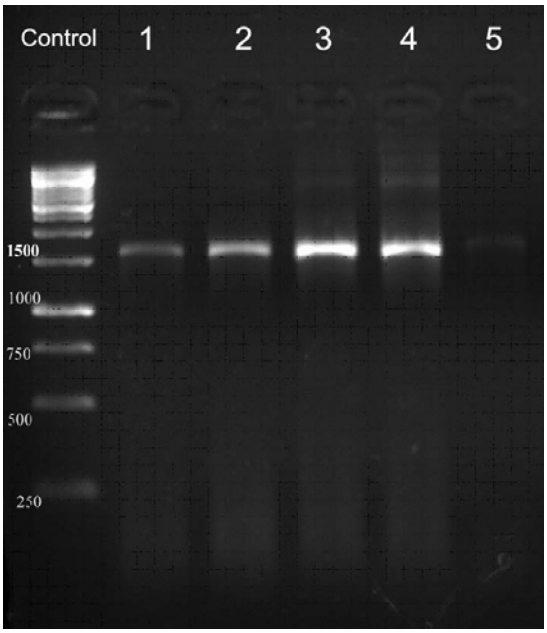


Fig. 3: PCR amplification of 16 S RNA for *Staphylococcus aureus* (MRSA)

In general, the zones of inhibition in antibiotic per plant extract plates were in the range of 05-10 mm wider than the zones of inhibition in the control plates. Result revealed positive synergism between drug cefoxitin when used with methanolic extracts of *N. sativa* seeds. There was significant increase of 9 mm in the diameter of zone of inhibition when cefoxitin and *N. sativa* used in combination (Fig. 5). Clear zone for black seed were of sizes 24 mm, augmentin 38 mm and cefoxitin 16 mm when used alone and increased to 30 mm for black seed plus cefoxitin and 40 mm augmentin plus black seeds. In case of paracetamol negative synergism was observed. Size of zone of inhibition reduced from 11-8 mm when used in combination with black seeds (Fig. 5). Combining effects of cefoxitin with tested extracts showed the inhibition zones 8-9 mm wider.

**Phase contrast microscopy:** *Nigella sativa* extract treated log phase cells of *S. aureus* were observed under phase contrast microscope and time-lapse images were captured. Two significant morphological changes were observed. When cells of *S. aureus* (ATCC33591) were treated with this compound complete lysis was observed immediately after treatment. Whereas, when cells of *S. aureus* were treated with sub-lethal concentration of *N. sativa* extract, morphology of treated cells was not similar to that of the bacteria in the control culture and some very large cells were observed (Fig. 6, 7).

**Time kill kinetic assay:** Time kill kinetics showed a dose dependent bactericidal effect on methicillin-resistant and sensitive *S. aureus* strains, indicating strong anti-Staphylococcal activity of methanolic extract of black seeds of *N. sativa* irrespective of the spectrum of resistance against other antimicrobial agents (Fig. 8). The data clearly indicate strong antimicrobial activity of *N. sativa* against Gram-positive organisms, particularly *Staphylococcus* species.

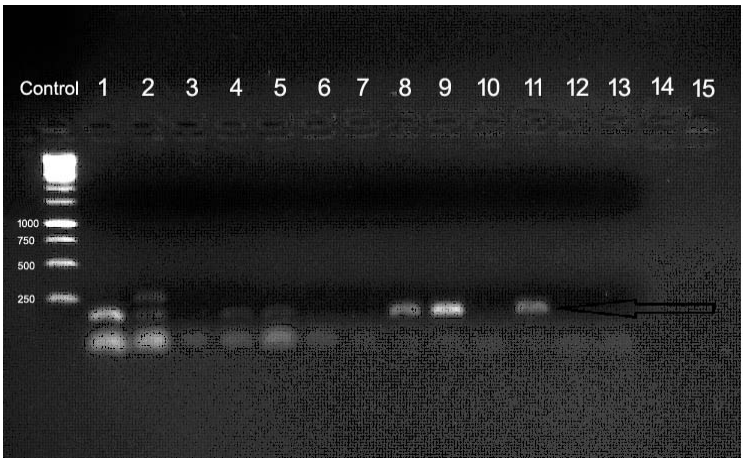


Fig. 4: Mec A gene amplification by PCR with 1 kb DNA ladder
Control: Contained 1 kb DNA ladder, Lane 1-6,8,9 and 11: Mec A positive and were confirmed MRSA, Lane 7, 8, 11, 13 and 14: Mec A negative and MSSA, Lane 1: Two bands for Mec A, Size of Mec A gene was 163 bp

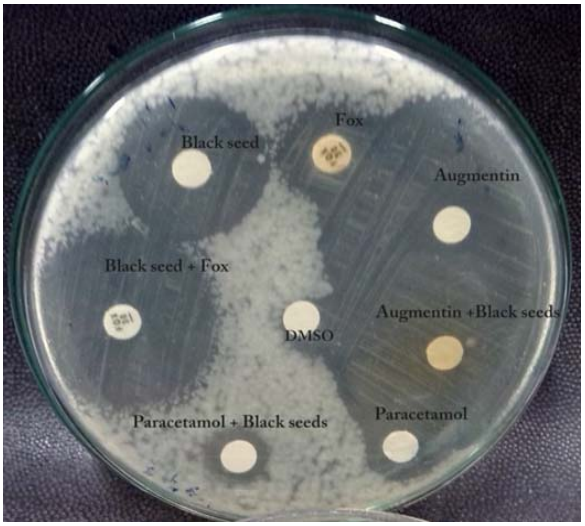


Fig. 5: Synergism between black seed methanolic extracts with antibiotics (Cefoxitin and augmentin) and non-antibiotics (Paracetamol)

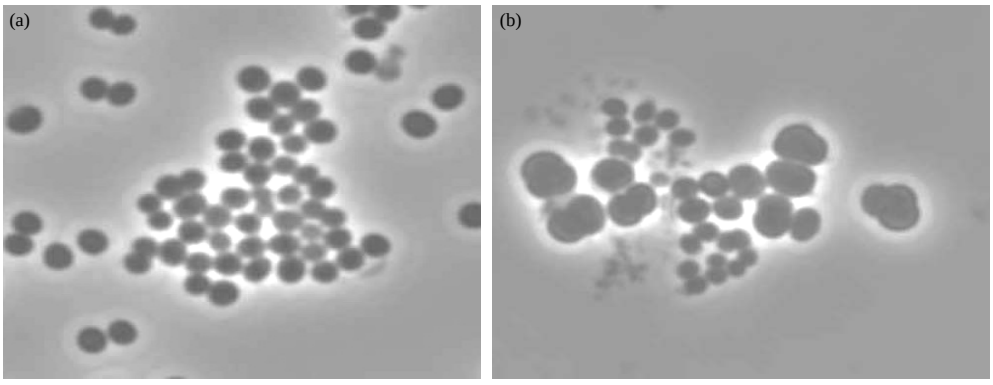


Fig. 6(a-b): Phase contrast images live cells of *S. aureus* (ATCC33591) treated with sub lethal concentration (1/2 MIC) of *Nigella sativa* (a) Control cells without treatment and (b) Cells treated with *Nigella sativa*

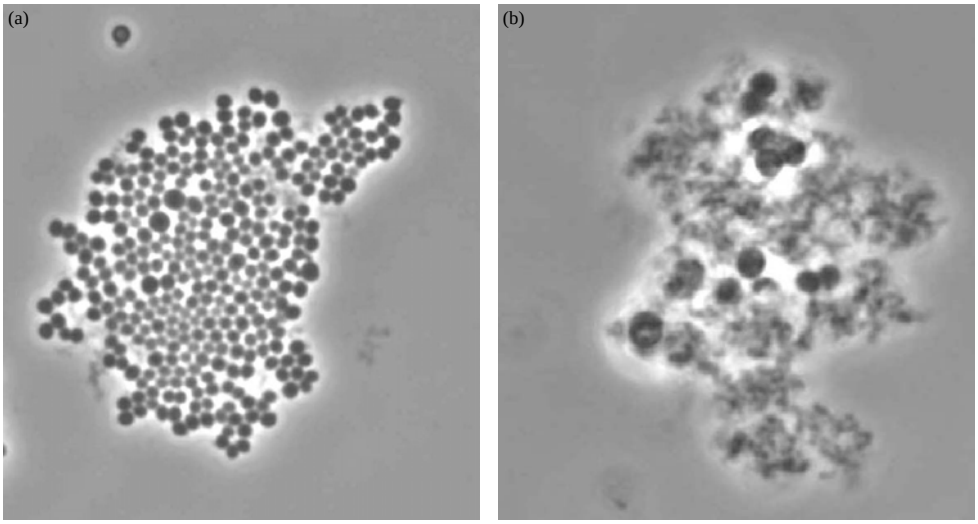


Fig. 7(a-b): Phase contrast images of live cells of *S. aureus*. Live cells of *S. aureus* treated with MIC concentration of methanolic extract of *Nigella sativa* (a) Control cells without treatment and (b) Cells treated with extract (lysis observed)

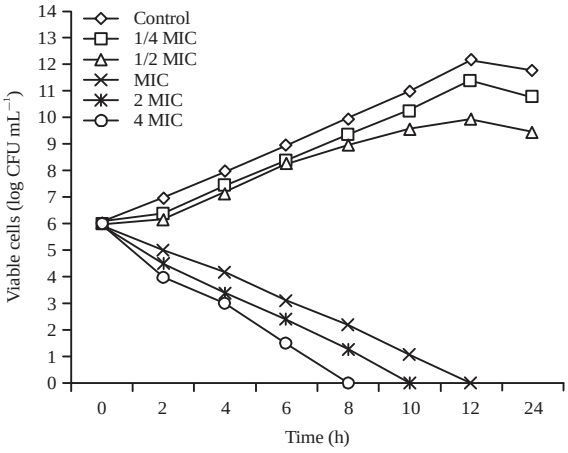


Fig. 8: Effect of crude extract of *Nigella sativa* seeds on bacterial growth kinetics

## DISCUSSION

The present study focused on evaluation of the pre-identified potent herbal extract of seeds of *N. sativa* in combination with cefoxitin, a third-line antibiotics and Augmentin a β-lactamase inhibitor, antibiotic against Mec A gene positive *S. aureus*. In this study methanolic extract of *N. sativa* showed good activity against Mec A gene positive *S. aureus* (Fig. 3). There were considerable alternative sources of natural antimicrobials from plants with different mode of actions, some of them are employed in traditional medicine for centuries and was found to have competitive effects compared to some commercial antibiotics[23,39]. It was also reported in a review by Naz[40] that *N. sativa* extracts were shown to have outstanding *in vitro* antibacterial activity against Gram positive bacteria[40]. Other researchers also reported antibiotic synergism with medicinal plants[18,40]. Development of synergistic herbal-synthetic combinations is a major tool to augment the activity of existing antibiotics thereby reducing, alleviating or delaying the resistance process[41-46].

Although, the antimicrobial activity of *N. sativa* plant's extract has been previously reported[47], but little is known about their interaction with antimicrobial agents which are commonly prescribed to treat MDR bacterial infections. Here the synergistic activity of *N. sativa* extract with a wide range of antimicrobial agents was tested. *Nigella sativa* showed synergistic activity with cefoxitin (third line antibiotic) and amoxicillin against MDR *S. aureus* strains. Since *N. sativa* is bacteriostatic for Gram positive cocci, so it is hypothesized that this combination might prevent the recurrence of bacterial growth caused by antibiotic selective mutants towards the end of the disease's course. Therefore, it is worthwhile to test *N. sativa* and its compounds for synergistic activity with third line antibiotics *in vivo*.

It was very surprising to see that *N. sativa* was able to reverse augmentin resistance in MRSA. Augmentin is a β-lactam antibiotic that inhibits cell wall peptidoglycan through binding and competitive inhibition of Penicillin Binding Proteins (PBPs)[12]. Activation of Mec A gene and gene variants led to the formation of PBP2a, which binds with β-lactam antibiotics in lower affinity, resulting in drug

resistance[11,12]. Therefore, unavailability of PBP2a might resume antimicrobial action of oxacillin in MRSA strains. Already it has been shown that *N. sativa* alone induces ultrastructure changes in MRSA, indicating significant changes in the molecular machinery of bacterial cells. It is speculated that the addition of *N. sativa* might interfere in the synthesis or transportation of PBP2a levels on the cell membrane. Previous studies also showed similar effects on oxacillin resistance by different compounds such as phenothiazine, chlorpromazine, thioridazine, inducing physiological damage to bacterial cell membranes[48,49]. Further studies are warranted to understand the exact mechanism of action. This study is the first to report the synergistic antimicrobial effects of *N. sativa* in combination with β-lactam antibiotics. In a separate study, these extracts were tested for toxic effects on human RBCs. *Nigella sativa* extract was found to be non-toxic on the concentrations tested for antimicrobial activity. The synergistic antimicrobial effects of crude methanolic extract in combination with β-lactam antibiotics provided the evidence to advance the study by separating, purifying and analyzing the different bioactive components present in crude extract of *N. sativa*. Then structural characterization of the compounds that are responsible for anti-MRSA activity should be carried out by using cutting edge technologies.

## CONCLUSION

On the basis of results of antibacterial assay of this study *S. aureus* was found to be more sensitive to methanolic extract of *N. sativa* as compared to aqueous extract. It was also concluded that black seeds extract worked well in combination with augmentin and cefoxitin while the anti-MRSA activity is reduced when black seeds extract was used in combination with a non-antibiotic paracetamol.

## SIGNIFICANCE STATEMENTS

Development of synergistic herbal-synthetic combinations is a major tool to augment the activity of existing antibiotics thereby reducing, alleviating or delaying the resistance process. Ethnobotanical studies showed it is a medicinal plant which is essential in the treatment of microbial infections. Data obtained in the study clearly indicated strong antimicrobial activity of methanolic crude extract of seeds of *N. sativa* against Gram-positive organism, particularly methicillin resistant *S. aureus* and multiple drug resistant *S. aureus*. This study is the first to report the synergistic antimicrobial effects of *N. sativa* in combination with β-lactam antibiotics.

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