Activity Guided Isolation and Purification of Antimicrobial Peptides from *Nigella Sativa* Seeds

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In present investigation, isolation and purification of antimicrobial peptides/proteins was carried out from *Nigella sativa* (Kalongi) seeds. Bioactivity of seed extracts was tested against a set of multi-drug resistant bacterial (*Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pasturella multocida*) and fungal (*Aspergillus niger, Alterneria alterneria, Aspergillus flavis and Rhizopus solani*) strains. *Nigella sativa* showed promising antibacterial activity against *B. subtilis, P. multocida* and *E. coli*, whereas poor antibacterial activity was found against *S. aureus*. Further all extracts showed excellent antifungal activity against *A. alterneria, A. flavis and Rhizopus solani* while minimum zones of inhibition were observed against *A. niger.* Purification of the bacterial peptides/proteins was carried out by dialysis, ammonium sulphate ($(NH_4)_2SO_4$) precipitation, gel filtration using Sephadex G-100 gel and ion-exchange on DEAE-Sephadex A-50. Antibacterial and antifungal activities were determined after each purification step. SDS-polyacrylamide gel electrophoresis revealed that the purified protein was a monomer with molecular mass of 14 kDa which possess a strong and broad antimicrobial spectrum.

Keywords*: Nigella sativa* seed, peptides/protein, purification, antimicrobial activities

INTRODUCTION

Nigella sativa Linn. (*Rannumculaceae*) vernacular name; Kalongi (Urdu), Nigella (English), is native to Southwest Asia, Southern Europe, Western and Middle Asia and North Africa. The seeds of *N. sativa are* commonly known as black cumin having pungent bitter taste like mustard-seeds (Sharma *et al*., 2009). Previous studies revealed that *N. sativa* plant parts had anti-inflammatory (Chehl *et al*., 2009), analgesic (Bashir and Qureshi, 2010), antioxytocic activity (Burits and Bucar, 2000), antioxidant activity

(Alenzi *et al*., 2010), antidiabetic activity (Benhaddou-Andaloussi *et al*., 2010), antimicrobial (Landa *et al.*, 2009), antiasthmatic activity (Boskabady *et al*., 2010), antihypertensive effect (Dehkordi and Kamkhah, 2008), antitumor activities (Mbarek *et al.*, 2007) and anti-helicobacter activity (Salem *et al.*, 2010). Today most of the plants extracts are in use commercially due to their preservative and medicinal activities. In order to promote the use of medicinal plants as potential sources of antimicrobial agents, it is pertinent to thoroughly investigate their composition and activity because phytochemicals produced as a defense against environmental stresses which lead to developments of new antimicrobial drugs (Nair and Chanda, 2006). Previously, the antimicrobial activities of *N. sativa* proteins extracts have been reported. However, there are lack of studies highlighting the extraction, purification and bioactivity of *N. sativa* peptides/protein extracted from seeds. Therefore, the present investigation was focused on extraction, isolation and purification of antimicrobial peptides extracted from *N. sativa* seeds.

MATERIALS AND METHODS

Plant material. The *N. sativa* seeds were purchased from local market, Faisalabad and authenticated form Department of Botany, University of Agriculture, Faisalabad, Pakistan.

Extraction procedure. The dirt free *N. sativa* seeds were air dried followed by oven drying to a constant weight and extracted in 10 m*M* sodium acetate buffer (pH 8) (Terras *et al.*, 1993) in the presence of 1 m*M* phenylmethylsulfonylflouride (1:2) followed by the addition of 2 n*M* thiourea addition (1:2) (Nair and Chanda, 2006). The contents were homogenized and blended in a blender (Mamrelax, Fait Common, France), centrifuged at 10,000 x *g*, 4^{o}C for 10 min, and supernatant was stored at 4^{o}C till further analysis.

Partial purification. The proteins/peptides was precipitated with $(NH_4)_2SO_4$ at 80% saturation level, centrifuged at 10,000 x *g* at 4°C for 10 min, and dialyzed (Regente *et al.*, 1997). The supernatant and residues were tested for antimicrobial activity. The residues with maximum antimicrobial activity were purified by gel filtration on Sephadex G-100 column (4 × 21 cm), equilibrated and eluted with 10 m*M* Tris-HCl buffer (pH 8). The absorbance was recorded at 280 nm. The fractions with maximum protein contents were pooled and assayed for antimicrobial activity, the samples that exhibited antimicrobial

activity were further purified by the ion-exchange chromatography, on DEAE-Sephadex A-50 (Sigma Chemical Co., St. Louis, MO 63178). The eluent was reconstituted in a mixture containing 500 mmol/L of NaCl and 30 mM/L of Tris (pH: 7.15) and extensively washed with 30 mmol/L Tris buffer (pH 7. 15). The elution was done by the gradient method with 0.2-1 *M* sodium chloride buffer (pH: 8), absorbance was recorded at 280 nm[13]. Each fraction was tested for antimicrobial activity. The characterization of the peptides/proteins was done by SDS-PAGE (Laemmli, 1970).

Antimicrobial activity assay. The antibacterial and antifungal activities of crude extract and different chromatographic fractions were checked by disc diffusion method (CLSI, 2007), MIC was also estimated (Sarker *et al.,* 2007) against different fungal (*A. niger* ATCC 10575, *A. alterneria, F. solani, R. solani*) strains and bacterial (*P. multocida* B2, *E. coli*, *B. subtilis* JS2004, *S. aureus*) which were procured from the Institute of Microbiology and Department of Clinical and Medical Surgery (CMS), University of Agriculture, Faisalabad, Pakistan. Sabouraud dextrose agar (SDA) and nutrient agar (NA) media (oxoid, UK) were used for the growth of fungal and bacterial strains, respectively. Chloramphenicol solution (10 mM) was added to avoid the bacterial growth on the medium plates. Fungal spores and bacterial cultures were inoculated in Petri plates containing the respective media and the Petri plates were incubated at 27°C and 37°C for 48 and 24 h, respectively. The antimicrobial activities were checked according to the method reported elsewhere, whereas the MIC values were estimated following Sarker method. Rifampcin and distilled water were used as positive control and negative control, respectively.

Protein contents determination. The soluble proteins of the samples were determined by Bradford method (Bradford, 1976) using bovin serum albumin (BSA) as a standard.

RESULTS AND DISCUSION

The antimicrobial activity of *N. sativa* seed crude extracts was tested against different bacterial and fungal strains (Table 1). 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. Figure 1 shows antibacterial activity of various extracts and fractions of *N. sativa* against *E. coli*. For purification, crude extract of *N. sativa* seeds were subjected to $(NH_4)_2SO_4$ precipitation at 80% saturation level. The supernatant did not show any antibacterial activity except a weak effect against *E. coli* (Table 2). 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).

Table 1: 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)	-	-

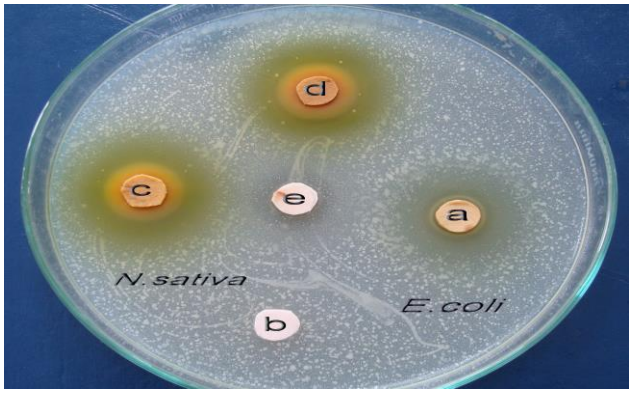


Fig. 1: Antibacterial activity of *N. sativa* seed extracts against *E. coli:* (A) supernatant (B) crude extracts (C) residue (D) gel filtration Peak III (E) ion exchange peak II

Table 2: Antibacterial activity of different samples of *N. sativa* against *E. coli*

Sample	Bacterial strain *E. coli*
*Crude extract	++
*Residue after (NH4)2SO4 ppt	++
Supernatant after (NH4)2SO4 ppt	+
Peak I (5-8 Fraction) of gel filtration	+
Peak II (12-16Fraction) of gel filtration	++
*Peak III (17-20 Fraction) of gel filtration	++
Peak I (5–8 Fraction) of ion exchange column	+
*Peak II (17–20 Fraction) of ion exchange column	++
Positive Control (rifampcin against bacteria)	+++
Negative Control (Autoclaved water)	-

The antimicrobial activity was determined by disc diffusion method and reported in mathematical terms. Zone size (mm) of 0, 1-12, 13-20 and 21-40 correspond to – (no or poor activity), + (moderate activity), ++ (strong activity) and +++ (very strong activity), respectively. ciprofloxacin against bacteria), Negative Control (Autoclaved water) no activity (-).The fractions and the extracts that contained the antimicrobial protein are marked with *.

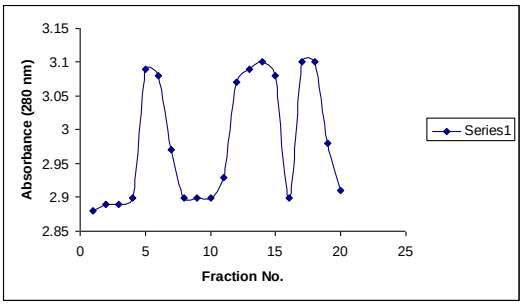


Fig. 2: Gel filtration pattern of ammonium sulphated residues of *N. sativa* seed extracts.

The residue obtained after ammonium sulphate precipitation was re-suspended in buffer and then applied to gel filtration column (Sephadex-G100). Fractions (1 mL) were collected and absorbance was noted at 280 nm (Fig. 2). Activity-directed antibacterial activity revealed no effect or a little effect for the peaks I, II and III, whereas peak II exhibited strong effect against *E. coli* which was pooled. The pooled fractions were subjected to antimicrobial assay and it was found that the 14th fraction (data not shown) had a maximum activity that was subjected to DEAE- Sephadex chromatography (Fig. 3). A major peak was observed detected constituting fractions 17-20 that also showed strong antibacterial activity against *E. coli*. Minor peaks (fraction 1-3, 5-7 and 12-14) were also observed which did not show any antimicrobial activity. The fraction 14 of gel filtration and subsequently fraction 18 of the ion-exchange chromatography was electrophoresed on SDS-polyacrylamide gel. It was observed that the antimicrobial protein moved as a single coomassie stain band for the ion-exchange sample.

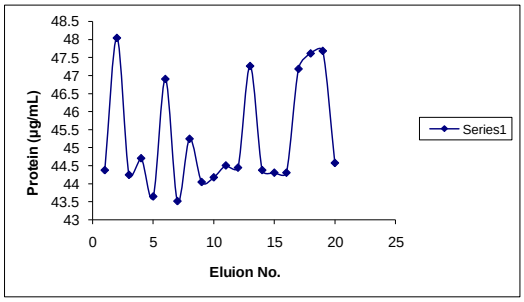
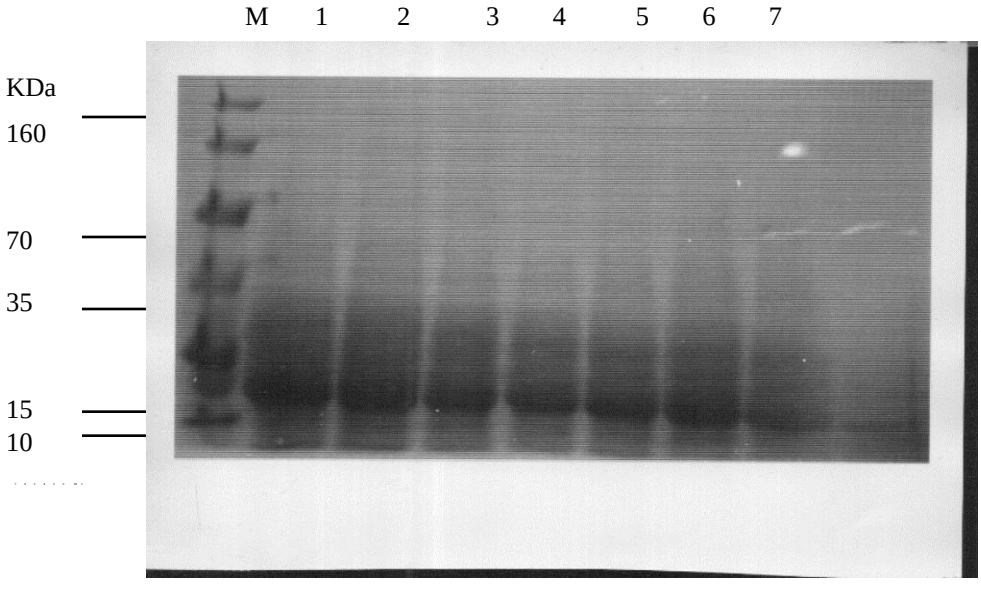


Fig. 3: Ion-exchange separation pattern of the 14th fraction from the gel filtration of *N. sativa* seed extracts. The sample was applied to DEAE-Sephadex ion-exchanger A-50 and eluted using a gradient of NaCl (0.2-1*M*) in 30 m*M* Tris-HCl buffer (pH : 7.15).

Antimicrobial activities of the pooled fractions (5–8 and 17–20) were determined by disc diffusion method. The molecular mass of the protein was estimated to be 14 kDa. SDS-

PAGE of the crude extract showed various protein bands which were disappeared after gel filtration and finally, a single protein band corresponding to 14 kDa was observed. The purified protein (named Ng-14) exhibited considerable antimicrobial activity against a set of fungal and bacterial strains. In mixed lymphocyte cultures (MLC), whereas *N. sativa* and its purified proteins were found stimulatory as well as suppressive. The antimicrobial proteins with similar molecular mass have been isolated by other researchers from other plant species. An antifungal protein (allivin) was isolated from bulbs of the round-cloved garlic (*A. sativum*) using ion-exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, ion-exchange chromatography on CM-Sepharose and FPLC-gel filtration on Superdex 75. Allivin exhibited a molecular weight of 13 kDa in gel filtration and SDS-polyacrylamide gel electrophoresis (Wang and NG, 2002). A wide range of biological activities of garlic *in vitro* and *in vivo* have been also verified including its antioxidant, antitumor and anti-inflammatory effects. They further reported that the modulatory effect of 14 kDa molecule mass isolated from garlic on IDO induction was recorded. Cultures of mononuclear cells were exposed to 14 kDa garlic fraction on-reducing conditions having two polypeptide chains of 12.5 ± 0.5 kDa (Nikoo *et al.,* 2008). A protein with molecular mass of 30 kDa possessing strong and broad antifungal activity has been reported from the leaf extracts of *Engelmannia pinnatifida* (Huynh *et al.,* 2001). The results of present investigation indicated that the *N. sativa* have strong and broad spectrum of antibacterial and antifungal activity which was due to protein of molecular mass 14 kDa.



SDS-PAGE of the seeds extracts at different stages of purification of *N. sativa*. molecular mass markers. Lanes 1 and 2 are crude extracts, lanes 3 and 4 are gel filtration extract and lanes 5, 6 and 7 are for ion-exchange chromatographic fraction. Samples containing approximately 100 ug protein were boiled for 5 min. in Laemmli buffer (2% SDS, 5% 2-mercaptoethanol, 0.002% bromophenol blue, 0.0625M Tris HCl pH 6.8) and loaded onto gel. The gel was run at 120 V for 1 h, stained with coomassie blue and analyzed on gel documentation system after de-staining.

CONCLUSION

The isolation and purification of antimicrobial peptides/proteins was investigated from *Nigella sativa* seeds. Activity of extracts was tested against a set of bacterial and fungal strains. It was concluded that the plant extracts exhibited high antimicrobial potential. A 14 KDa peptide with antifungal and antibacterial activity was isolated and reported.

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