Research Article

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Antimicrobial Activity of Purified Peptides/Proteins Isolated from *Nigella sativa* Seeds

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Abstract

***Objective**:*
To study the antimicrobial activity of purified peptides/proteins isolated from *Nigella sativa* (Kalongi) seeds extracts.

Material and Methods:
Peptides/proteins isolates of *N. sativa* was screened for their antimicrobial potential against the bacterial strains including *Escherichia coli, Pasturella multocida, Bacillus subtilis* and *Staphylococcus aureus,* and fungal strains including *Aspergillus flavis, Aspergillus niger*, *Rhizopus solani* and *Alterneria alternata*. Purification was done through precipitation with ammonium sulphate ($(NH_4)_2SO_4$), dialysis, size exclusion chromatography by using DEAE-Sephadex A-50, and the fractions were eluted using the gradient buffered NaCl (0.2-1 M).

Results:
Nigella sativa exhibited significant antibacterial potential against *E. coli* and *S. aureus* while poor antifungal activity against *Rhizopus solani* and minor inhibition zones were recorded against fungal strain *A. niger*. It was revealed by electrophoresis (SDS-PAGE) gel that the monomeric protein (purified) has 14 kDa molecular mass which has broad-spectrum and strong antimicrobial activity.

Conclusion:
Peptides/proteins contained by the *N. sativa* showed good antimicrobial activities.

Key Words: *N. sativa*, antimicrobial, peptides, proteins

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INTRODUCTION

Nigella sativa commonly known as Kalongi which is native to North Africa, Southwest Asia, Western and Middle Asia and Southern Europe. *N. sativa* seeds are usually called as black cumin or black seeds. It tastes pungent and bitter (Sharma et al., 2009). Published reports on *N. sativa* seeds revealed its antioxidant (Alenzi et al., 2010), antioxytocic (Burits and Bucar, 2000), antimicrobial (Landa et al., 2009), antihypertensive (Dehkordi and Kamkhah, 2008), antidiabetic (Benhaddou-Andaloussi et al., 2010), antiasthmatic (Boskabady et al., 2010), antitumor properties (Ait Mbarek et al., 2007), analgesic (Bashir and Qureshi, 2010) and anti-inflammatory (Chehl et al., 2009) properties. Various crude extracts of *N. sativa* were screened for their effectiveness against various microbial strains including 6 Gram positive and 16 Gram negative representatives. Multiple resistances were observed against Gram negative strains (Morsi, 2000, Landa et al., 2009). Even today in many developing countries, medicinal plants are playing significant role in primary health care as therapeutic remedies. Physiologically active principles are found in medicinal herbs that have been exploited over years in traditional medicines for treating various ailments because of their antimicrobial properties (Pirbalouti et al., 2010). Scientific experimentations on the plants antimicrobial activities have been documented in the late 19th century (Raja et al., 2009). A large number of drugs even in this modern era have been isolated from medicinal plants and other natural agents due to their uses in traditional medicinal system. To endorse the medicinal plants usage as antimicrobial agents due to their phytoactive constituents (Nair and Chanda, 2006). The research work was planned to study the antimicrobial potential of purified peptide isolated from *N. sativa* seeds.

MATERIALS & METHODS

The present research work was carried out at Medicinal Biochemistry Research Lab, Biochemistry department, Agriculture University Faisalabad, Pakistan. The seeds of *N. sativa* were purchased from Faisalabad seed market. The seeds were identified and authenticated from Botany department of the same University.

Extraction procedure

The *N. sativa* seeds were cleaned with tap water followed by cleaning with distilled water to make dirt free and dried to remove excess moisture. The seeds of *N. sativa* were extracted in 10 mM Na acetate buffer. 1mM Phenylmethylsulfonylflouride (PMSF as protease inhibitor) was added to final concentration in 1:2 in mortar and pestle and 2Mm Thiourea i.e 100g seeds in 200 mL extraction buffer (Terras et al., 1993). The samples were homogenized and mixed in blender (Mamrelax, Fait Common, France) for the breakdown of cells and centrifuged through using high speed refrigerated centrifuge machine at 10,000g for 10 minutes at 4 ºC, the

mixture were separated into residues and supernatants; the residues were discarded and the supernatant of centrifuged samples collected was filtered for the removal of remaining residue of seed found in supernatant and the extract was placed at 4 ºC for storage until further examination (Turrini et al., 2004).

Partial purification

Peptides & protein in the filtrate were precipitated at 80% saturation with $(NH_4^-)_2SO_4$ (HUYNH et al., 1996). After precipitation, centrifuged the filtrate at 4°C and 10,000 g for 10 minutes to separate residue and supernatant from crude precipitated extracts. Dialysis was performed for the removal of ammonium sulphate (Regente et al., 1997). The residue was again suspended in minimum quantity of appropriate buffer. The supernatant and the residue were examined for antimicrobial properties. Gel filtration was used for the purification of re-suspended residues with maximum antimicrobial activity (Deutcher, 1990, Marty et al., 2002). Gel filtration was performed for purification of antifungal peptides and protein using Sephadex G-100 (Yang et al., 2006) column (4 × 21 cm), equilibrated and each diffused sample was eluted by Tris-HCl buffer (10 mM, pH: 8). Absorption of eluants was measured at 280 nm. Antimicrobial activity was determined after pooling the fractions of higher protein contents. Further purification of fractions with antimicrobial activity were carried out on DEAE-Sephadex A-50 by ion exchange chromatography and reconstituted in 500 mmol NaCl followed by extensive washing in Tris HCl buffer (pH 7.15; 30 mmol/L). The gradient elution was carried out with 0.2-1 M NaCl buffer (pH 8). Optical density was measured for all the fractions at 280 nm (Terras et al., 1993) and then antimicrobial activity was performed for each fraction. The peptides/proteins were characterized on SDS-PAGE (Laemmli, 1970).

Determination of antimicrobial effect

Disc diffusion assay was utilized for the determination of antimicrobial activity of crude extract and chromatographic fractions (Fritsche et al., 2007) and MIC following the method of Sarker et al. (2007) in the samples collected at every step against selected fungal strains, *Alterneria alternata, Fusarium solani, Rhizopus solani* and bacterial strains *Escherichia coli, Pasturella multocida* B2, *Staphylococcus aureus* and *Bacillus subtilis* JS2004) strains procured from NIAB (Nuclear Institute for Agriculture & Biology), Faisalabad and Microbiology Department of Agriculture University Faisalabad. Nutrient agar (NA) and Sabouraud dextrose agar (SDA) media were used to grow bacteria and fungi, respectively. Chloramphenicol (10 mM) was added in Sabouraud dextrose agar (SDA) media to avoid the growth of bacteria (Emmons et al., 1970). The spores of fungi and culture of bacteria were inoculated in corresponding medium, followed by incubation at 27 ºC and 37 ºC for fungal and bacterial growth for 48 and 24

hours, respectively. Small discs of filter paper were placed on the media containing microbial (bacterial or fungal) growth and then the test extracts (100 µL) were added on each disc and incubated for microbial growth. The extracts form a clear zone around the disc called the inhibition zone showing antimicrobial activity. Inhibition zones were measured in millimeter with the help of zone reader (HUYNH et al., 1996).

Determination of protein contents

The soluble proteins in samples were measured with the help of Bradford assay (Bradford, 1976). Protein concentration was determined from standard curve plotted with bovine serum albumin (BSA) through its varying concentrations.

RESULTS AND DISCUSSION

N. sativa extracts were tested for their antimicrobial potential using different microbial strains including Gram positive; *S. aureus & B. subtilis* and Gram negative; *P. multocida* & *E. coli*. The antifungal activity was tested against *A. alternata, A. flavis, A. niger* and *F. solani* having strong inhibitory effects (Table 1).

The antimicrobial effect was reported by measuring inhibition zones (mm) as 21-40, 12-20, 1-12 and 0 corresponding to +++ (very strong activity), ++ (strong activity), + (moderate activity) and – (no or poor activity), respectively. For MIC, reading was recorded in µquant for bacteria at 500 nm and for fungus at 620 nm.

Tested samples showed pronounced activity against Gram-positive bacteria as compared to their activity against Gram negative bacteria. The results showed highest activity of *N. sativa* extracts against *P. multocida* (16 mm zone of inhibition).

Pharmacological effects of *N. sativa* seeds include antiviral, antibacterial, antifungal, antioxidant, anti-parasitic and anti-inflammatory activities as reported in published studies (Ali and Blunden, 2003). The antibacterial potential of *N. sativa* was reported against bacterial strains in literature (Landa et al., 2009, Hannan et al., 2008, Morsi, 2000). The minimum inhibitory concentration (MIC) of *N. sativa* seeds were studied by microdilution method. The extracts were considered of possessing good antimicrobial activity at MIC <100µg/mL, moderate activity (100 to 500µg/mL), weak activity (500 to 1000µg/mL), and inactive (over 1000µg/mL) (Holetz et al., 2002). The MIC value against bacteria was recorded at 500 nm (Sarker et al., 2007).

N. sativa exhibited good antimicrobial potential with 5.85 mg/mL MIC against *E. coli*, & also showed activity against *P. multocida* and *B. subtilis*. The extract also showed antifungal activity against *A. niger* and *A. alternata.* It was revealed that the activity of extracts against fungal strains was improved by increasing the extract concentration. Earlier studies reported that medicinal plants tannins showed remarkable toxicity against fungi and bacteria (Banso and Adeyemo, 2007).

Table 1: Antibacterial and Antifungal Activity of *N. sativa* crude extract by Disc Diffusion Method and Minimum Inhibitory Concentrations (MIC)

Microorganism	Zone of inhibition (mm)	MIC (mg/mL)
Bacterial strains		
Escherichia coli	+	23.43 ± 0.0234
Bacillus subtilis	++	5.85 ± 0.005
Pasturella multocida	++	46.87 ± 0.0468
Staphylococcus aureus	+	23.43 ± 0.023
Rifampicin (Positive control)	+	4.38±0.0048
Fungal strains		
Alterneria alterneria	++	5.85 ± 0.0058
Aspergillus flavis	++	11.71 ± 0.011
Aspergillus niger	+	23.43 ± 0.0234
Rhizopus solani	+	1.464 ± 0.0014
Terbinafin (Positive control)	+	5.56±0.0055
Autoclaved water (-ve control)	-	172.5±0.172

Table 2: Antibacterial activity of different samples of *N. sativa* against *E. coli* by disc diffusion method

Sample	Bacterial strain (*Escherichia coli*)
*Crude extract	++
*Residue after $(NH_4)_2SO_4$ ppt	++
Supernatant after $(NH_4)_2SO_4$ 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 (rifampicin)	+++
Negative Control (Autoclaved water)	-

The proteins from the N. sativa extracts were isolated through activity-guided fractionation. Figure 1 showing the *N. sativa* extracts and fractions activity against *E. coli* strain. *N. sativa* seeds crude extract was purified by subjecting through ammonium sulphate $(NH_4)_2SO_4$ precipitation at 80% saturation level. No antibacterial activity was observed in the supernatant with the exception of weak antimicrobial activity against *E. coli* (Table 2). The residue showed strong activity against the tested microbial strains most likely because of peptides or proteins found in the sample. Proteinase K treatment to the residue diminished the antimicrobial activity confirming that peptides or proteins in the extract were responsible for the antimicrobial effects of *N. sativa* seeds (Jamil et al., 2007).

Following precipitation with ammonium sulphate, the residue was again placed in buffer & applied to Sephadex-G100 column in gel filtration. The 1mL fractions were obtained followed by measuring the optical density at 280 nm (Fig. 2).

Antibacterial assay revealed a little or no effect determined for I, II and III peaks, whereas II peak showed significant activity against *E. coli*. The fractions were pooled, analyzed for antimicrobial activity and maximum activity was observed 14th fraction (data not shown). The obtained proteins through gel filtration column from 14th fractions were analyzed through DEAE-Sephadex chromatography (Fig. 3).

Major peak detected through chromatographic analysis constitute he fractions 17-20 showing strong antibacterial potential against *E. coli*. A minor peak was also observed constituting fractions 1-3, 5-7 and 12, but have no significant potential against the microbes. The filtrate (crude extract) and the fraction 14 of size exclusion chromatographic sample and fraction 18 of ion exchange chromatography were analyzed through electrophoresis using SDS-PAGE. It was found that protein with antimicrobial potential travelled as single coomassie stained band for ion exchange sample.

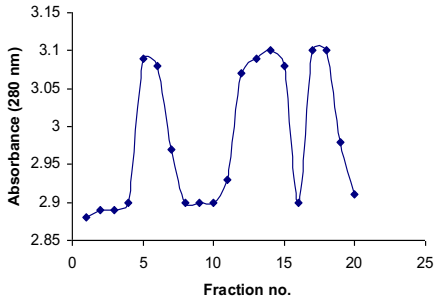

Figure 1: Pattern of ammonium sulphate residues of *N. sativa* in gel filtration.

Ion-exchange chromatography using DEAE-Sephadex ion-exchanger A-50 was performed for samples and the elution was carried out using 0.2-1M NaCl gradient in 30mM buffer solution (Tris HCl; pH 7.15). The protein concentration at 595 nm was determined by Bradford method and plotted. Disc diffusion method was used for the

determination of antibacterial effects of pooled fractions (5–8 and 17–20).

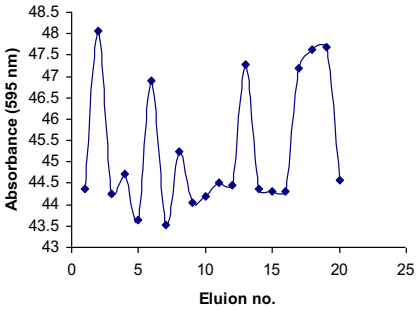

Figure 2: Pattern of separating the 14th fraction by ion-exchange chromatography from the gel filtration of *N. sativa*.

The protein molecular mass was 14 kDa. Many protein bands were observed on SDS-PAGE analysis of the extract and disappeared on gel filtration resulting in a single band of protein which corresponds to 14 kDa that was obtained through ion-exchange chromatographic method. The purified protein called as Ng-14, showed significant effects against the tested microbial strains. A number of protein bands (10-94 kDa) were exhibited by whole *N. sativa*.

Present investigations clearly indicate that *N. sativa* possess the broad-spectrum antimicrobial activity probably because of the presence of protein (14 kDa) purified from the plant by SDS-PAGE. Additional studies such as sequencing and characterization of the purified protein are required to determine the types of antimicrobial protein/peptides responsible for the antimicrobial effects of *N. sativa* seeds.

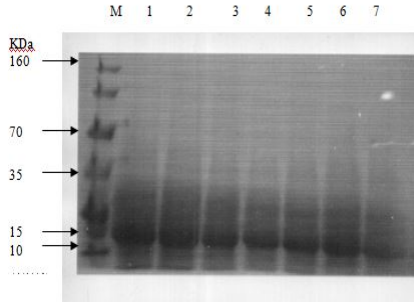

Figure 3: Seeds extract at various stages of *N. sativa* purification on SDS-PAGE. Molecular marker (Lane M), Crude extracts (Lanes 1 and 2), extract of gel filtration (lane 3 and 4) and ion-exchange chromatographic fractions (lanes 5, 6 and 7).

CONCLUSION

N. sativa seeds are rich source for antimicrobial peptides/protein and have great potential as antimicrobial compounds against pathogenic microbial strains. These peptides/proteins can be used in treating infectious diseases and may find the place in pharmaceutical industries for medicinal preparations.

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Conflict of interest

Authors declare that there is no conflict of interest.

Ethical approval

Ethical and other necessary approvals were taken from Institutional Review Board of the University of Agriculture, Faisalabad, Pakistan.

Consent for Publication

All authors approved manuscript for publication.

REFERENCES

Ait Mbarek, L., Ait Mouse, H., Elabbadi, N., Bensalah, M., Gamouh, A., Aboufatima, R., Benharref, A., Chait, A., Kamal, M. & Dalal, A. 2007. Anti-tumor properties of blackseed (*Nigella sativa* L.) extracts. *Brazilian Journal of Medical and Biological Research*, 40, pp.839-847.

Alenzi, F., El-Bolkiny, Y. E.-S. & Salem, M. 2010. Protective effects of *Nigella sativa* oil and thymoquinone against toxicity induced by the anticancer drug cyclophosphamide. *British journal of Biomedical Science*, 67, pp.20-28.

Ali, B. & Blunden, G. 2003. Pharmacological and toxicological properties of *Nigella sativa*. *Phytotherapy Research*, 17, pp.299-305.

Banso, A. & Adeyemo, S. 2007. Evaluation of antibacterial properties of tannins isolated from *Dichrostachys cinerea*. *African Journal of Biotechnology,* 6.

Bashir, M. U. & Qureshi, H. J. 2010. Analgesic effect of *Nigella sativa* seeds extract on experimentally induced pain in albino mice. *J Coll Physicians Surg Pak,* 20, pp.464-7.

Benhaddou-Andaloussi, A., Martineau, L., Vallerand, D., Haddad, Y., Afshar, A., Settaf, A. & Haddad, P. 2010. Multiple molecular targets underlie the antidiabetic effect of *Nigella sativa* seed extract in skeletal muscle, adipocyte and liver cells. *Diabetes, Obesity and Metabolism*, 12, pp.148-157.

Boskabady, M., Mohsenpoor, N. & Takaloo, L. 2010. Antiasthmatic effect of *Nigella sativa* in airways of asthmatic patients. *Phytomedicine*, 17, pp.707-713.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, pp.248-254.

Burits, M. & Bucar, F. 2000. Antioxidant activity of *Nigella sativa* essential oil. *Phytotherapy Research,* 14, pp.323-328.

Chehl, N., Chipitsyna, G., Gong, Q., Yeo, C. J. & Arafat, H. A. 2009. Anti-inflammatory effects of the *Nigella sativa* seed extract, thymoquinone, in pancreatic cancer cells. *HPB(Oxford),* 11, pp.373-381.

Dehkordi, F. R. & Kamkhah, A. F. 2008. Antihypertensive effect of *Nigella sativa* seed extract in patients with mild hypertension. *Fundamental & Clinical Pharmacology*, 22, pp.447-452.

Deutcher, M. 1990. Guide to protein purification. *Methods in Enzymology,* 182.

Emmons, C. W., Binford, C. H., Utz, J. P. & Kwon-chung, K. J. 1977. Medical Mycology 3rd edition. Medical Mycology, Henry Kimpton Publishers, London.

Fritsche, T. R., Mcdermott, P. F., Shryock, T. R. & Walker, R. D. 2007. Agar dilution and disk diffusion susceptibility testing of Campylobacter spp. *Journal of Clinical Microbiology*, 45, pp.2758-2759.

Hannan, A., Saleem, S., Chaudhary, S., Barkaat, M. & Arshad, M. U. 2008. Anti bacterial activity of *Nigella sativa* against clinical isolates of methicillin resistant *Staphylococcus aureus*. *J Ayub Med Coll*, 20, pp.72-74.

Holetz, F. B., Pessini, G. L., Sanches, N. R., Cortez, D. A. G., Nakamura, C. V. & Diasfilho, B. P. 2002. Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. *Memórias do Instituto Oswaldo Cruz*, 97, pp.1027-1031.

Huynh, Q. K., Borgmeyer, J. R., Smith, C. E., Bell, L. D. & Shah, D. M. 1996. Isolation and characterization of a 30 kDa protein with antifungal activity from leaves of *Engelmannia pinnatifida*. *Biochemical Journal*, 316, pp.723-727.

Jamil, A., Shahid, M., Khan, M. & Ashraf, M. 2007. Screening of some medicinal plants for isolation of antifungal proteins and peptides. *Pakistan Journal of Botany*, 39, pp.211-221.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

Landa, P., Marsik, P., Havlik, J., Kloucek, P., Vanek, T. & Kokoska, L. 2009. Evaluation of antimicrobial and anti-inflammatory activities of seed extracts from six *Nigella* species. *Journal of Medicinal Food*, 12, pp.408-415.

Marty, K. B., Williams, C. L., Guynn, L. J., Benedik, M. J. & Blanke, S. R. 2002. Characterization of a cytotoxic factor in culture filtrates of *Serratia marcescens*. *Infection and Immunity*, 70, pp.1121-1128.

Morsi, N. M. 2000. Antimicrobial effect of crude extracts of *Nigella sativa* on multiple antibiotics-resistant bacteria. *Acta Microbiologica Polonica*, 49, pp.63-74.

Nair, R. & Chanda, S. 2006. Activity of some medicinal plants against certain pathogenic bacterial strains. *Indian Journal of Pharmacology*, 38, 142.

Pirbalouti, A. G., Malekpoor, F., Enteshari, S., Yousefi, M., Momtaz, H. & Hamedi, B. 2010. Antibacterial activity of some folklore medicinal plants used by Bakhtiari tribal in Southwest Iran. *International Journal of Biology*, 2, 55.

Preethi, R., Devanathan, V. V. & Loganathan, M. 2010. Antimicrobial and antioxidant efficacy of some medicinal plants against food borne pathogens. *Advances in Biological Research*, 4, pp.122-125.

Raja, R. V., Ramanathan, T. & Savitha, S. 2009. Studies on wound healing property of coastal medicinal plants. *Journal of Bioscience and Technology*, 1, pp.39-44.

Regente, M. C., Oliva, C. R., Feldman, M. L., Castagnaro, A. P. & De La Canal, L. 1997. A sunflower leaf antifungal peptide active against *Sclerotinia sclerotiorum*. *Physiologia Plantarum*, 100, pp.178-182.

Sarker, S. D., Nahar, L. & Kumarasamy, Y. 2007. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. *Methods*, 42, pp.321-324.

Sharma, N., Ahirwar, D., Jhade, D. & Gupta, S. 2009. Medicinal and phamacological potential of *Nigella sativa*: a review. *Ethnobotanical Leaflets,* 2009, 11.

Terras, F. R., Torrekens, S., VAN Leuven, F., Osborn, R. W., Vanderleyden, J., Cammue, B. P. & Broekaert, W. F. 1993. A new family of basic cysteine-rich plant antifungal proteins from *Brassicaceae* species. *FEBS Letters*, 316, pp.233-240.

Turrini, A., Sbrana, C., Pitto, L., Ruffini Castiglione, M., Giorgetti, L., Briganti, R., Bracci, T., Evangelista, M., Nuti, M. & Giovannetti, M. 2004. The antifungal Dm-AMP1 protein from Dahlia merckii expressed in *Solanum melongena* is released in root exudates and differentially affects pathogenic fungi and mycorrhizal symbiosis. *New Phytologist,* 163, pp.393-403.

Yang, X., Li, J., Wang, X., Fang, W., Bidochka, M. J., She, R., Xiao, Y. & Pei, Y. 2006. Psc-AFP, an antifungal protein with trypsin inhibitor activity from *Psoralea corylifolia* seeds. *Peptides*, 27, pp.1726-1731.