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Identification and Characterization of Antimicrobial peptide

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Abstract

The present study and experimentation was conducted to identify and to characterize AMP (antimicrobial peptide) from a plant source. N.sativa's seeds were used as a source for this study. Protein fractions of seeds were purified by ammonium sulfate precipitation and gel filtration chromatography (GFC). The protein concentration was measured by Bradford assay. Broth assay and antimicrobial assay was done using well plate method against *E. coli* and *S.aureus* using all the protein fractions simultaneously. 80% ammonium sulfate cut dialyzed fraction depicted best result showing the presence of antimicrobial peptide. This peptide was further purified from the fraction using GFC and Molecular weight analysis (MW) was done on SDS-PAGE and characterize by antimicrobial activity assay. The seed extract can be pharmaceutically and clinically important antimicrobial agent. Further studies are required to identify the active mechanism of these antimicrobial peptides on microbes.

Keywords

Nigella sativa, Antimicrobial peptide, E.coli, SDS-PAGE, GFC, protein chracterization

1. Introduction

Many plants, herbs and their seeds have been used as drugs and natural curing ingredient for a lot of ailments and as a therapeutic agent to treat human diseases. Despite the major advancement in the medicine field, the development of new drugs from natural sources is still considered more authentic. Therefore the evaluation of rich heritage of traditional medicine is essential (Warrier P.R et al; 2004).Nigella Sativa Linn, Belongs to the family Ranniculanceae commonly known as Kalvanjika , Kalvanji, Black Cumin, Ajaji etc. It is mostly found and cultivated in areas of Pakistan & India and also in Syria, Lebanon and Israel & South Europe. It is an annual herb. Seeds are small, trigonous dicotylednous. Internally White, Externally Black, bitter in taste & odour is slightly aromatic (Tutin et al; 1964).Historically seeds were recorded to be prescribed by ancient Egyptians & greek physicians to treat nasal, congestion, headache, toothache & intestinal worms as well as diuretic to promote mensuration & increase in milk production (Gareja WG etal; 2003) (El-Dakhakhny M; 1965).These were discovered in Tutankhamen's tomb implying that it played an important role in ancient Egyptian practices. These seeds remained also important in Muslim and Chinese history and also in middle and Far East countries where it was known as habatul-barakh & was important in treatment of many ailments. This herbal seed is in the list of natural drugs of "Tib-e -Nabwi". According to Islamic teachings these seeds have all the cures except death (Gareja WG etal; 2003). Hazrat Muhammad (P.B.U.H) said, "Hold on to the use of black seed for it has the cure for every illness except death". *N.sativa* contains 31.94 % carbohydrates, 38.20 % fats, 4.37% ash, 7.94% fibers and mineral elements & 20.85 % proteins including 8 essential amino acids such as butamic acid, argnine , aspartic acid, methionine etc (Hak.A etal ; 1999)Nigella Sativa seeds have many uses related to human health. It can be used as astringents, stimulant and diuretic, anthelmintic for the treatment of jaundice, fever, dyspepsia, paralysis, piles and skin disease. There is some evidence to suggest that black seed might help boost the immune system, fight cancer, prevent pregnancy & lessen allergic reactions by acting as anti-histamine. But there is not enough information in humans yet. As plants have been exposed continuously to pests & pathogens infection however is the exception rather than norm. During their evolution plants, like all multicellular organisms have developed enough mechanisms to defend themselves against such assaults and for that

purpose they contain antimicrobial peptides in them. (Kombrink E. et al; 2001). Antimicrobial peptides are diverse group of molecules that are usually less than 50 amino acid long. Their chemical structure enables them to slip into the lipid bilayer surrounding bacteria to cause a variety of microbial effect such as membrane disruption. They can be chemically synthesized. (Scott et al ; 2008).According to electrical charges AMPs can be divided into anionic and cationic peptides. So far 1500 AMPs have been identified. (Wang G. et al; 2009)Mechanism is that, positively charged CAMPs interacts with negatively charged microbial surface and interactions disrupts the membrane barrier functions via pore formation or unspecific membrane permeabilization (Wilmes et al; 2011).Of all the plant antimicrobial peptides that have been characterize to date, a large proportion share common characteristics. They are generally small < 10 kDa, highly basic proteins and often an even number of cysteine residues. A variety of AMP classes have been discriminated which include Defensins, Thionine, Lipid transfer protein, Heviein & knottin like peptides (Scott et al; 2008).Among these Defensins belong to largest AMP family. (Mendez. E et al; 1990).All Plant Defensis are small 45-54 amino acids basic peptides containing 4 disulfide bridges with a single exception of flower defensins which possesses 5 disulfide bonds. Plant defensins show structural and functional similarity to defensin of insects, mammals and fungi. (B.J.C. Janssen et al; 2003) (M. E.Selset A.J et al; 2005).

2. Materials and Methods

Aqueous seed extract:

Seeds of Nigella sativa were purchased from local herbal store, washed, dried, weighed, grounded maintain temperature at -70◦C. Powder was dissolved in Tris-Cl 20mM buffer (pH 7.4) in the ration 1:10. Aqueous extract of seed proteins obtained after filtration and centrifugation and stored at -20◦C. The whole process of extraction took 48 hours. Centrifugation was done on 100G force for 10 minutes per fraction.

Protein precipitation and isolation:

the aqueous extract of proteins was then fractioned by ammonium sulfate precipitation into 20%, 40%, 80% cuts. Whole process was done at 4◦C giving each cut 4 hours for protein precipitation. Each fraction was dissolved in the minimum volume of 20mM Tris-Cl buffer (pH 7.4) and then carried to dialysis for the removal of any contamination from the protein fraction. For the quantification of protein in each fraction Bradford assay was done.

Antimicrobial activity assay:

Each protein fraction was analyzed simultaneously for the highest antimicrobial activity in two types of assays. Broth assay and inhibition zone assay (well plate method). These assays were done against *E.coli* and *S.aureus* separately and a standard antibiotic "ampiciline" was used for this purpose after setting and making the desired and calculated dosage. For *E.coli* L.B agar and broth (pH 7.2) was used and for *S.aureus* MSA (pH 8) was used. In inhibition zone assay small wells were made using vacuum pumps.

Analysis of protein fraction:

SDS-PAGE analysis was done to relate the fraction proteins with the antimicrobial activity and it was analyzed that a < 10 kDa protein shows antimicrobial activity. 10% polyacrylamide gel was made for the analysis. Then protein fractions were concentrated by acetone precipitation using ice chilled acetone.

Purification of antimicrobial peptide:

After the SDS-PAGE analysis the antimicrobial peptide was purified by gel filtration chromatography (GFC) using sephadex 50 as resin. Fractions of 2ml were collected using 20mM Tris-Cl buffer (pH 7.4) and absorbance was taken. Then all the fractions were concentrated by lyophillization and stored in minimum volume of buffer.

MW determination of antimicrobial peptide:

Each lyophillized and chromatographed fraction was analyzed using SDS-PAGE and fractions were analyzed for MW (molecular weight) in relation to the molecular weights of the antimicrobial peptides.

Characterization of purified AMP (antimicrobial peptide):

AMP was characterized using antimicrobial zone assay. Using all the lyophilized and purified fractions (sub fractions of 80% cut fraction) against *S.aureus*, the fraction having AMP showed 100% antimicrobial effect.

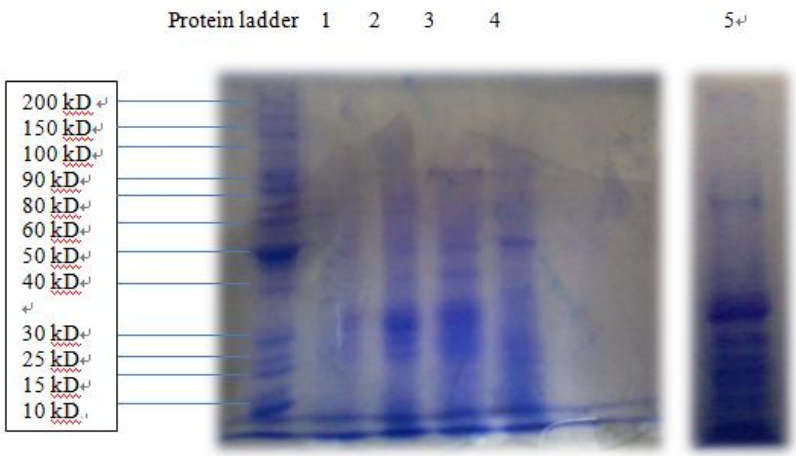


Figure 1. SDS-PAGE analysis of aqueous Extract of Nigella sativa seeds after ammonium precipitation and dialysis. Lane 1(20% cut), Lane 2(40% cut), Lane 3(60% cut), Lane 4(80% cut) and Lane 5(whole seed protein cut).

Table 1. Protein concentrations of different dialysed fractions of N.sativa seed

Fractions of Nigella sativa's seeds	**Protein concentration (µg/ml)**
20 % cut fraction	1.6
40 % cut fraction	5.4
60 % cut fraction	9.21
80 % cut fraction	5.8
Whole protein fraction	5

Table 2. pH of different fractionos of N.sativa's seeds

Fractions of Nigella sativa's seeds	**pH of fractions (in range of)**
20 % cut fraction	6
40 % cut fraction	7
60 % cut fraction	5
80 % cut fraction	7
Whole protein fraction	5.5 to 7
Purified fraction	6 to 7

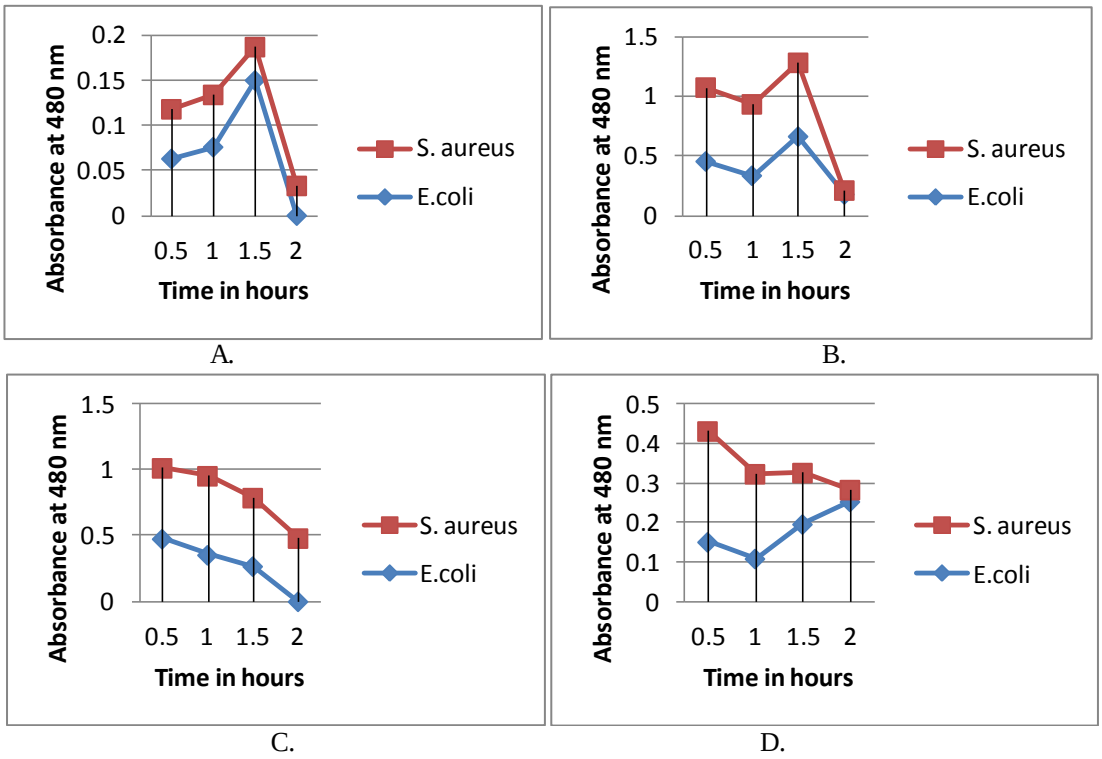
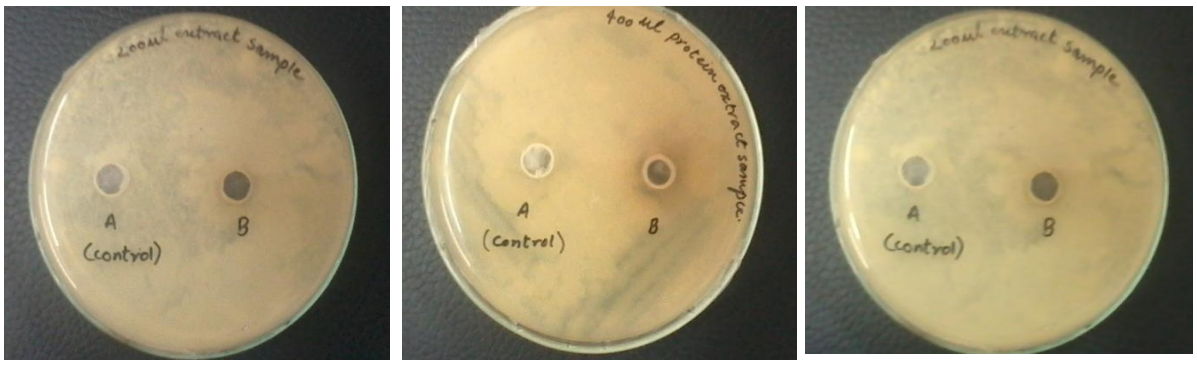


Figure 2. **A.**Graphical representation of antimicrobial effect of 20% cut fraction against *E.coli* and *S.aureus* by broth assay, shows that inhibition effect increases upto 1.5 hours then it decreases. **B**. Graphical representation of antimicrobial effect of 40% cut fraction against *E.coli* and *S.aureus* by broth assay, shows that inhibition effect increases irregularly upto 1.5 hours then it decreases. **C.** Graphical representation of antimicrobial effect of 60% cut fraction against *E.coli* and *S.aureus* by broth assay, shows that inhibition effect first decreases in 1 hour then it increases in next 1 hour. **D**. Graphical representation of antimicrobial effect of 80% cut fraction against *E.coli* and *S.aureus* by broth assay, shows that inhibition effect decreases gradually in two hours.

Table 3. Absorbance of different fractions for *E.coli* and *S.aureus* at OD 600nm

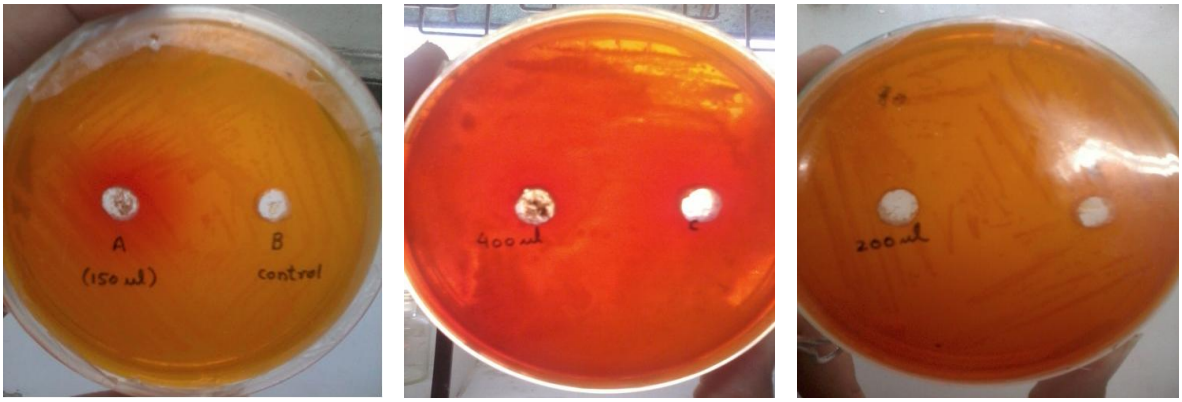
Fractions of Nigella sativa's seeds	**Absorbance at (600 nm) Against *E.coli***	**Absorbance at (600 nm) Against *S.aureus***
Control	0.57	0.68
20 % cut fraction	0.38	0.57
40 % cut fraction	0.545	0.532
60 % cut fraction	0.43	0.548
80 % cut fraction	0.261	0.284
Whole protein fraction (100 % cut)	0.452	0.395
Purified fraction	0.181	0.201

This table shows optical densities of all the ammonium sulfate dialysed fractions from Nigella sativa seeds and also of the fraction that was purified and have antimicrobial peptide in it, At 600nm against *E.coli* and *S.aureus*.



A B C

Figure 3. shows antimicrobial effect of *Nigella sativa* seed extract against *E.coli* using L.B agar as medium. (**A**)Shows the zone of inhibition around the well labeled as B which contain 200 µl of sample A contain distill water and taken as control. **(B)** shows the zone of inhibition 0.5 cm around the well labelled as B containing 400 µl sample and A contains distill water taken as control. **(C)** shows the zone of inhibition around well labelled as B containing 100 µl sample and A containing distill water taken as control



A B C

Figure 4. shows antimicrobial effect of *Nigella sativa* seed extract against *S.aureus* using MSA as medium. **(A)** Shows the zone of inhibition 1.3cm around the left well which contain 150 µl of sample right well contains distill water and taken as control. **(B)** shows the zone of inhibition around the left well containing 400 µl sample and right well contains distill water taken as control. **(C)** shows the zone of inhibition around the left well containing 200 µl sample and right well contains distill water taken as control.

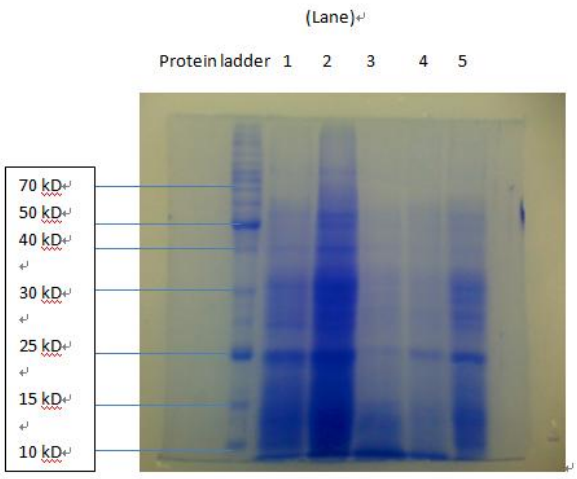


Figure 5. SDS-PAGE analysis for the purified peptide of Nigella sativa extract.
Lane 1(GFC fraction) 1-2, Lane2 (GFC f.) 3-5, Lane3 (GFC f.)19-22, Lane4 (GFC f.)11-17 and Lane5 (GFC f.) 6-7. Molecular Weight of purified peptide <10 kDa

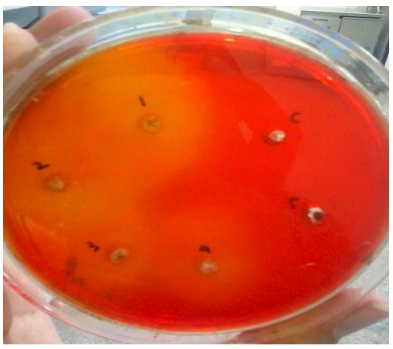


Figure 6. Antimicrobial activity determination of purified fraction after gel filtration chromatography well#1=1-2 GFC fraction, well#2=3-5GFC f. well#3= 19-22GFC f, well#4=6-7 GFC f. and well#5 = 11-17 GFC f. c=control. only well# 5 showed no bacterial growth showing maximum antimicrobial effect

Discussion

This work was done to identify the presence of antimicrobial peptide and to prove antimicrobial effect using N.sativa's seeds. Antimicrobial effect was mainly shown by 80% ammonium sulfate precipitation fraction. The purification and characterization of antimicrobial peptide was preceded with this fraction in which the presence of antimicrobial peptide was identified. This work is also of immense importance as the antimicrobial activity was analyzed against pathogenic clinical isolates *E.coli* and *S.aureus*. It is also previously been reported that Nigella sativa's seed oil shows potential antimicrobial activity against multi-drug resistant bacteria such as *S.aureus*. Black seed extract has been extensively studied for its antimicrobial activity against a wide range of bacterial, fungal and parasitic organisms. Limited data is also available so far regarding its efficacy against methicillin resistant *S.aureus*. SDS-PAGE results showed different banding patterns most important of which is of < 10kDa band and band that according to previous studies is the antimicrobial protein defensins and in this indicated that the antimicrobial activity is the protein present in the 80 % cut fraction. This is also proved as no other fractions showed that much antimicrobial effect except that whole seed protein which also contain that < 10kDa band. Gel Filtration Chromatography of that 80% cut fraction further purified the peptide and the antimicrobial Assay of the Gel Filtration Chromatography fraction then showed the presence of single band <10kDa in some fraction which was analyzed by SDS-PAGE. The Antimicrobial Assay of that fraction set proves and characterized the antimicrobial peptide defensin with MW < 10kDa which according to previous literature is ~ 5.5 kDa. The antimicrobial peptide in the 80% cut fraction has different effects on different strains of bacteria. On E.coli it has less effect and for shorter period and on *S.aureus* it has greater antimicrobial effect and for longer time period. But all these indications and identification are still as to need to be move confirmed. The antimicrobial peptide may block some signaling path way or May replace certain enzymes essential for microbial growth. But all these are just prediction for different studies that are not completely proved by specific experimental procedure. There is also an urgent need that a standard method may be devised for extract preparation. Acid extraction methods are also been used for the isolation and identification of antimicrobial peptides from the Nigella sativa seeds.

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