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ACTIVITY-GUIDED ISOLATION OF A NOVEL PROTEIN FROM *FOENICULUM VULGARE* WITH ANTIFUNGAL AND ANTIBACTERIAL ACTIVITIES

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ABSTRACT

This study describes the activity-guided isolation and purification of antimicrobial proteins from the seeds of *Foeniculum vulgare*. Purification was carried out by $(NH_4)_2SO_4$ precipitation, gel filtration and ion-exchange chromatography. Antifungal and antibacterial activities were determined by disc diffusion assay method. SDS-polyacrylamide gel electrophoresis revealed that the purified protein was a monomer with molecular mass of 60 kDa. This is a first report on purification of a protein from *Foeniculum vulgare*, which possesses a strong and broad spectrum antimicrobial activity. Our results suggest that the antimicrobial properties of this plant seed may be further investigated to explore the possibility of using them in the treatment of candidal or bacterial infections.

KEYWORDS

Foeniculum vulgare, gel filtration, antifungal, antibacterial, electrophoresis

1. INTRODUCTION

Fennel is an annual, biennial or perennial plant, depending on the variety, belonging to *Apiaceae* family and is native to the Mediterranean area [1]. It has been cultivated and introduced into many regions outside that zone; it is grown commercially in some of them, such as Russia, India, China and Japan [2]. Mature fennel fruit and essential oil are used as flavoring agents in food products such as liqueurs, bread, pickles, pastries, and cheese. They are also used as a constituent in cosmetic and pharmaceutical products [3]. Extracts from fennel plant has been used as an antispasmodic, diuretic, analgesic and antipyretic and has antimicrobial properties; it can also be used for skin disorders, conjunctivitis and blepharitis of the eye [4].

Herbs and spices are amongst the most important targets to search for natural antimicrobials from the point of view of safety [5]. They may provide an alternative to currently used pest control agent [6]. In East Asia, work has been done with plant extracts and essential oils to control stored food mites [7].

Foeniculum vulgare, vernacular name; Saunf (Urdu), Fennel (English) is an aromatic plant. The essential oils, compounds and their antimicrobial, insecticidal as well as repellent activities of this medicinal plant have been reported [8]. They have the potential of being acute ovicidal, fumigant, insect growth regulatory and insecticidal against various insect species [9].

Despite the enormous number of studies conducted on the antimicrobial properties of aromatic plants, very little work has been published on the antimicrobial properties of Foeniculum vulgare (fennel). It is hoped that the present report will contribute to the existing state of knowledge about the antimicrobial properties of medicinal plants.

2. MATERIALS AND METHODS

Seeds of *Foeniculum vulgare* (Voucher No. 11365, 26-05-1929,), Herbarium Punjab Agriculture College, Lyallpur, Shelf No. 6/35) were obtained and taxonomically identified from the Department of Botany, University of Agriculture, Faisalabad.

2.1 Extraction

Seeds were made dirt free. Seeds were extracted in potassium phosphate buffer (pH: 7.0) following the method [10]. The plant seeds were mixed with extraction buffer, by a ratio of 1:2 i.e., 100 g of sample was mixed with 200 mL of extraction buffer. Then this mixture was ground at 4°C. The next step was to centrifuge the mixtures at 10,000 x*g* at 4 °C for 15 minutes. The residues were discarded, and the supernatant was separated and filtered to remove particles. The resultant filtrate (crude extract) was stored at 4°C till further analysis [11]. Protein contents at each step were determined by a researcher [12].

2.2 Purification

The proteins in the crude extract were precipitated with $(NH_4)_2So_4$ at 80 % saturation by using a method given by some researchers [13]. The precipitated crude extract was centrifuged at 10,000 x*g*, 4 °C for 15 minutes. The residue was re-suspended in the extraction buffer. The residue and supernatants were tested for antimicrobial activities.

2.3 Dialysis of the sample

The re-suspended residues were dialyzed against distilled water to remove ammonium sulphate. The dialyzed samples were stored at 4° C in 100 mL sterilized bottles till further analysis [14].

2.4 Gel filtration and anion exchange chromatography

The dialyzed sample of *Foeniculum vulgare* was further purified by gel filtration, using Sephadex G-100 [13]. Absorbance of the eluents was recorded at 280 nm and 595 nm. The fractions with maximum protein contents were pooled out and tested for antimicrobial activities. The fraction showing high activity was further purified by ion-exchange chromatography on DEAE-Sephadex A-50 column. The elutions were carried out in a gradient of sodium chloride of different molarity ranging from 0.2-1 M [15]. Molecular mass of the crude protein was determined by SDS-PAGE [16].

2.5 Antimicrobial activities

2.5.1 Microbial strains

The fennel seed extracts were tested against a panel of microorganisms, including four bacteria, *Escherichia coli* B10, *Bacillus subtilis* JS2004, *Pasturella multocida* local isolate, *Staphylococcus aureus* API Staph tac 6736152 and four pathogenic fungi, *Aspergillus niger, Rhizopus solani, Alterneria alterneria* and *Aspergillus flavus.* The pure bacterial and fungal strains were obtained from the Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan. Bacterial strains were cultured overnight at 37°C using nutrient agar (Oxoid, Hampshire, UK) while fungal strains were cultured overnight at 27 °C on sabouraud dextrose agar (Oxoid, Hampshire, UK).

2.5.2 Disc diffusion method

The antimicrobial activity of the fennel seed extracts from different steps was determined by the disc diffusion method. Fungal spores and bacterial cultures were inoculated in Petri plates containing the respective medium and incubated at 27 ºC and 37 ºC for fungi and bacteria, for 24 and 48 h, respectively. Wicks paper discs 6 mm in diameter were then laid flat on the medium having fungal or bacterial growth and 100 µL of the sample was applied on each disc. The Petri plates were incubated for 48 h, for the growth of microbes. The antimicrobial activity was shown by the appearance of the clear zones around the growth.

The zones of inhibition were measured in millimeters with a zone reader [17]. Ciprofloxacin (Bacteria) and Terbinafine-HCl (Fungi) were used as standard. Disc without samples having autoclaved water was used as negative control. Minimum Inhibitory Concentrations (MIC) of crude extracts was performed using microtiter plate-based assay. For bacterial strains and for fungal strains a Florentine method was used given by a group researchers [18,19].

2.6 Statistical Analysis

All the experiments were conducted in triplicate unless stated otherwise and statistical analysis of the data was performed by analysis of variance (ANOVA), using STATISTICA 5.5 (Stat Soft Inc, Tulsa, Oklahoma, USA) software. A probability value of $p \leq 0.05$ was considered to denote a statistically significance difference. All data are presented as mean values ± standard deviation (SD).

3. RESULTS AND DISCUSSIONS

Antimicrobial activity of the extracts of *Foeniculum vulgare* were tested against fungal (*Aspergillus niger, Rhizopus solani, Alterneria alterneria* and *Aspergillus flavus)* and bacterial strains (*Escherichia coli, Bacillus subtilis, Pasturella multocida and Staphylococcus aureus)* with strong inhibitory effect (Table 1); hence activity guided fractionation was carried out to isolate the respective protein. After performing antimicrobial assay, MIC of seed extracts of fennel was performed. The result is represented in table 2.

Table 1: The antimicrobial activity of the different samples collected at different steps of purification of *Foeniculum vulgare*

Tested organisms	Crude Extract	Supernatant after $(NH_4)_2SO_4$ ppt	Residue after $(NH_4)_2SO_4$ ppt	Peak I	Peak II	Peak III	Peak IV	Standard
Diameter of Inhibition Zone (mm) including disc diameter of 6 mm								
Pasturella multocida	14 ± 1	13±1	14±1	14 ±1	14 ±1	16 ±1	16 ±1	22±1
Escherichia coli	14 ± 1	12±1	14±1	16 ±1	14 ±1	16 ±1	16 ±1	22±1
Bacillus subtilis	20 ±1	14±1	16±1	14 ±1	14 ±1	14 ±1	16 ±1	22±1
Staphylococcus aureus	12 ± 1	14±1	16±1	12 ±1	14 ±1	14 ±1	14 ±1	22±1
Aspergillus niger	12 ± 1	12±1	14±1	12 ±1	12 ±1	14 ±1	14 ±1	22±1
Alternaria alternaria	13 ± 1	13±1	14±1	12 ±1	13 ±1	14 ±1	14 ±1	22±1
Aspergillus flavus	22 ± 1	16±1	18±1	16 ±1	16 ±1	16 ±1	18 ±1	22±1
Rhizopus solani	14 ± 1	14±1	16±1	12 ±1	12 ±1	12 ±1	14 ±1	22±1

No activity was recorded in negative control disc. As the experiment was conducted in triplicate and means are given in the table. For purification, crude extract of *F. vulgare* seeds was subjected to ammonium sulphate precipitation to 80% saturation level. Strong antifungal and antibacterial activities were observed for the residue, showing that the activity was most likely due to some proteins and peptides.

Table 2: Minimum inhibitory concentration (mg/mL) of *Foeniculum vulgare* against fungal and bacterial species.

Microorganisms	Seed Extract (mg/mL)	Positive Control (mg/mL)
Bacterial Species:		
Pasturella multocida	5.79±0.05	23.43±0.02
Escherichia coli	5.78±0.05	11.71±0.01
Bacillus subtilis	1.45±0.01	93.75±0.09
Staphyloccocus aureus	1.75±0.07	46.87±0.04
Fungal species:		
Aspergillus niger	0.36±0.03	1.46±0.01
Alternaria alternaria	0.46±0.03	22.45±0.22
Aspergillus flavus	0.78±0.02	93.75±0.09
Rhizopus solani	1.449±0.01	11.71±0.01

The residue, re-suspended in buffer after ammonium sulpahte precipitation, was applied to gel filtration column (Sephadex-G 100). One milliliter fractions were collected, and the absorbance was noted at 280 nm and 595 nm using Bradford reagent (Figure 1).

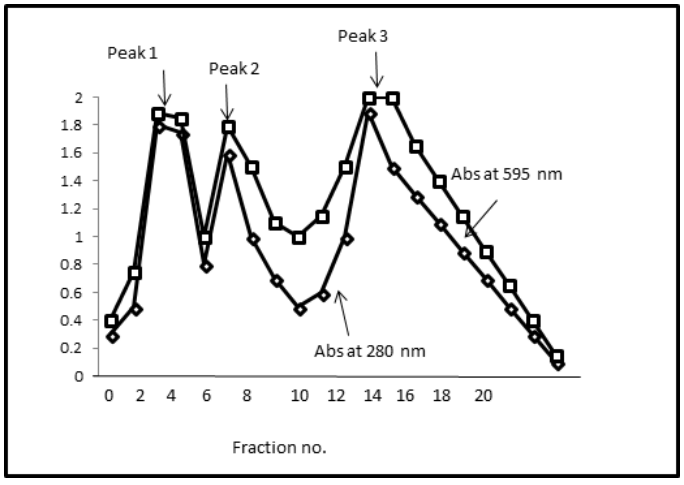

Figure 1: Gel filtration pattern of ammonium sulphate residues of *Foeniculum vulgare*.

Activity-directed antimicrobial effect revealed that no or little effect could be observed for the peak 1 (fraction 3 and 4), peak 2 (fraction 6), while peak 3 (fraction 13) exhibited strong effect against *Pasturella multocida, Escherichia coli* and *Aspergillus flavus.* It was concluded that fraction 13 had maximium effect. The proteins obtained from the fraction 13 of the gel filtration column were subjected to DEAE Sephadex chromatography (Figure 2). Gradient molar solutions of NaCl (0.2-1 M) were used for elution of sample.

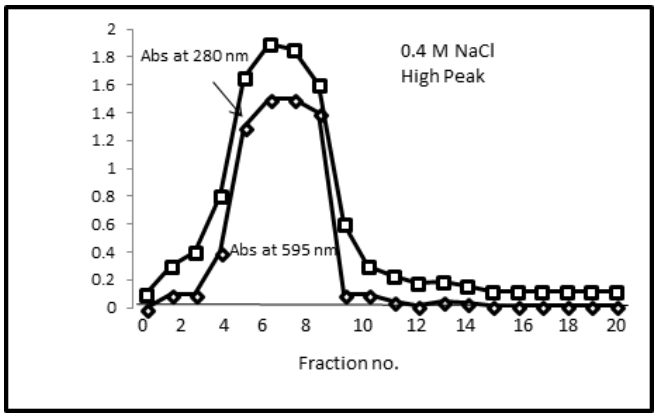

Figure 2: Ion exchange separation pattern of the 13th fraction from gel filtration of *Foeniculum vulgare*.

A major peak was detected constituting fractions 5-8 with NaCl concentration of 0.4 M. The crude extract, fraction 13 of gel filtration and fraction 5 of the ion-exchange chromatographic sample were electrophoresed on SDS-polyacrylamide gel. It was observed that the antimicrobial protein migrated as a single coomassie stain band for the ion-exchange sample.

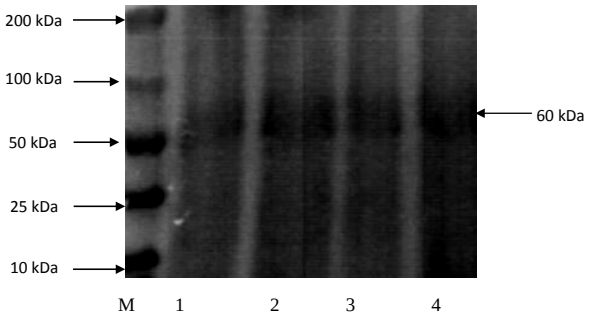

Figure 3: SDS-PAGE of seeds extracts at different stages of purification of *Foeniculum vulgare*. Lane M molecular mass markers. Lane 1 and 2 crude extracts, lane 3 is gel filtration extract and lane 4 is for ion-exchange chromatographic fraction.

The estimated molecular mass of the protein was 60 kDa. SDS-PAGE of the crude extract showed many bands that disappeared after gel filtration, and finally a single protein band corresponding to 60 kDa was obtained after ion-exchange chromatography. No report to our knowledge exists in the literature for purification of *Foeniculum vulgare.* However, antimicrobial proteins have been isolated by some researchers. A group researchers reported that the Gram-negative strains of bacteria, especially *E. coli*, have less sensitivity to fennel extracts [20]. In other study, they found that fennel exhibit an inhibitory effect against a wide range of *Bacillius* species [21]. There also reported that the extracts of fennel are active against *Aspergillus* species [22].

4. CONCLUSION

The results of this study show that *Foeniculum vulgare* is a good source of antimicrobial proteins. A 60 kDa protein was purified from the plant that exhibited strong and broad spectrum antimicrobial activity. However, further studies like sequence analysis and characterization of the purified protein are necessary before its use as antimicrobial against pathogenic fungi and bacteria. Due to antimicrobial activity, seed extracts of *F. vulgare* can be used as supplement in pharmaceutical industries. These extracts are valuable not only for increasing shelf life of foodstuffs, but it could be a future target for replacing synthetic antibacterial agents.

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