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Full Length Research Paper

Effect of the purification of antidermatophytic proteins from *Nigella sativa* on four zoophilic species

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The antidermatophytic activities of proteins which are extracted from four plant species (*Carum carvi*, *Cymbopogon citratus, Moringa oleifera,* and *Nigella sativa*) on four zoophilic dermatophytes (*Microsporum canis, Microsporum equinum, Trichophyton mentagrophytes,* and *Trichophyton verrucosum)* were evaluated in this study. The crude proteins of *N. sativa* had the broadest significant spectrum of antidermatophytic activity on the tested dermatophytes as well as the greatest antioxidant activity (95.11%) and the highest protein content (82 mg/ml). 17 amino acids were found in the four tested plant proteins with *N. sativa* protein having the highest content of amino acid (347.21 µg/ml). *N. sativa* protein had the greatest effect on the fungal cell permeability of all the tested zoophilic dermatophytes. Purification of *N. sativa* protein on Sephadex G-100 column showed two peaks of protein (Pr1 and Pr2) as well as increasing antidermatophytic activities. Complete purification of the most active fraction (Pr2) on diethylaminoethyl (DEAE)-Sephadex eluted one single peak with increasing antidermatophytic activity (7.6 cm) against the most sensitive pathogen (*M. canis*), representing 1.43 fold purification of the crude protein. The molecular weights of the purified *N. sativa* proteins (Pr1 and Pr2) were 42.7 and 31 kDa, respectively. The highest antidermatophytic activity of Pr2 was observed at a pH of 7 and temperature of 20°C. Na^+, K^+, Ca^{2+} and Mg^{2+} decreased the antidermatophytic activity of the pure protein of *N. sativa*.

Key words: Dermatophytes, plant, protein, purification.

INTRODUCTION

Dermatophytes are keratinophilic fungi capable of causing dermatophytosis (commonly known as tinea or ringworm) in animals (Liu et al., 1997). Dermatophytes (*Trichophyton* and *Microsporum*) constitute a serious problem, especially in tropical and subtropical developing countries (Raza, 1998). Conant (2004) reported that the most common species of dermatophytes are *Trichophyton* and *Microsporum* which cause superficial fungal infections of animal skin. Zoophilic dermatophytes are found primarily in animals and cause marked inflammatory reactions in humans who have contact with infected cats, dogs, cattle, horses, birds, or other animals (Rochette et al., 2003).

The overuse of antibiotics in the treatment of infectious diseases and the appearance of multi-drug resistant fungal strains as well as lack of efficacy and side effects have driven researchers to the study of antimicrobial agents of plant (Dorman and Deans, 2000). Subramanian and Saratha (2010) stated that drug resistance to pathogenic fungal species has been reported all over the world, emphasizing that the situation is alarming in developing countries due to indiscriminate use of antibiotics. Another concern raised about synthetic drugs and antibiotics is that, they may have interactions with the body system, which disturb the metabolic processes. However, the judicious use of medicinal herbs can even cure deadly diseases that have long defied synthetic drugs (Bhattacharjee, 2001). Consequently, there has been an increased demand for medicinal plants by modern pharmaceutical industries (Jamil et al., 2007). Scientists are working on the extraction of anti-infection compounds, including antifungal proteins from natural

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sources like plants. The anti-infection compounds show broad-spectrum bioactivity against infection-causing agents such as bacteria, fungi, protocists, protozoans, viruses and yeasts (Conlon et al., 2003). The demand for proteins for medical and industrial use is increasing rapidly and plants are now recognized as a safe, efficient and inexpensive means of their production. Plants also offer other advantages which include: rapid scalability, the absence of human pathogens and the potential for direct oral administration of unprocessed or partially processed plant material. Plants, over the past years, have shown promise as bioreactors for the large-scale production of various proteins and biopharmaceuticals (Conley et al., 2011).

Plants produce a wide range of selective antifungal compounds either in a constitutive or an inducible manner (Cammue et al., 1992). Among these compounds, several low molecular weight proteins with antifungal activity have been isolated from various plants (Terras et al., 1992). Numerous studies have been carried out to extract various natural products for screening antimicrobial activity, but attention has not been given to the isolation of small proteins from the plants for the examination of antimicrobial activity (Hudson et al., 1993).

Antimicrobial peptides are found in all kingdoms of life, ranging from plants through insects to animals (Kaneda and Kajimura, 2002). These peptides are termed antimicrobial because they display an unusually broad activity spectrum. The defense mechanism against phytopathogenic fungi functions by inhibiting the microorganism's growth through diverse molecular modes such as binding to chitin or increasing the permeability of the fungal membranes or cell wall (Maher et al., 1994). Moreover, the synthesis of many presumed defense related proteins are induced when plants are confronted with pathogens (Linthorst, 1991).

In terms of antimicrobials, several of the amphibian peptides were purified to homogeneity and they displayed potent antimicrobial activity (Hearst et al., 2010). The activities of plant derived antimicrobial peptides may include the ability to kill or neutralize not only bacteria but also fungi, parasites, cancer cells, and even enveloped viruses like HIV and herpes simplex virus. Also, most of the antimicrobial peptides are quite selective about microbes (Kmysz et al., 2003).

New technological advances have led to huge progress in the study of protein complexes in plants (Pflieger et al., 2011). Consequently, the present study focused on the purification of proteins which are extracted from some local potential medicinal plants to homogeneity and examined the antidermatophytic activities of the purified proteins on four zoophilic dermatophytes. Antioxidant and individual amino acids constituents were determined in all the tested plant protein extracts. The effect of the plant proteins on the permeability of the tested pathogens was determined. The complete purification steps of the most effective plant protein were investigated and the molecular weight and characterization of the most effective purified protein were recorded.

MATERIALS AND METHODS

Zoophilic dermatophytes

Four zoophilic dermatophytes (*Microsporum canis, Microsporum equinum, Trichophyton mentagrophytes,* and *Trichophyton verrucosum*) were obtained from the Faculty of Veterinary, Cairo University. Cultures were grown on Sabouraud dextrose agar (SDA) slants. Sterile saline solution (0.85%) was added to the slants and the culture was gently swabbed with a cotton-tipped applicator to dislodge conidia from the hyphal mat. The suspension was transferred to a sterile tube and the volume was adjusted to 5 ml with sterile phosphate buffered saline solution with a pH of 7. The resulting suspension was used for the subsequent experiments.

Plant source

Four plant samples were collected from Sekem's company farms at Belbes, 60 km, Egypt; they were selected from the field after harvest. Two spices (fruits of *Carum carvi* and seed grains of *Nigella sativa*) and two herbs (leaves of *Cymbopogon citratus* and *Moringa oleifera*). The plant samples were collected in sterile polyethylene bags. For each species sample, a minimum of 10 replicates were taken, and mixed to prepare one composite sample.

Preparation of crude extract

Plant samples were dried at room temperature and were ground into fine powder with mortar and pestle. The dried powder was defatted with 150 ml of diethyl ether at room temperature for 8 h and filtered. The soluble protein was extracted from the tested plant species in accordance with the method recommended by Shewry et al. (1995). Protein concentration was determined spectrophotometrically at 260 nm by the method of Segel (1968).

Antidermatophytic activity

The four plant extracts (*C. carvi*, *C. citratus*, *M. oleifera* and *N. sativa*) were examined. Known volumes of SDA medium were sterilized by autoclaving and then aliquots of about 15 ml of this medium were dispersed into sterile Petri dishes, 9 cm in diameter. A hole about 1 cm in diameter was made at the center of solidified medium using a sterilized cork borer. One milliliter of each plant extract protein was put in the hole. Petri dishes were inoculated with 1 ml spore suspension of the four tested zoophilic dermatophytes *(M. canis*, *M. equinum*, *T. mentagrophytes* and *T. verrucosum)*. The diameters of inhibition zones (cm) were measured (mean of three diameters at right angles to each other) 5 to 10 days after the incubation period at 30°C. Triplicate plates for each plant extract concentration were used (Serey et al., 2007).

Antioxidant activity

The free radical scavenging activity was measured for the four tested plant proteins using 1,1- diphenyl-2-picryl-hydrazyl (DPPH) assay. The tested plant samples were added to 1 mM DPPH in the reaction mixture. The reaction mixtures were taken in Eppendorf

Table 1. Antidermatophytic activities (cm) of the tested plant proteins (*Carum carvi, Cymbopogon citrates, Moringa oleifera* and *Nigella sativa*) on the tested zoophilic dermatophytes (*Microsporum canis, Microsporum equinum, Trichophyton mentagrophytes* and *Trichophyton verrucosum).*

Zoophilic dermatophyte	*C. carvi*	*C. citratus*	*M. oleifera*	*N. sativa*
M. canis	1.8	3.5	0.5	5.3
M. equinum	1.0	1.7	0.4	3.8
T. mentagrophytes	0.7	1.0	0.3	1.8
T. verrucosum	1.3	1.5	1.2	2.2
LSD at 0.05	0.41	1.13	0.08	1.17

LSD, Least significant difference.

tubes and incubated at 37°C for 30 min. Ascorbic acid was used as control. The absorbance was measured at 517 nm. The DPPH radical concentration was calculated using the following equation: Scavenging effect (%) = 100 − A0 − A1 / A0; where, A0 is the absorbance of the control reaction and A1 is the absorbance in the presence of the tested plant extracts (Al-Fatimi et al., 2007).

Effect of the tested plant proteins on membrane permeability of the tested zoophilic dermatophytes

The membrane permeability of the tested zoophilic dermatophytes to protein contents was determined by the method of Segel (1968). The total sugars in the culture filtrate of the tested dermatophytes were determined according to phenol sulfuric acid reagent. The absorption was measured at 485 nm (Dubois et al., 1956). The determination of DNA was done quantitatively according to the method of Burton (1968) by measuring the color development after treating the extracted DNA with diphenylamine reagent and the absorbance was measured at 600 nm. The colorimetric analysis of ribose sugar using orcinol reaction (Ashwell, 1957) was done for quantitative determination of RNA. The plant protein with the most effective antidermatophytic activity on the most sensitive dermatophyte was selected for further experiments.

Partial purification of the most effective plant protein

Partial purification of the extracts was performed at the 80% saturation level of ammonium sulphate. After precipitation, the extracts were centrifuged at 12,000 r.p.m. and 4°C and the supernatants were dialyzed (Huynh et al., 1992). The antidermatophytic activity of the partially purified plant protein was tested.

Purification by gel filtration of the most effective plant protein

A complete purification process was carried out by gel filtration using Sephdex G-100 column (10 x 1.25 cm) with10 mM Tris HCl buffer. The pooled active fractions obtained from the previous column was concentrated with ammonium sulfate, dialyzed and applied in small amounts of buffer to diethylaminoethyl (DEAE) - Sephadex column and the exchanged material was eluted in succession with the stepwise gradient of 0.0 to 0.2 M of NaCl prepared with the same buffer. The antidermatophytic activity of the purified plant protein was tested (Dahot, 1998).

Estimation of the molecular weight of the most effective purified plant protein

The homogeneity of pooled pure protein fractions were checked by sodium dodecyl-sulphate-polyacrylamide gel electrophoesis (SDS-PAGE). The Laemmli SDS-PAGE discontinuous system with homogenous gel was used (Laemmli, 1970). Proteins were visualized by silver staining. Standard marker proteins (Maltose-binding proteins, 42.7 kDa; carbonic anhydrase, 31 kDa; trypsinogen, 24 kDa; lysozyme, 14.4 kDa) were used.

Characterization and antidermatophytic activities of the most effective purified plant protein

The antidermatophytic activity of the effective purified protein was recorded at pH values of 3, 5, 7, 9 and 11 and temperatures values of 0, 5, 20, 30, 40, 80, and 100°C. Four metallic salts, namely NaCl, KCl, $MgSO_4$ and $CaCO_3$ were tested. Each of these salts was incubated in a concentration of 10^{-2} M for 60 min with the purified protein solution. Antidermatophytic activity was tested according to method recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2002).

RESULTS AND DISCUSSION

Antidermatophytic activity

The results indicate that two (*N. sativa* and *C. citratus*) out of the four plant proteins had a broad spectrum of antifungal activity by forming wide clear zones of inhibition, while the rest had smaller zones of inhibition against the tested dermatophytes. *N. sativa* showed the most significant and highest antifungal activity (5.3 cm) on the most sensitive dermatophyte (*M. canis)*, while *M. oleifera* showed the lowest antidermatophytic activity (0.5 cm) (Table 1). This shows that the antidermatophytic activity was due to the proteins present in the tested plant extract, and not due to any other compounds. Similarly, the extract of *Psoralea corylifolia* also showed significant antidermatophytic activity on some fungal species and the antifungal activity was mainly due to proteins or peptides, and not because of some other compounds (Jamil et al., 2007). The fact that *N. sativa* can serve as a potential source of antidermatophyte drugs and can be used in folk medicine for the treatment of fungal skin infections was recorded by Aljabre et al. (2005).

The antidermatophytic activities of *N. sativa* seeds on 20 fungi, including pathogenic and industrial strains and the great potential of *N. sativa* for being used tropically in

Table 2. Antioxidant activity (%) and protein content (mg/ml) of the tested plant proteins (*Carum carvi, Cymbopogon citrates, Moringa oleifera* and *Nigella sativa).*

Plant protein	Antioxidant activity (%)	Protein (mg/ml)
C. carvi	80.41	53.00
C. citratus	87.50	65.10
M. oleifera	72.33	50.83
N. sativa	95.11	82.00
LSD at 0.05	5.31	17.31

Antioxidant activity of ascorbic acid (control) = 90.53%. LSD, Least significant difference.

treating fungal diseases was tested by Nazrul et al. (1989). Terras et al. (1992) isolated an antifungal protein from the seeds of *Brassica* species. Also, Ye and Ng (2002) isolated a variety of antifungal proteins from some leguminous plants. *N. sativa* seeds extract exhibits inhibitory effect against candidiasis, which validates the traditional use of the plant in fungal infections (Khan et al., 2003). Tzortzakis and Economakis (2007) stated that lemongrass' antifungal activities were only fungistatic. Also, Kekuda et al. (2010) recorded that the antifungal activities of the compound isolated from *M. oleifera* were moderate against microbes.

Antioxidant activity and protein contents

The data in Table 2 reveals that all the tested plant protein (*C. carvi*, *C. citratus*, *N. sativa* and *M. oleifera*) have different values of antioxidant activities, with the most effective antidermatophytic protein (*N. sativa*) showing the most significant antioxidant activity (95.11%) as well as the highest protein content (82 mg/ml), while *M. oleifera* extract showed the lowest antioxidant activity (72.33%). The results obtained are similar to Ramadan et al., (2003) who stated that the crude *N. sativa* extract showed potent *in vitro* radical scavenging activity.

The results obtained reveal that the highest antidermatophytic activity of *N. sativa* was accompanied with the highest antioxidant activity which probably implies that some active constituents which were found in the extracts of *N. sativa* may have been responsible for both the antidermatophytic and antioxidant activities. The higher antioxidant activity of the tested protein may be attributed to its higher protein content but its greater specific antioxidant activity may not be attributed to the same factor. There is a close relationship between the hydrophobicity and antioxidant activity of proteins and peptides (Saiga et al., 2003). Shahid (2011) stated that *N. sativa* has strong antifungal activity as well as a strong antioxidant activity, showing great promise of antimicrobial properties which indicates that its potential for discovery of antifungal principles and inductions played an important role in detecting the gene of bioactive proteins by biotic and abiotic stress.

The antioxidant activity of *N. sativa* was recorded by Yoruk et al. (2010). Proteins and peptides have excellent potential as food antioxidants (Elias et al., 2008). The plant, as a natural antioxidant, is readily accepted by consumers and does not require safety tests (Arcan and Yemenicioğlu, 2007). Many plant proteins have been reported to have antioxidant activity (Chang et al., 2007). Hu et al. (2003) reported that the cationic characteristics of proteins are also important for their antioxidant activity since positively charged groups help the inhibition of lipid oxidation by electrostatic repulsion of transition metals away from the lipid droplets.

Other advantages of using plant proteins as antioxidant substances are their additional functions such as nutritive value and antimicrobial activity (Fukuzawa et al., 2005). Several proteins showed both antioxidant and antimicrobial activities (Satue-Gracia et al., 2000). Medicinal plants prevent diseases as a result of their antioxidant activities; one of the potential properties of *N. sativa* seeds is the ability of one or more of its constituents to reduce microbial toxicity due to its antioxidant activities (Salem, 2005).

Determination of individual amino acids

The profile of individual amino acids in the tested plant proteins is recorded in Table 3. The data reveals that 17 amino acids in the tested plant samples were recorded, with *N. sativa* protein having the highest antidermatophytic activity as well as the most significant amino acid contents (347.21 µg/ml) followed by *C. citratus*, *M. oleifera* and *C. carvi* (249.21, 234.19 and 181.56 µg/ml, respec-tively). The obtained data is similar to that of Ibrahim et al. (2010) who stated that amino acids protect plants against pathogens. The antioxidant activity of proteins is associated with their constituent amino acids, whereas the antioxidant activities of aromatic amino acids such as tyrosine, phenylalanine, tryptophan and the sulfur-containing amino acid, cysteine, are due to their ability to donate protons to free radicals and the antioxidant activity of basic amino acids such as lysine and arginine, and acidic amino acids such as aspartate and glutamate are carried out by chelating metal ions (Lundstrom, 2004).

When *N. sativa* protein was tested, it was found that

Table 3. Individual amino acids (µg/ml) of the tested plant proteins (*Carum carvi, Cymbopogon citrates, Moringa oleifera* and *Nigella sativa).*

Amino acid	*Carum carvi*	*Cymbopogon citratus*	*Moringa oleifera*	*Nigella sativa*
Alanine	6.53	12.96	8.00	14.62
Arginine	7.95	14.21	22.13	16.55
Asparatic acid	22.71	20.56	20.51	22.96
Cystine	2.82	19.02	13.53	20.21
Glutamic acid	27.62	14.50	27.52	32.76
Glycine	12.41	10.72	15.11	16.91
Histidine	2.70	3.35	1.15	4.51
Leucine	13.72	19.85	16.03	23.94
Lysine	2.33	8.35	2.16	9.51
Methionine	3.61	4.53	3.11	6.44
Phenyl alanine	20.81	27.93	30.70	55.75
Proline	10.53	11.59	12.95	14.92
Serine	14.27	10.35	18.62	20.51
Theronine	9.72	5.28	6.55	11.93
Tryptophan	2.14	19.56	13.85	20.44
Tyrosine	11.14	33.50	11.83	40.38
Valine	10.55	12.95	10.44	14.87
Total	181.56	249.21	234.19	347.21
LSD at 0.05	6.12	5.57	8.90	15.43

LSD, Least significant difference.

the proportion of phenylalanine amino acid was the highest (55.75 µg/ml) followed by tyrosine (40.38 µg/ml). The dominance of phenylalanine and tyrosine in *N. sativa* may emphasize their role in the antidermatophytic and antioxidant activities on the tested dermatophytes. The high antioxidant activity of the tested protein fractions may be due to the higher frequency and/or proper positioning of antioxidant amino acids (Arcan and Yemenicioğlu, 2007).

It was reported that the basic amino acid, histidine, may act both as a radical-scavenger and a metal-chelator due to its imidazole ring (Je et al., 2004). Novel antifungal defensins peptides from *N. sativa* seeds were isolated and the peptides differed by a single amino acid residue, while the defensins displayed strong antifungal activity on a number of phytopathogenic fungi; thus, high antifungal activity of *N. sativa* defensins makes them promising candidates for engineering pathogen-resistant plants (Slavokhotova et al., 2011).

Effect of the tested plant proteins on membrane permeability of the tested dermatophytes

The present study indicated that the treatment of the tested dermatophytes with *N. sativa* protein was more effective in decreasing the productivity of protein contents in the culture media, with *M. canis* causing the most significant decrease (271 µg/ml) compared with the non-treated control (300 µg/ml) (Table 4). On the other hand, *C. carvi*, *C. citratus* and *M. oleifera* lead to an increase in the diffusion of protein contents into the culture media of all tested zoophilic fungi. From the results of previous studies, it was concluded that some plant extracts such as those *C. carvi*, *C. citratus*, and *M. oleifera* may accelerate nitrogen absorption and metabolism to synthesize protein which plays a protective role in the plants against microbes, while other plant extracts such as the one from *N. sativa* could inhibit nitrogen absorption and microbial metabolism leading to low protein followed by minimum microbial growth (Kraus et al., 2003). The obtained results are similar to Ravikumar et al. (2010) who stated that the antimicrobial activity exhibited by some plant parts could be due to the presence of phytochemicals in the plant extracts, which results in the inhibition of cell protein synthesis.

Plant extracts can act against microorganisms by potentially acting as either protein denaturing agents or dehydrating agents. Plant compounds may interfere with biological processes, reacting with vital nitrogen components (proteins) and thereby inhibit the growth of microorganisms (Deans, 2002, 2007). Kaur and Arora (2009) found out that the antimicrobial ability of a plant extract forms a complex with extracellular soluble proteins and cell walls. Antimicrobial phytochemicals (polypeptides) mechanisms thought to be responsible for the action of phytochemicals against microorganisms and their modes of action may include inactivation of the protein and loss of function as well as their ability to form a complex with extracellular and soluble proteins (Abu-

Table 4. Effect of the tested crude plant proteins (*Carum carvi, Cymbopogon citrates, Moringa oleifera* and *Nigella sativa*) on membrane permeability of the tested zoophilic dermatophytes (*Microsporum canis, Microsporum equinum, Trichophyton mentagrophytes* and *Trichophyton verrucosum)*.

Zoophilic dermatophyte	Control	*C. carvi*	*C. citratus*	*M. oleifera*	*N. sativa*
Protein (µg/ml)					
M. canis	300	320	412	347	271
M. equinum	318	353	320	402	310
T. mentagrophytes	412	420	465	437	363
T. verrucosum	392	401	431	400	320
LSD at 0.05	8.30	12.51	15.45	3.11	10.37
Sugars (µg/ml)					
M. canis	340	417	420	392	530
M. equinum	305	384	415	371	432
T. mentagrophytes	218	274	300	250	370
T. verrucosum	335	351	400	408	450
LSD at 0.05	18.33	23.01	20.07	21.00	18.00

Nucleic acids (µg/ml)	**DNA**	**RNA**	**DNA**	**RNA**	**DNA**	**RNA**	**DNA**	**RNA**	**DNA**	**RNA**
M. canis	200	350	192	312	150	263	175	325	131	205
M. equinum	295	317	215	300	173	285	238	302	150	226
T. mentagrophytes	328	385	271	318	242	295	300	350	275	320
T. verrucosum	305	350	259	316	201	270	250	320	185	201
LSD at 0.05	23.00	15.36	12.55	4.75	24.10	7.91	12.86	5.60	19.00	20.91

LSD, Least significant difference.

Shanab et al., 2008). Antimicrobial peptides from plants permeabilize fungal membranes to cause death (Jenssen et al., 2006).

In Table 4, all tested plant proteins induced the tested zoophilic species to produce more sugars in the culture filtrates, with the most significant accumulation of sugars (530 µg/ml) being shown by the treatment with *M. canis* and *N. sativa* compared with the non-treated control, which may have caused the disturbance in the permeability of the plasma membrane of the tested microbes. Such accumulation of sugar could be due to the failure of sugar uptake by the microbes under the effect of plant extract, resulting in the inhibition of microbial growth. Antimicrobial peptides are very effective against microbes; some of them carry out their action by lysis, by binding to and disrupting the outer membrane of microbes, while others penetrate the membrane and interact with specific internal targets or cause pore formation which results in the leakage of important intracellular contents (Haung et al., 2000).

The results of this study reveal that there was a significant decrease in the nucleic acid components of all the tested pathogens when the tested plant proteins were applied; the decrease in the nucleic acid components reached minimum values of 131 and 205 µg/ml for DNA and RNA, respectively when *M. canis* was treated with *N. sativa* (Table 4). This finding may be due to the fact that the primary mode of inactivation by plant proteins is nucleic acid damage. Electronegative compounds in plants may interfere with biological processes, reacting with vital nitrogen components (proteins and nucleic acids) and thereby inhibit the growth of microorganisms (Rajeswari et al., 2011). Based on the foregoing results, the plant proteins (*N. sativa)* which had the most anti-dermatophytic effect on the most sensitive dermatophyte (*M. canis*) were selected for further experiments.

Purification of *N. sativa* protein

Antidermatophytic assay of partially purified *N. sativa* protein

The antidermatophytic activity of partially purified protein fractions of *N. sativa* dialyzed by 80% ammonium sulphate improved to 5.9 cm, representing 1.11 fold purification over the crude protein (Table 5). Many of the antimicrobial proteins were extracted from plant seeds (Chrisrtenes et al., 2002; Thevissen et al., 2003; Peter et al., 2004; Rengente et al., 2005).

Antidermatophytic assay of pure *N. sativa* protein

The results of antidermatophytic activity of purified protein fractions of *N. sativa* are shown in Table 5. The

Table 5. Purification scheme of *N. sativa* protein.

Purification step	Total volume (ml)	Total protein (mg)	Antidermatophytic activity (cm) on *M. canis*	Yield (%)	Purification fold
Crude protein	100	82.00	5.3	100	1.00
Ammonium sulphate precipitate	9	45.62	5.5	55.63	1.03
dialyzed protein	1.2	25.17	5.9	30.69	1.11
Sephadex G-100 column chromatogram	Pr1	15.23	6.2	18.57	1.16
	Pr2	13.50	7.0	16.46	1.32
DEAE-Sephadex	Pr1	14.11	6.5	17.21	1.22
	Pr2	12.42	7.6	15.14	1.43

DEAE, Diethylaminoethyl.

separation of *N. sativa* protein on Sephadex G-100 column chromatography showed two peaks of proteins (Pr1 and Pr2) which eluted from the column with increasing antidermatophytic activities (6.2 and 7.0 cm, respectively) (Figure 1). The antidermatophytic protein fractions (Pr1 and Pr2) obtained from the previous column were applied to DEAE-Sephadex column and the antidermatophytic activity increased, reaching 6.5 and 7.6 cm inhibition zones against *M. canis,* respectively representing 1.22 and 1.43 fold purification, respectively over the crude protein.

The increase in bioactivity may have been as a result of purification and this increase in antidermatophytic activity presumably involves the removal of low molecular weight inhibitors, possibly the phenolic substance released from the growth substrate, and the higher concentration of bioactive protein, while purification may help in the unmasking of the antimicrobial activity of proteins (Figures 2 and 3).

This observation provides strong empirical evidence that small proteins play an important role in antifungal defense system of plants (Terras et al., 1995). It is also possible that the antidermatophytic activity of plant extracts after gel filtration is due to the presence of bioactive pure protein. The endogenous antimicrobial peptides of plants are typically cationic amphipathic molecules consisting of 12 to well over 100 residues; this amphipathic structural arrangement is believed to play a key role in the action of the antimicrobial mechanism, while the hydrophilic and strongly positively charged domain is believed to initiate peptide interaction with the negatively charged fungal surface, such that many cationic antimicrobial peptides have been described as membrane-active agents (Kim et al., 1998).

Estimation of the molecular weights of the purified *N. sativa* proteins

The homogeneity of pure protein fractions (Pr1 and Pr2) was checked by SDS polyacrylamide disc gel electrophoresis; the pure protein fractions were found homogeneous by showing single bands. The molecular weights of the purified *N. sativa* proteins (Pr1 and Pr2) were 42.7 and 31 kDa respectively (Figure 4). The low molecular weight protein fraction (Pr2) may be suitable for high antidermatophytic molar activities on pathogens or might be explained by the higher specific adsorption of protein on pathogens (Sterho et al., 1997). The obtained results are similar to that of Sakıyan et al. (2004) who stated that the antimicrobial activities tended to decrease with the increasing amount of the amino acid residues. The fractionation of whole *N. sativa* seeds using SDS-PAGE showed a number of protein bands ranging from 94 to 10 kDa molecular mass (Salem, 2005). The purification of 29-kDa protein, a microbe inhibiting protein, from *Quercus infectoria* was highly effective in inhibiting the mycelial growth of *Fusarium oxysporum, Cochliobolus miyabeanus, Macrophomina phaseolina, Colletotrichum gloeosporioides, Magnaporthe salvinii, Cochliobolus lunatus, Alternaria solani, Pythium aphanidermatum* and *Colletotrichum falcatum* (Yamunarani et al., 2005).

Characterization and antidermatophytic activities of the purified *N. sativa* protein

The data in Figure 5 shows that the maximum antidermatophytic activity (7.8 cm) of purified *N. sativa* protein (Pr2) was observed at a pH of 7 and a temperature of 20°C (Figure 6). At high temperature, the activity decreased and was finally lost, which pointed to the fact that the antidermatophytic compounds may be regarded as proteins which may cause membrane permeabilization, resulting in the binding of cationic proteins to the negatively charged membrane surface, and subsequent pore-formation; with an increase in temperature, these proteins may get degraded (Thevissen et al., 1996). The effect of thermal processing on purified

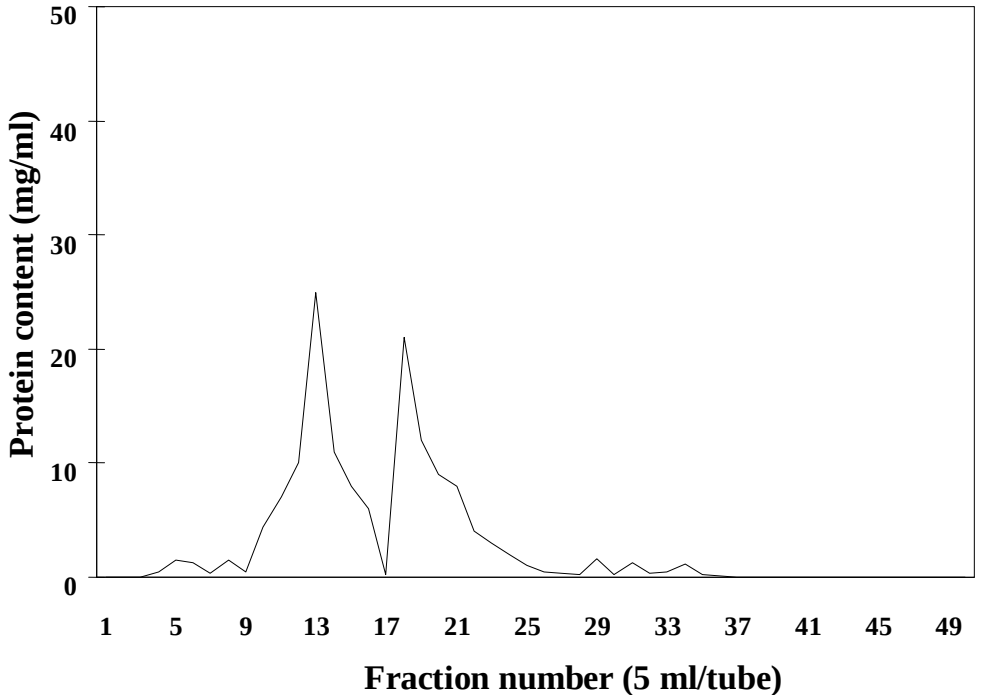

Figure 1. Typical elution profile of the behavior of *N. sativa* protein on Sephadex G-100.

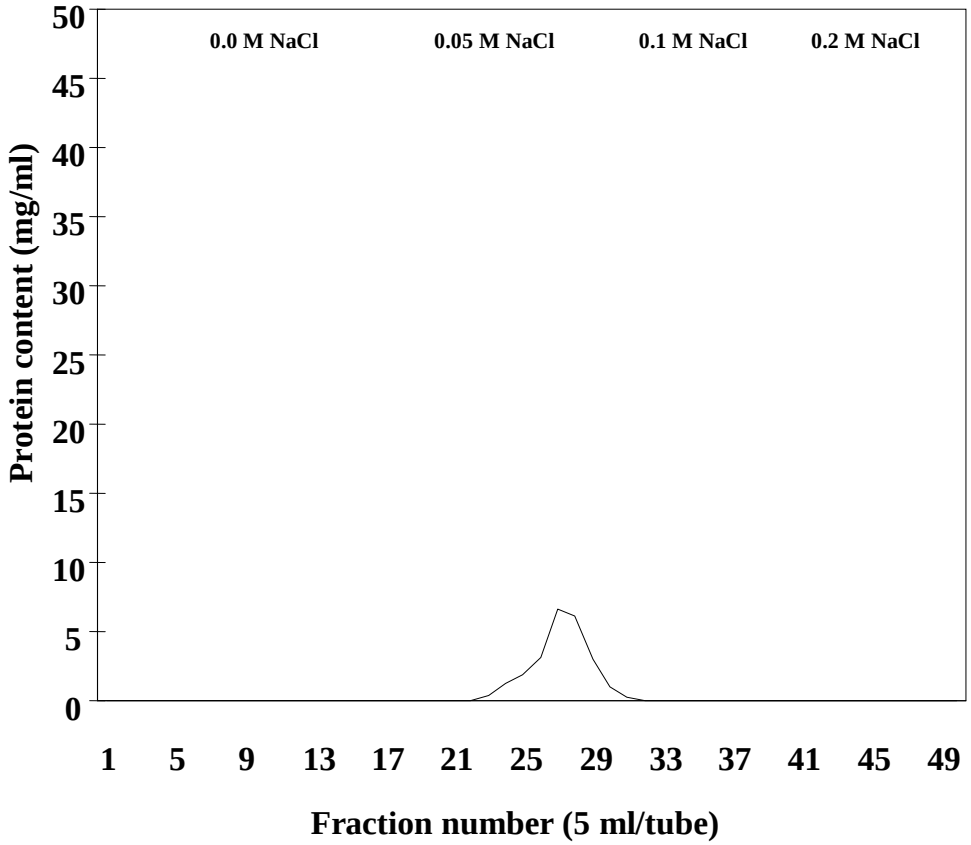

Figure 2. Typical elution profile of the behavior of protein (Pr1) on DEAE-Sephadex. DEAE, Diethylaminoethyl.

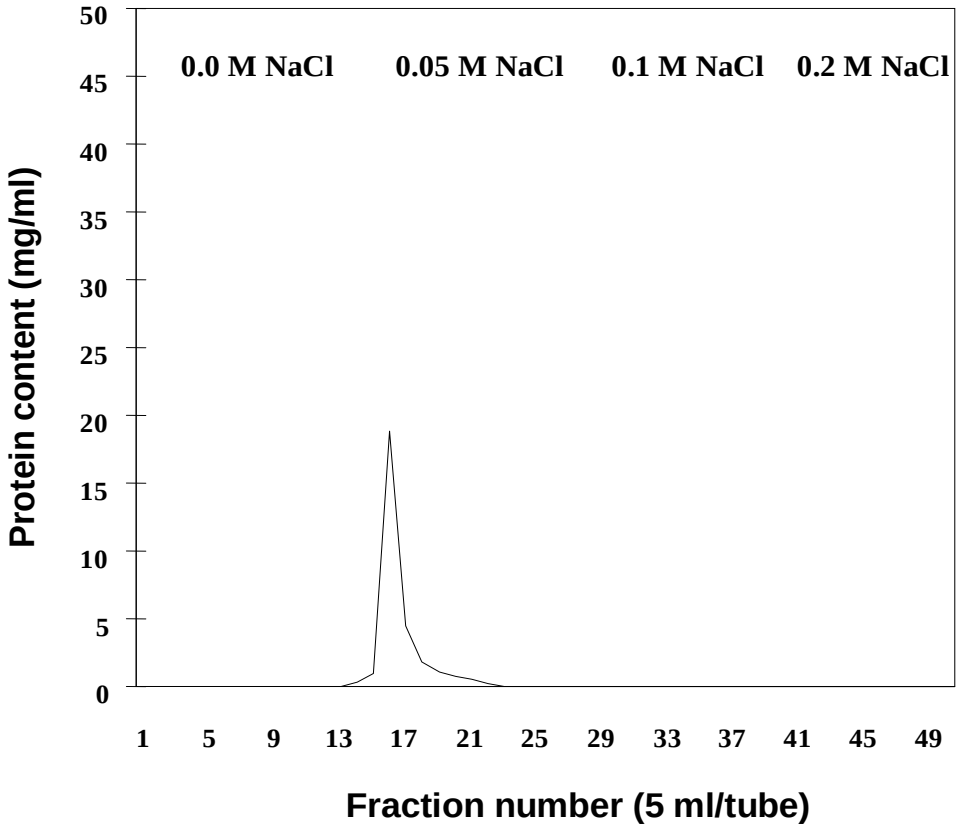

Figure 3. Typical elution profile of the behavior of *N. sativa* protein (Pr2) on DEAE-Sephadex. DEAE, Diethylaminoethyl.

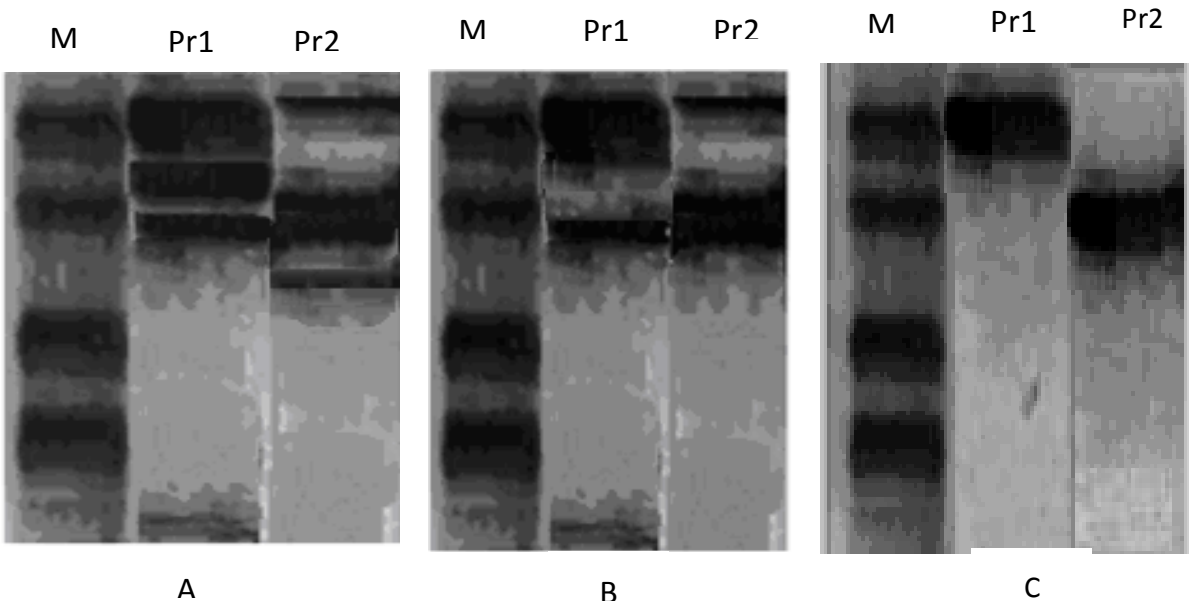

Figure 4. Estimation of the molecular weight of the purified *N. sativa* proteins (Pr1 and Pr2) by SDS-PAGE profile. M, Marker (maltose-binding proteins, 42.7 kDa; carbonic anhydrase, 31 kDa; trypsinogen, 24 kDa, lysozyme, 14.4 kDa). (A) Dialyzed protein, (B) chromatography on Sephadex G-100 column, (C) chromatography on DEAE-Sephadex column. DEAE, Diethylaminoethyl.

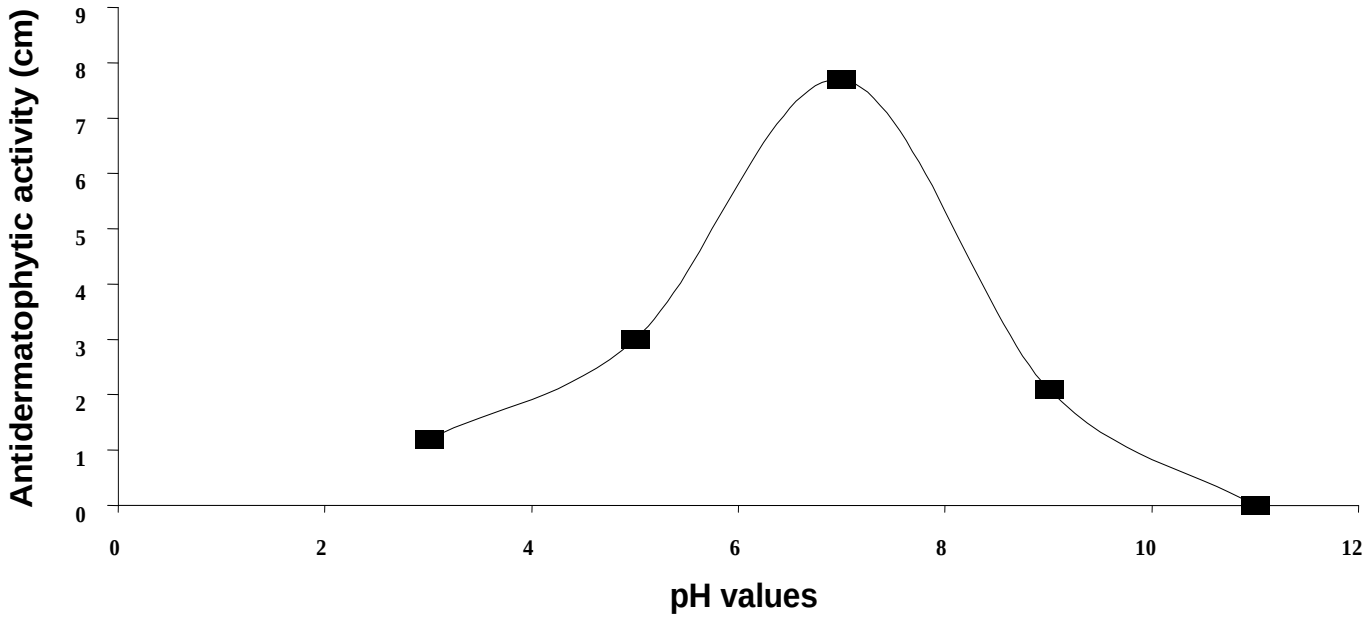

Figure 5. Effect of pH values on the antidermatophytic activity of the purified *N. sativa* protein (Pr2) on *M. canis*.

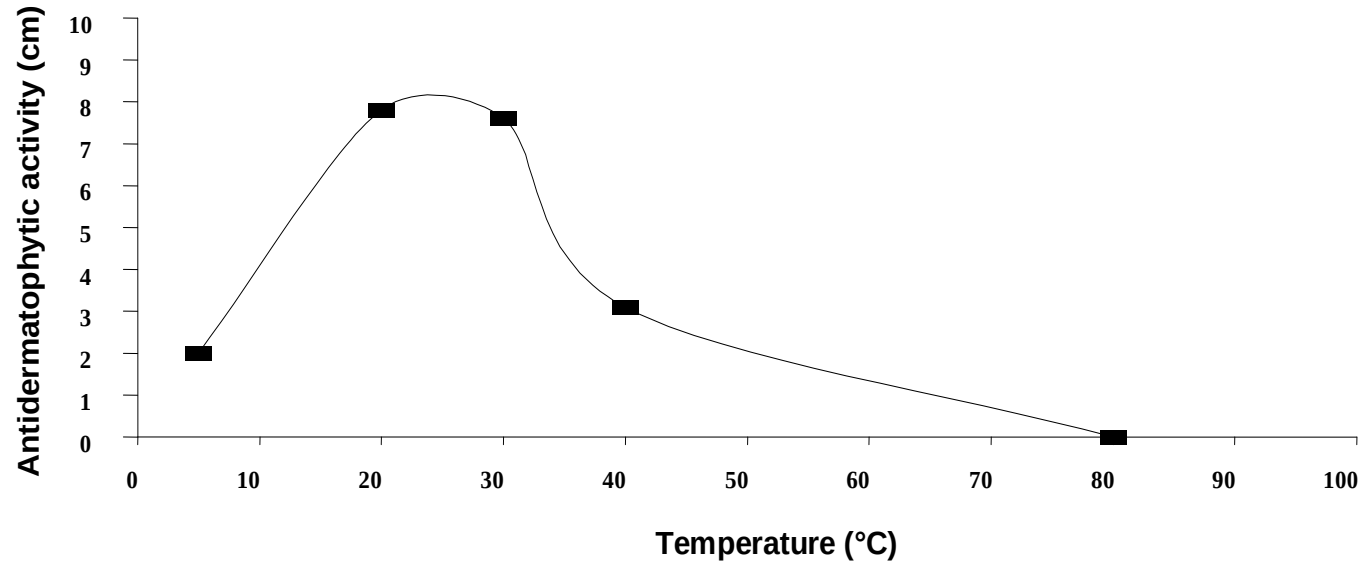

Figure 6. Effect of temperature on the antidermatophytic activity of the purified *N. sativa* protein on *M. canis.*

plant protein was investigated by Arcan and Yemenicioğlu (2007).

Our results in Figure 7 demonstrate that the purified protein (Pr2) was sensitive to different ions (Na^+, K^+, Ca^{2+} and Mg^{2+}) which decreased its antidermatophytic activity. This might be due to the effect of these ions on the coagulation properties of plant extracts (Okuda et al., 2001), or the stabilization of membrane phospholipid structures (Thevissen et al., 1999). This result is similar to that of Jabeen et al. (2008) who found that small amounts of mono- and divalent cations (up to 50 mM) were shown to severely decrease the potency of antifungal plant proteins, possibly through the stabilization of membrane phospholipid structures.

Conclusion

The results obtained from this study suggest that *N. sativa* proteins are not only remarkable sources of anti-

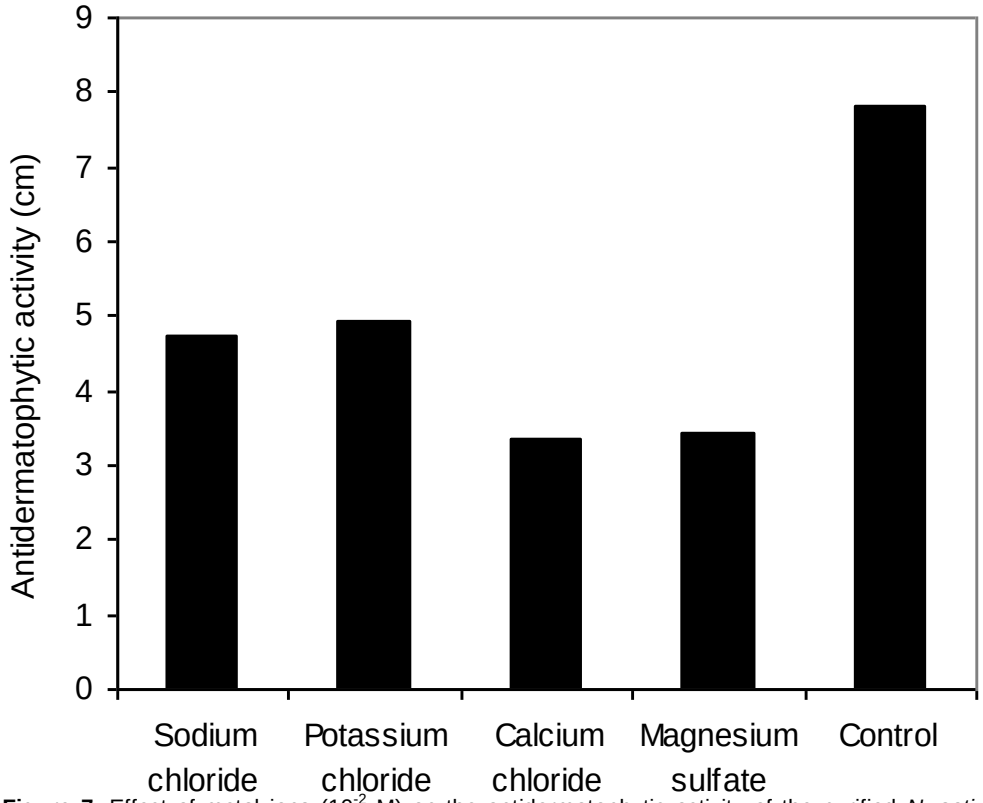

Figure 7. Effect of metal ions (10^{-2} M) on the antidermatophytic activity of the purified *N. sativa* protein (Pr2) on *M. canis.*

dermatophytic agents but also potential sources of antioxidants. The complete purification of *N. sativa* antidermatophytic proteins eluted low molecular weight proteins (Pr2) with the highest antidermatophytic activity. This observation provides strong empirical evidence that small proteins (Pr2) may play an important role in the defense system of antidermatophytic pharmaceutical plants.

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