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Isolation and Purification of an Antifungal Protein from Kiwi Fruits and Demonstration of Its Antifungal Activity

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Authors' contributions

This work was carried out in collaboration between all authors. Author AW performed the extraction, assay of antifungal activity and wrote the manuscript. Author NR designed the study, performed the purification part. Author VR provides the samples of kiwi and also provides the laboratory facilities and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A single polypeptide low molecular weight 25 KDa thaumatin-like antifungal protein was purified from Hayward variety of kiwifruits. It was purified to apparent homogeneity involving saline extraction, $(NH_4)_2SO_4$ precipitation, anion exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-100, respectively. In present work, simple chromatography procedure was described for purifying antifungal activity form the pulp of Hayward kiwifruit. It furnishes evidence of existence of antifungal activity against *Fusarium oxysporum* (83.72%) and *Rhizoctonia solani* (33.78%) and did not show any inhibitory effect against *Dematophora necatrix*. The proteins have low molecular weight and would be potential importance for increasing disease resistance in plants through genetic engineering and can be used as bio control for controlling fungal diseases in plants.

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1. INTRODUCTION

Fungal diseases have been one of the principle causes of crop losses ever since human started to cultivate plants. To date, epidemic spread of fungal diseases is controlled by various crop husbandary techniques, such as crop rotation, fungus resistant cultivars and applications of agrochemicals. Unfortunately, in many cases we have come to rely almost exclusively on the use of chemical control practices. These chemicals are costly and have become less effective after evolution of pathogens. Moreover their use is potentially harmful to the environment. The growing concern about the environment together with a strong motivation to lower production cost, encourages the development of cultivars of that require few chemicals. Therefore, there is need to find innovative ways of controlling fungal diseases without using chemicals. Newly developed technologies in plant breeding such as Restriction Fragment Length Polymorphism and gene transfer technology can be used to develop disease resistant cultivars [1]. Thus, there is an extensive search for genes that encode enzymes/protein involved in synthesis of compounds toxic to fungi or for genes that encode proteins with a direct inhibitory effect on growth of fungi [2].

In order to combat fungal attack, some plants elaborate a variety of inhibitory molecules that comprise phenols, melanin, tannins, phytolexins and pathogenesis related proteins with the capability of acting directly on fungi to inhibit growth [3,4]. The antifungal proteins are structurally diverse. Antifungal proteins and peptides have drawn the attention of a large number of researchers by virtue of their potential value in protecting economically important crops from fungal attack. It is well documented that there are many types of antifungal proteins and peptides with different amino acid sequences and antifungal potencies [5]. Thaumatin-like proteins [6-8], Chitinases [9,10], Ribosome inactivating proteins [11], Cyclophilin like proteins [12] and miraculin like protein [13] are the members of the family of antifungal proteins.

The kiwifruit *(Actinidia chinensis)* is a popular fruit very rich in Vitamin C. There are four cultivars growing in mid hills of Himachal Pradesh (India) viz., Allision, Bruno, Hayward and Monty. The aim of present paper was to ascertain whether kiwifruit elaborated any antifungal proteins. The objectives of this study were to isolate and purify antifungal protein from the green flesh of unripened kiwifruits.

2. MATERIALS AND METHODS

2.1 Extraction and Partial Purification

Four cultivars of kiwifruits viz., Allision, Monty, Bruno and Hayward were procured from Department of Fruit Science, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan. H.P. India. One fruit (125 g) was homogenized in a grinder with 0.9% saline solution and centrifuged at 15,000 rpm for 30 min at 4. The supernatant was precipitated with ammonium sulphate. The precipitates were dissolved in Tris-HCL buffer (10 mM, pH 7.0). The protein was then, used for antifungal assay [8].

2.2 Purification of Antifungal Protein

Throughout the course of protein purification the relative absorbance of fractions was monitored at 280 nm. The crude protein was dialyzed against the same buffer using dialysing tubing with an 10 KDa cut off at 4℃ for 24 hour with three changes of dialysis buffer. Manually-packed DEAE-cellulose column (5×8 cm) (Genei Bangalore, India) pre equilibrated with 10 mM Tris-HCl buffer pH 7.0 containing 1mm DTT (dithiothreitol) and 1 mM EDTA (ethylenediaminetetraacetic acid) was used for purification. After loading the dialysed protein sample, column was eluted with start buffer (10 mM Tris-HCl buffer, pH 7.0 containing 1 mM DTT and 1 mM EDTA) to remove the unbound/unabsorbed protein and bound/absorbed protein eluted with 1M NaCl in the same buffer at a fast flow rate of 1 ml/min and fraction size was 3 ml. Both absorbed and unabsorbed fraction tested for antifungal activity.

The fractions which shows antifungal activity was concentrated and was directly loaded on to Sephadex G-100 gel filtration column (2×30 cm) (from GE healthcare). The flow rate was adjusted to 1 ml/min and fractions of 3 ml size were collected. Antifungal activity and protein concentration was estimated in each fraction. The fraction having high antifungal activity were collected and pooled.

2.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular mass of the purified antifungal protein was estimated by SDS-PAGE, which was performed using 12% polyacrylamide gel by the method described by Laemmli and Favre [14]. After electrophoresis the gel was stained with Coomassie Brilliant Blue R-250. The molecular mass of kiwifruit antifungal protein was determined by comparison of electrophoretic mobility with those of molecular mass markers.

2.4 Assay of Antifungal Activity

The agar diffusion assay was used for antifungal activity toward *Fusarium oxysporum*, *Rhizoctonia solani* and *Dematophora necatrix* carried out in 100 mm × 15 mm petriplates containing 20 ml of malt extract agar (MEA). Inhibition of the fungi was studied by adding (10%) partially purified extract of kiwi protein in MEA before plating. Mycelial disc of 5 days old culture of the test fungal pathogen was placed in the middle of the plates and incubated at 24±1℃ for seven days. In control, only nutrient broth was added in place of the partially purified extract of kiwi protein (three replicate plates per each bio-control agent-pathogen). Fungal growth inhibition was recorded after seven days of incubation and antifungal activity was calculated according to Vincent [15] using the formula:

$$I = \frac{C-T}{C} \times 100$$

Where, I = Per cent growth inhibition
C = Growth of fungus in control
T = Growth of fungus in treatment

3. RESULTS AND DISCUSSION

The kiwifruit antifungal protein was subjected to sequential ammonium sulphate saturations from 0 to 80%. Most of the kiwifruit antifungal protein was precipitated between 30-80% saturation of ammonium sulphate, which contained 652.0 mg protein. Below and above 30–80% saturation of ammonium sulphate, antifungal activity of the protein was significantly low. Based on the results of this experiment, 30-80% ammonium sulfate saturation cut was given to precipitate antifungal protein of kiwifruit. Whenever required in subsequent experiments, protein samples between 20-80% ammonium sulfate cut were pooled, concentrated and applied onto anion exchange chromatography.

The fractions were pooled according to the absorbance at 280 nm and assayed for antifungal activity [16]. Table 1 showed the comparison of protein content after ammonium sulphate precipitation (30-80%) among four cultivars of kiwifruit. The highest protein content (330 mg /100 g) and antifungal activity was recorded in Hayward followed by Monty (252 mg/100 g) and Allision (232 mg/100 g) and minimum was found in Bruno (149 mg/100 g). The Hayward antifungal protein was again purified on DEAE-cellulose column, only the unabsorbed fractions contained antifungal activity (Fig. 1). The antifungal protein was eluted in the fraction 81-102 with the NaCl gradient of 0.5 M. The maximum antifungal activity was observed in the 30th fraction (Fig. 1). However, fractions 27-34 had very high antifungal activity and therefore, these samples were pooled and lyophilized to concentrate the sample and subjected to gel-filtration. Similar result have also been reported in green flesh of kiwifruits [8,17]. Thaumatin like antifungal proteins have been isolated from a variety of species including grape berries [18], tomato [19], maize seeds [20], tobacco leaves [21], wheat, sorghum and barley [22,23].

The fraction was pooled and loaded on to Sephadex G-100 column for gel chromatography pre-equilibrated with 10 mM Tris-HCl (pH 7.0). The protein rich sample (4.0 ml) obtained from the anion exchange chromatography was applied to the column. The fraction number 20-25 exhibited antifungal activity (Fig. 2). Maximum antifungal activity was observed in 23rd fraction with a large peak. These fractions were pooled and lyophilized to concentrate the sample and used for SDS-PAGE. The protein content and antifungal activity was assayed after lyophilized the sample. Table 2 summarise the result of purification of protein. The active fraction then loaded on SDS page which appeared as a single band with molecular mass of 25 KDa (Fig. 3). The antifungal protein yield was 49 mg from 100 g of Hayward kiwi fruit. Hexiang and Tzi [8] reported 21 KDa antifungal protein isolated from Kiwifruit with 96 mg yield from 3 Kg of fruits. Antifungal proteins from seeds of the Kweilin Chestnut Castanopsis chinensis have molecular mass of 30 KDa which is higher than the isolated from kiwifruit [17]. Whereas, antifungal protein from French bean legume reported to be 21 KDa [24].

The antifungal activity of protein was compared using agar diffusion assay method. The purified

protein arrested mycelia growth six days after incubation and no overgrowth was observed even after eight days of fungal growth (Table 3, Plates 1, 2). Partially purified protein showed comparatively higher growth inhibition against *Fusarium oxysporum* (83.72%) than against *Rhizoctonia solani* (33.78%) and showed no antagonistic activity against *Dematophora necatrix.* Similar antifungal protein has also been reported in French bean legumes and kiwifruits [8,10]. The French bean protein exerted antifungal activity against *Fusarium oxysporum,*

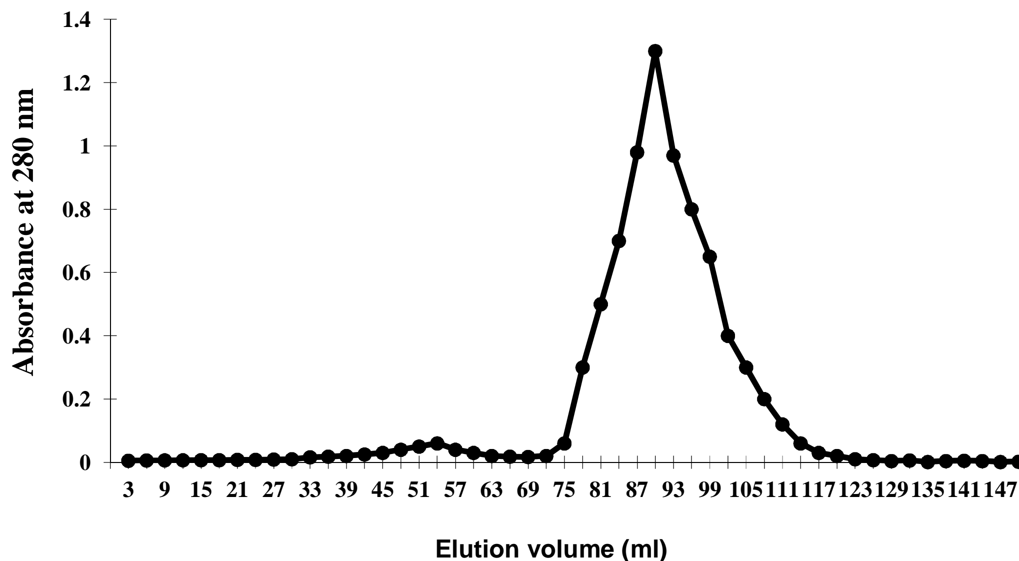


Fig. 1. Anion exchange chromatography of the fraction of kiwi fruit extract on DEAE-cellulose column. Antifungal activity was observed in fractions corresponding to elution volume of 81-105 ml were pooled and subjected to gel filtration chromatography for further purification

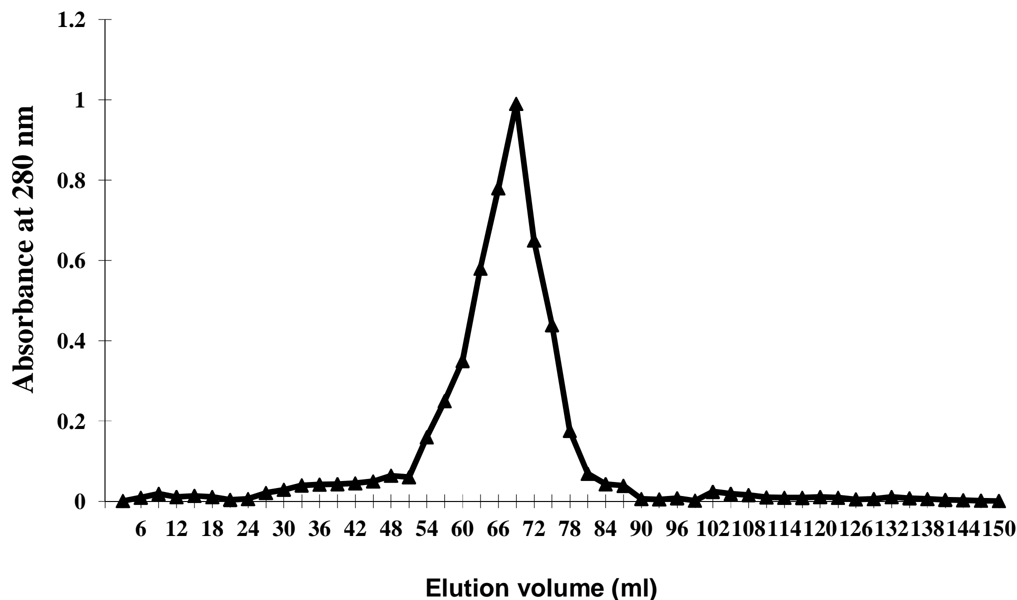


Fig. 2. Gel filtration of pooled fractions from ion exchange chromatography by fast protein liquid chromatography on a Sephadex G-100 column. Antifungal activity was observed in fractions corresponding to elution volume of 60-75 ml

Pleurotus and *Coprinus comatus* but not against *Rhizoctonia solani*. Antifungal protein from coconut displayed antifungal activity against *Fusarium oxysporum*, *Mycosphaerella arachidicola* and *Physalospora piricola* [26]. However, Kiwifruit protein has been reported to be inhibitory against *Botrytis cinera* and suppressive to *Sphaerella arcchidicola* and *Coprnicus comatus*. It is also reported that kiwifruit TLP manifested an inhibitory activity against HIV-1 reverse transcriptase. Further, these proteins are thaumatin like protein (TLP) which are pathogenesis related protein (PRP) and have inhibitory effect on various fungal pathogens [8,25].

Table 1. Comparison of antifungal protein in kiwifruit cultivars

Cultivars	Partially purified protein (mg*)
Allision	232.0
Bruno	149.0
Hayward	330.0
Monty	252.0

Protein obtained from 100 g starting material

Table 2. Summary of purification of antifungal protein from kiwifruit

Steps	Protein (mg*)
Crude extract	1210
$NH_4SO_{4\ (30\text{-}80\%)}$	615
DEAE-Cellulose	96
Sephadex G-100	49

Protein obtained from 100 g starting material

Table 3. Effect of purified protein on the growth of *Fusarium oxysporum, Rhizoctonia solani* in mixed culture on malt extract medium

Fungus	Per cent (%) growth inhibition
Fusarium oxysporum	83.72
Rhizoctonia solani	33.78
Dematophora necatrix	0.00

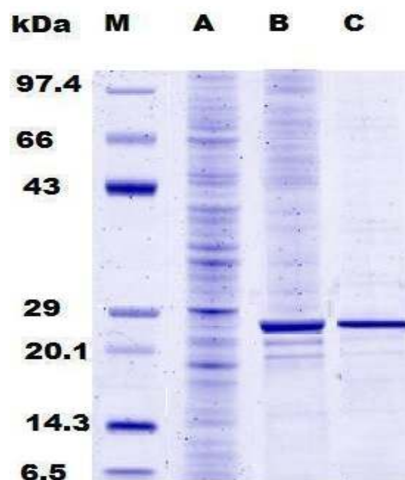
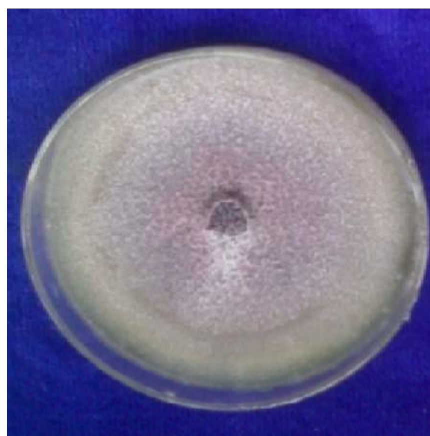


Fig. 3 SDS-PAGE (12%) of antifungal protein preparations at various stages of purification. M- Low range molecular weight marker; Lane A: Crude protein (10 µg), Lane B: DEAE column chromatography pooled fractions (10 µg), Lane C: Sephadex G-100 column chromatography pooled fractions (10 µg)



Control

Test

Plate 1. Effect of purified protein on the growth of *Fusarium oxysporum*

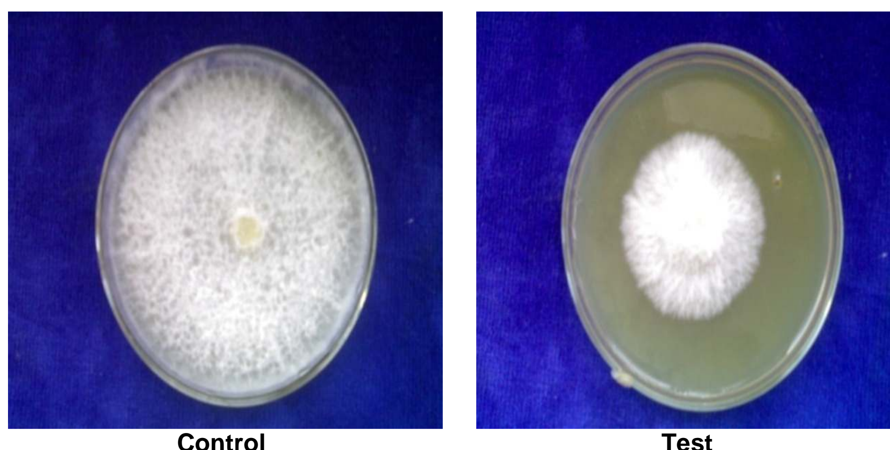


Plate 2. Effect of purified protein on the growth of *Rhizoctonia solani*

This study thus indicated that kiwifruit contains good amount of antifungal activity. By using only one fruit (125 g) and with purification of protein have shown good result against two tested fungus. These results indicate that in kiwifruits antifungal proteins present in high enough level to be isolated. Thus there is need to sequence the protein and test it against other fungi under laboratory and field conditions.

4. CONCLUSION

In the results of this study, it was concluded that simple chromatography procedure is used for purifying antifungal activity form the pulp of Hayward kiwifruit. It furnishes evidence of existence of antifungal activity. The proteins have low molecular weight and potential importance for increasing disease resistance in plants through genetic engineering and can be used as bio control for controlling fungal diseases in plants.

HIGHLIGHTS

Low molecular weight of 25 KDa thaumatin-like protein isolated from Hayward variety of kiwifruit which showed antifungal activity against *Fusarium oxysporum* and *Rhizoctonia solani.* This study could be useful for obtaining biocontrol against fungal diseases in plants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Radhajeyalakshmi R, Velazhahan R, Balasubramanian P, Doraiswamy S. Overexpression of thaumatin-like protein in transgenic tomato plants confers enhanced resistance to *Alternaria solani*. Archives of Phytopathology and Plant Protection. 2005;38:257–265.
2. Kamran Safavi, Reza Zareie, Badraldin Ebrahim, Sayed Tabatabaei. Constitutive expression of thaumatin-like protein (TLP-3) in transgenic tobacco plants leads to enhance resistance to *Alternaria alternate*. Archives of Phytopathology and Plant Protection. 2012;45:161–169.
3. Bowles DJ. Defense-related proteins in higher plants. Annual Review of Biochemistry. 1990;59:873-907.
4. Linthorst HJM. Pathogenesis-related proteins of plants. Critical Review in Plant Science. 1991;10:123-150.
5. Vincent SM, Ho Jack, H Wong, Ng TB. A thaumatin-like antifungal protein from the emperor banana. Peptides. 2007;28:760-766.
6. Del Campillo E, Lewis LN. Identification and kinetics of accumulation of proteins induced by ethylene in bean abscission zone. Plant Physiology. 1992;98:955-961.
7. Vu L, Huynh QK. Isolation and characterisation of 27 KDa antifungal protein from the fruits of *Diospyros texana*. Biochem. Biophysics Research Communication. 1994;202:666-672.
8. Hexiang W, Tzi BN. Isolation of an antifungal thaumatin like protein from kiwi fruits. Phytochemistry. 2002;61:1-6.

9. Vogelsang R, Barz W. Purification, characterization and differential hormonal regulation of a c-1, glucanase and two chitinases from chickpea (*Cicer arietinum* L.). Planta. 1993;189:60-69.
10. Ye XY, Wang HX, Ng TB. Sativin, a novel antifungal miraculin-like protein isolated from the legumes of the sugar snap *Pisum sativum* var. *Macrocarpon*. Life Science. 2000a;67:775–781.
11. Leah R, Tommerup H, Svendesen I, Mundy J. Biochemical and molecular characterization of three barley seed proteins, alpha and beta pisavins from seeds of garden pea *Pisum sativum*. Biochemical Biophysics Research Communication. 1991;253:135-142.
12. Ye XY, Ng TB. Mungin, a novel cyclophilin-like antifungal protein from the mung bean. Biochemical and Biophysics Research Communication. 2000;273:1111–1115.
13. Ye XY, Wang HX, Ng TB. Dolchin a new chitinase like antifungal protein isolated from field beans. Biochemical Biophysics Research Communication. 2000b;269: 155-159.
14. Laemmli UK, Favre M. Maturation of the head of bacteriophage T4 1. DNA packaging events. Journal of Molecular Biology. 1973;50:575-599.
15. Vincent JM. Distortion of fungal hyphae in the presence of certain inhibitors. Nature. 1947;150:850.
16. Daniel M, Bollag S, Edelsten J. Concentrating protein solutions In: Protein methods. John Willey & Sons, INC Publication New York; 1991.
17. Chu KT, Ng TB. Isolation of a large thaumatin- like antifungal protein from seeds of the Kweilin chestnut *Ccastanopsis chinensis.* Biochemical Biophysics Research Communication. 2003;301:364-70.
18. Tattersall DB, van Heeswijck R, Hoj PB. Identification and characterization of a fruit-specific, thaumatin-like protein that accumulates at very high levels in conjunction with the onset of sugar accumulation and berry softening in grapes. Plant Physiol. 1997;114:759-769.
19. Pressey R. Two isoforms of NP24: A thaumatin-like protein in tomato fruit. Phytochemistry. 1997;44:1241-1245.
20. Huynh QK, Borgmeyer JR, Zobel JF. Isolation and characterization of a 30 kDa protein with antifungal activity from leaves of *Engelmannia pinnatifida*. Biochemical Biophysics Research Communication. 1992;182:1-5.
21. Rodrigo I, Vera P, Frank R, Conejero V. Cdna cloning of viroid-induced tomato pathogenesis-related protein P23: characterization as a vacuolar antifungal factor. Plant Physiol. 1991;102:939–945.
22. Vigers AJ, Roberts WK, Selitrennikoff CP. A new family of plant antifungal proteins. Molecular Plant-Microbe Interaction. 1999; 4:315-323.
23. Hejgaard J, Jacobsen S, Svendsen I. Two antifungal thaumatin-like proteins from barley grain. FEBS Letters. 1991;291:127-131.
24. Ye XY, Wang HX Ng TB. First Chromatographic Isolation of an antifungal thaumatin like protein from French Bean legumes and demonstration of its antifungal activity. Biochemical Biophysics Research Communication. 1999;263:130–134.
25. Punja ZK. Transgenic carrots expressing a thaumatin-like protein display enhanced resistance to several fungal pathogens. Canadian Journal of Plant Pathology. 2005;27:291–296.
26. Wang HX, Ng TB. An antifungal peptide from the coconut. Peptides. 2005;26: 2392-2396.

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