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Characterization of Antifungal Protein Isolated from the Leaf of *Solanum Trilobatum & Lepidagathis Cristata* and its Activity against Phytopathogenic Fungi.

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ABSTRACT

Microorganisms are responsible for great losses in economical crops, preventing effective food distribution worldwide. Although great amount of research is being performed to overcome the damage caused by fungal pathogens, an effective solution is still evasive. One of the methodologies is to use antifungal proteins. Different sources are available to obtain an antifungal protein, but, their efficiency varies with source. Medicinal plants are valuable resources of natural antimicrobial materials. *Solanum trilobatum* and *Lepidagathis cristata* are medicinal herbs used in Indian medicine of both Sidha and Ayurveda. Two closely related proteins with antifungal activities, purified from the leaves of *Solanum trilobatum* and *Lepidagathis cristata* are heat-stable proteins with a molecular mass of 31 KDa & 30 KDa respectively. Characterization of isolated proteins reveals that they are cation sensitive protein, highly heat stable proteins and are stable for a wide range of pH between 5 and 8.5. Antifungal protein from *Lepidagathis cristata* inhibits the spore formation and has the fungicidal action. Antifungal protein on the other hand from *Solanam trilobatum* delays the hyphal extension and has the fungi static action. The activities of antifungal proteins are less in *in- vivo* condition, when compared to *in-vitro* conditions.

Keywords: Antifungal protein, pathogen, *Solanum trilobatum*, *Lepidagathis cristata,* static action

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INTRODUCTION

About 250,000 species of fungi were extremely distributed in every diverse group of the ecosystem. The fungi are heterotrophic organisms possessing a chitinous cell wall, with the majority of fungal species growing as multi cellular filaments called hyphae forming a mycelium. Some fungal species also grow as single cells. Yeasts, molds, and mushrooms are examples of fungi. Most fungi are significant pathogens of plants, humans and other animals. The losses due to diseases of crops (e.g., rice blast disease) or food spoilage caused by fungi can have a large impact on human food supply and local economies. In 2007-2008 summers Africa, there was 70% to 100% loss in wheat due to a fungus on the crops. This huge loss in wheat has left the African people and their animals starving and moving in a mass exodus to the cities. South Africa had riots over the super inflation of the cost of wheat and other foods.

A fungus produces antifungal proteins that may contribute to both defenses against predators such as insects as well as pathogens. Number of antifungal peptides and proteins were synthesized by plants, bacteria, insects, mollusks, fungi and mammals. These proteins appear to be involved in resistance to fungal attack and these proteins and peptides have been classified, based on their function and structure. Those peptides and proteins were grouped as thaumatin like proteins, chitinases, glucanases, embryo abundant proteins, miraculin-like proteins, cyclophilin-like proteins, allergen-like proteins, thionins, and lipid transfer proteins. Different species of plants produce a number of lectins which act as protein, trypsin inhibitors, ribosome inactivating proteins, PR proteins, (1,3) b-glucanases, chitinases, chitin-binding proteins, thaumatin-like (TL) proteins, defensins, cyclophilin-like protein, glycine or histidine-rich proteins, lipid-transfer proteins (LTPs), killer proteins (killer toxins), protease inhibitors, etc., to protect themselves from both pathogens and their predators.

MATERIALS AND METHODS

MEDIA PREPERATION

Potatoes was peeled and sliced. 200 g of above mentioned potatoes was boiled for one hour in one liter of water. Filter and make up the volume to one liter. Add 3 g of glucose (dextrose), agar and heated until agar gets dissolved. The media should be sterilized using an autoclave. This media composition is used for the culture *Pyricularia oryzae* and *Alternari brassicae.*

SOURCE

Collection of fungal culture and maintenance

The fungal culture *Pyricularia oryzae* and *Alternari brassicae* were procured from CAS in Botany University of Madras.The fungal culture was maintained in Potato Dextrose Agar Medium (PDA) and were maintained at 4^{o}C in slants as a mother culture.

Pyricularia oryzae **-** Rice infecting fungi**,** present all over the world
Alternaria brassicae - infects rice, oil seed rape, cabbage, cauliflower present in south Asia

EXTRACTION OF ANTIFUNGAL PROTEIN

200 g of *Solanum trilobatum & Lepidagathis cristata* leaves were finely grounded using Braun coffee grinder and stirred for 1hr in 300 ml of cold 60 mM acetic acid. The suspension was centrifuged at 7400 rpm for 20 min and supernatant was collected. The supernatant was neutralized to pH 7.6 by adding 1M Tris base drop-wise and was placed at 4°C overnight. The precipitate formed was removed by centrifugation. Stirring the ground grain with dilute acetic acid was carried out to minimize the amount of contaminating protein, but it was not required for extraction of antifungal proteins. Stirring with 130 mM NaC1/10mM sodium phosphate (pH 7.4) produced an extract with equivalent amount of antifungal activity.

AMMONIUM SULFATE SATURATION AND DIALYSIS

The formation of precipitate at 50-70% $(NH_4)_2SO_4$ saturation was isolated from the *Solanum trilobatum* and *Lepidagathis cristata* leaf extract and dissolved in 50 mM sodium phosphate (pH 7.0) and the fraction (ii) was dialyzed against 50mM sodium phosphate (pH 7.0). Under 4°C, 14 KDa dialysis tubing was used for dialysis and the obtained protein was resuspended in 50 mM sodium phosphate (pH 7.0) and dialyzed.

SEPHADEX G-100 COLUMN CHROMATOGRAPHY

The fractions containing antifungal protein was purified using Sephadex chromatography. 10mM sodium phosphate (pH 7.0) was used to equilibrate the column and the fraction was passed through a Sephadex column (2.5 x 60cm) and washed through with additional 20ml of the sodium phosphate buffer. Contaminating proteins bound to the column were removed by elution with 10mM sodium phosphate (pH 7.0).

SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-Poly acryl amide gel electrophoresis was performed on slab gel with separating and stacking gels (10 & 5% w/v). The gel was removed, stained with silver stain and the gels were stored in 7% (v/v) acetic acid. The molecular mass of the purified antifungal protein was determined on SDS-PAGE. Purified protein samples were run on SDS-PAGE with the concurrent run of standard protein ladders (sigma chemicals). After separation, the gels were stained with silver nitrate.

pH OPTIMIZATION AND STABILITY

Optimum pH of purified antifungal protein towards stability was determined using 0.01M Sodium acetate buffer (pH 3 to 5.5), 0.01M Sodium phosphate buffer (pH 6 to 8) and Tris-HCl buffer (pH 8 to 9). The concentration and stability of protein at different pH was calculated after pre incubation of purified antifungal protein with pH ranging from 3 to 9. Concentration of the protein in the solution was determined by Bradford method using Bovine Serum Albumin fraction as standard.

TEMPERATURE OPTIMIZATION AND THERMAL STABILITY

The optimum temperature and thermal stability was determined at different temperature viz., 25, 30, 37, 40, 45, 55, 60, 65°C. 0.5ml of purified antifungal protein was dissolved in distilled water and incubated as above mentioned temperatures for 1hour (hr) and by using Bradford method employing Bovine Serum Albumin fraction as standard the protein concentration in the solution was determined.

***IN-VITRO* STUDIES**

HYPHAL EXTENSION-INHIBITION ASSAY

Antifungal activity was determined under sterile conditions using a hyphal extension-inhibition assay. Mycelia were placed in the centre of petri dishes which were harvested from actively growing fungal plates containing nutrient agar and incubated for 20-24hr at room temperature. Sterile filter paper discs were placed on the agar surface at four corners, and in front of the advancing fungal mycelium and 35µl of protein solution with different concentration (25, 50, 75, 100 µg/ml) was applied to each disc. The plates were incubated at room temperature for 20 hr and observed for zone of inhibition.

MORPHOLOGICAL ANALYSIS IN INHIBITED HYPHAE

After purification the antifungal activity against fungi was assayed on liquid cultures grown in test tubes. Different concentration (25, 50, 75, 100 µg/ml) of protein sample were mixed with 3 ml of either half-strength potato dextrose broth containing fungal spores at a concentration of 2×10^4 conidia ml^{-1}. After 24 hr incubation at 25°C fungal growth inhibition was routinely checked microscopically to find the difference in the morphology of hyphae.

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LETHAL EFFECT OF PROTEIN

The lethal effect of protein was identified in an experiment, where the protein concentration having highest antifungal activity (100µg/ml) was added to broth culture containing fungal spores at a concentration of $2x10^4$conidia ml^{-1}. After 48hr of incubation at 25°C, broth culture was analysed for the presence of viable cells by taking absorbance at 425nm for every 5hr. Then replacement of the medium (containing protein) by fresh PD broth (without protein), growth of mycelium was analyzed.

ANTI FUNGAL ACTIVITY OF PROTEIN IN THE PRESENCE OF METAL IONS

Potato Dextrose Agar (PDA) media supplement with 50 mM, 5 M of KCl and 5mM, 50 mM of $CaCl_2$ were plated on the petridishes. Mycelia were harvested from actively growing fungal plates and spread on the media. 35 µl of protein with concentration of 100 µg/ml was applied to sterile filter paper discs and placed on the agar surface at four corners of the plate. The plates were incubated at room temperature for 20 hr and the zone of inhibition of hyphae was compared to that of the results obtained in hyphal extension-inhibition assay.

***IN-VIVO* STUDIES**

INFECTION OF PLANTS WITH PHYTOPATHOGENIC FUNGI

The plants were grown in separate plastic bags under environmental conditions. Plant leaves to be tested were slightly wiped with alcohol in order to remove other contaminants present on the surface of leaves in *in-planta* condition. Then the surface of the leaves were scratched using sterile needle. 100 µl of fresh broth culture was applied on the scratched surface. The infection was made to spread for two days. Then the protein solution with concentration 100 µg/ml was applied to the zone of infection. Clearances of the zone of infections were observed every day for a week.

ANTIFUNGAL ACTIVITY OF PROTEIN

After infection and protein application, plant leaves under test, were picked from the plant and slightly washed with 0.5% sodium hypochlorite solution. Then the leaves were finely ground in a pestle and mortar with the drop wise addition of distilled water until homogenate of leaves were obtained. 100 µl of this homogenate was mixed with 2 ml of the sterile broth and then left for one day in order to resume the growth of mycelium. Then sterile broth was taken as blank for taking absorbance at 425 nm. Absorbance is directly proportional to the concentration of viable cell and the difference in the absorbance between test and control is inversely proportional to antifungal activity.

RESULT AND DISCUSSION

ISOLATION OF ANTIFUNGAL PROTEIN

The cell free extract of *Solanum trilobatum* and *Lepidagathis cristata* was collected using ammonium sulphate precipitation, dialysis. The relative recovery of antifungal protein from *Solanum trilobatum* and *Lepidagathis cristata* was found to be 13.7% & 14.43% respectively, though the amount of leaves initially taken for extraction was 200g (Table 1 & 2).

Table-1: Purification of antifungal protein from *Solanum trilobatum*

Purification stage	Volume (ml)	Total protein (mg)	Total antifungal unit ($Ux10^{-3}$)	Specific activity (U/mg)	Unit recovered (%)
Crude extract	100	1150	895	775	100
Ammonium sulphate	80	286	167	580	18.6
Sephadex G-100	30	85	123	1450	13.7

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Table-2: Purification of antifungal protein from *Lepidagathis cristata*

Purification stage	Volume (ml)	Total protein (mg)	Total antifungal unit(Ux10^{-3})	Specific activity (U/mg)	Unit recovered (%)
Crude extract	100	1150	850	740	100
Ammonium sulphate	80	640	180	280	21.18
Sephadex G-100	30	120	123	1003	14.43

Specific activity of both protein were calculated by dividing the total antifungal unit and total mg of protein. Specific activity of anti-fungal protein increases with purification fold.

SEPHADEX G-100 COLUMN CHROMATOGRAPHY

The concentrated proteins (dialyzed) from *Solanum trilobatum* & *Lepidagathis cristata* were loaded on Sephadex G-100 column and eluted with phosphate buffer, since this buffer is used widely to elute protein at maximum level. The antifungal protein fraction was eluted at a flow rate of 60ml/hr.

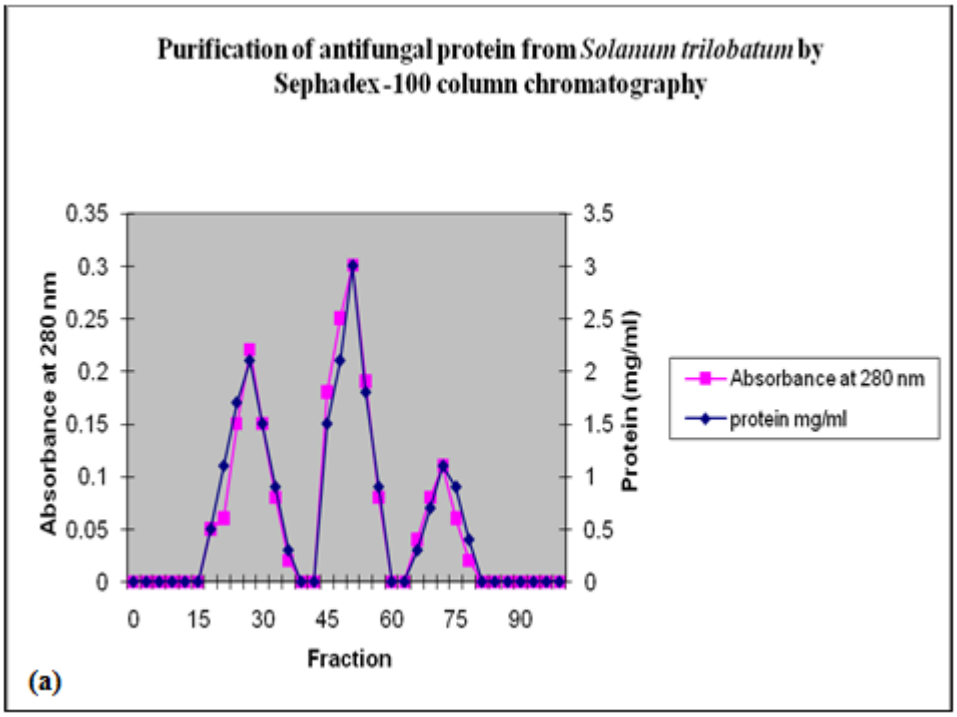

Fig-1 (a): Elution profile of antifungal protein from (a) *Solanum trilobatum*

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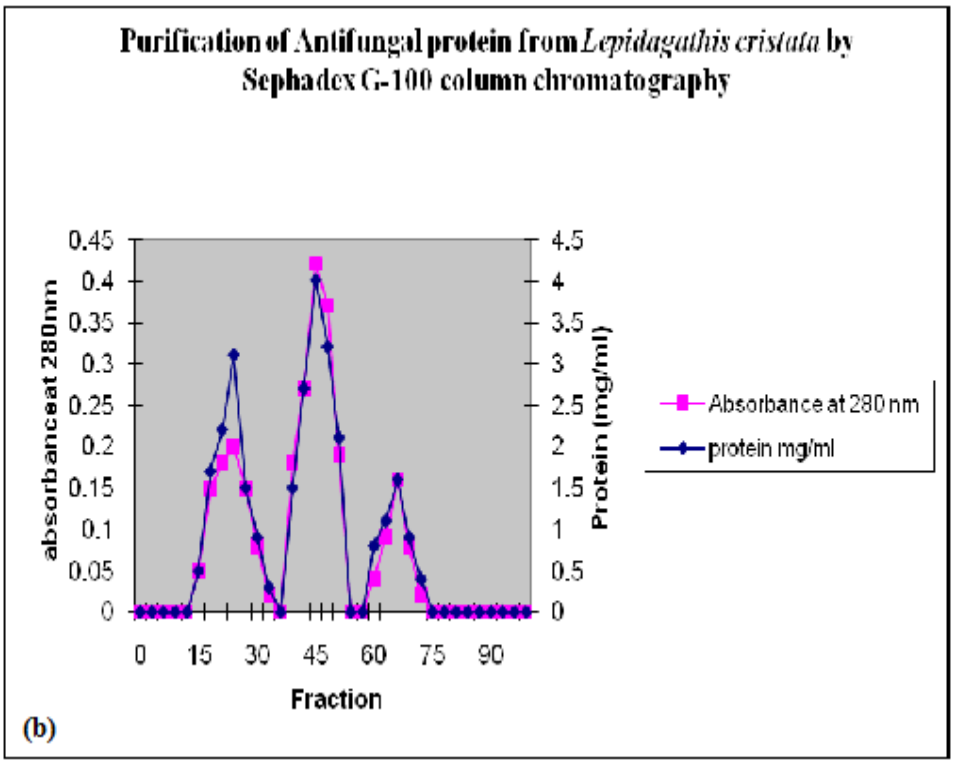

Fig-1 (b): Elution profile of antifungal protein from (b) *Lepidagathis cristata*

Solanum trilobatum

Each 2ml of 100 fractions were collected and protein concentration was analyzed by taking absorbance at 280nm using Bovine serum albumin as standard. Fractions showing similar absorbance were pooled together. From peak-I fractions from 15 to 39, peak-II fractions from 42 to 60 and peak-III fractions from 60 to 80 shows the absorbance of protein. But at peak-II fractions showed higher absorbance and higher protein concentration for *Solanum trilobatum*.

Lepidagathis cristata

Each 2ml of 100 fractions were collected and their protein concentration was analyzed by taking absorbance at 280nm using bovine serum albumin as standard. Elution profile was drawn using absorbance value and protein concentration of each fraction. Fractions showing similar absorbance were pooled together. From peak-I fractions from 14-32, peak-II fractions from 39 to 52 and peak-III fractions from 60 to 75 shows the absorbance of protein. But at peak-II fractions showed higher absorbance and higher protein concentration for *Lepidagathis cristata*.

DETERMINATION OF MOLECULAR MASS BY SDS- PAGE

The purified antifungal proteins (10 µl) were analysed on SDS- PAGE (10%w/v) and stained with silver nitrate. On sodium dodecyl sulfate-poly acryl amide gel electrophoresis the purified antifungal protein showed a single band indicating that it was electrophoretically homogeneous. The molecular mass of the purified antifungal protein *Solanum trilobatum* & *Lepidagathis cristata* was determined as 31 & 30 KDa respectively, by comparing with relative mobility of the molecular mass of protein ladder (Fig-2(a) and 2(b)).

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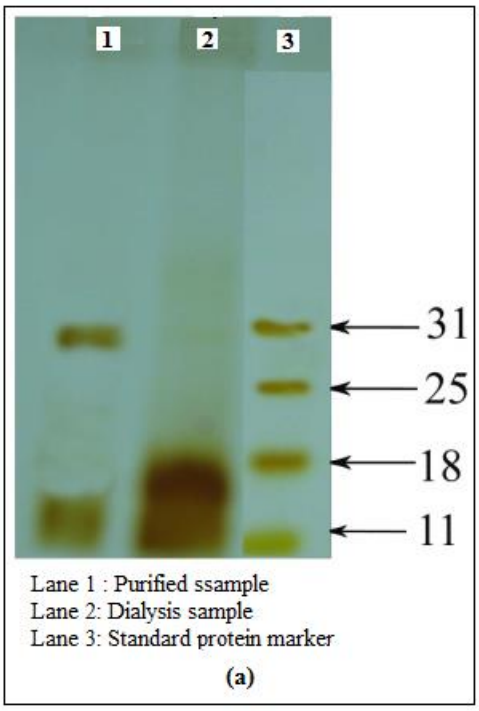

Fig-2 (a): Molecular mass of the purified antifungal protein (a) *Solanum trilobatum*

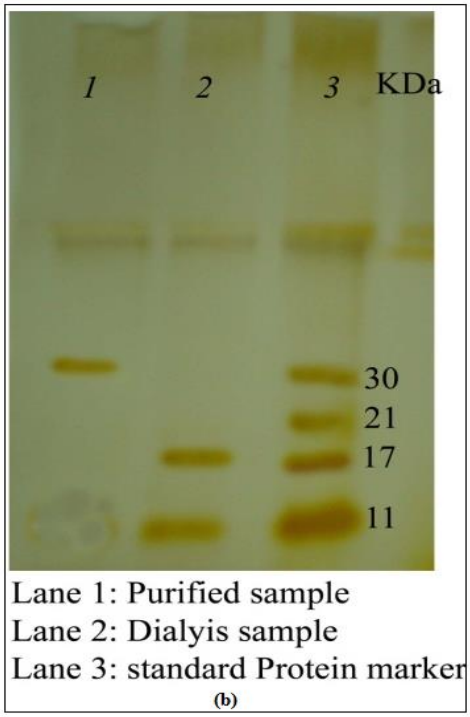

Fig-2 (b): Molecular mass of the purified antifungal protein (b) *Lepidagathis cristata*

PHYSICAL PROPERTIES OF ANTIFNGAL PROTEIN

pH optimization and stability of purified antifungal protein

The optimum pH for stability of antifungal protein from *Solanum trilobatum* & *Lepidagathis cristata* obtained from the absorbance was found to be pH 7 & 8 (Fig 3) respectively.

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The purified antifungal protein of *Solanum trilobatum & Lepidagathis cristata* showed maximum stability at 45 & 40˚C (Fig-4). So they are identified as heat stable protein.

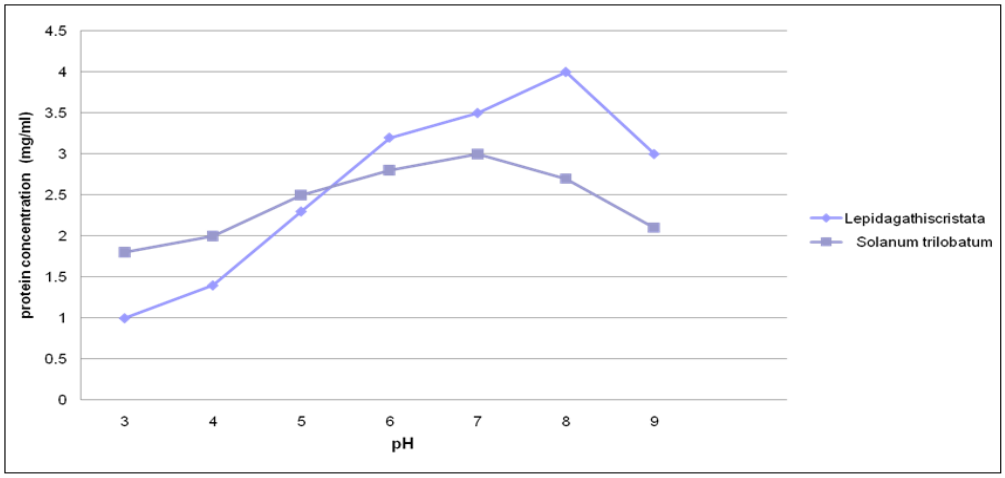

Fig-3: pH optimum and stability of purified antifungal protein Temperature optimization and thermal stability

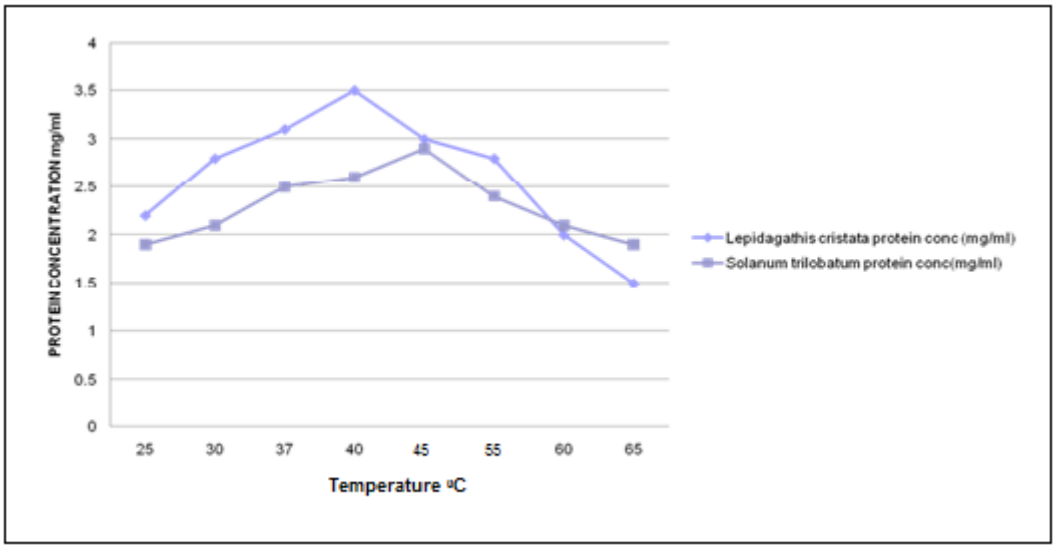

Fig-4: Temperature optimization and stability of antifungal protein

***IN-VITRO* STUDIES**

Antifungal protein on hyphal extension assay

Growth inhibition of *Alternia brassciae & Pyricularia oryzae* was noticed in the presence of *Solanum trilobatum & Lepidagathis cristata* purified protein (Fig-5 & 6). *Solanum trilobatum* purified and anti- fungal protein inhibited 10% growth of *Alternaria brassicae & 24%* growth of *Pyricularia oryzae*. *Lepidagathis cristata* purified anti-fungal protein inhibited 14% growth of *Alternaria brassicae* & 28% growth of *Pyricularia oryzae*, with concentration of purified protein 100 µg/ml. The lesser amount of inhibition was recorded in the concentration of 25, 50 and 75µg/ml respectively.

% zone of inhibition= Inhibition zone / the total plate zone

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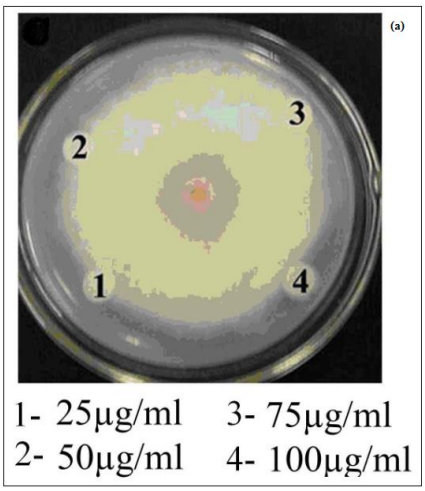

Fig-5 (a): Growth inhibiton of (a) *Alternaria brassicae* in the presence of *Solanum trilobatum* protein

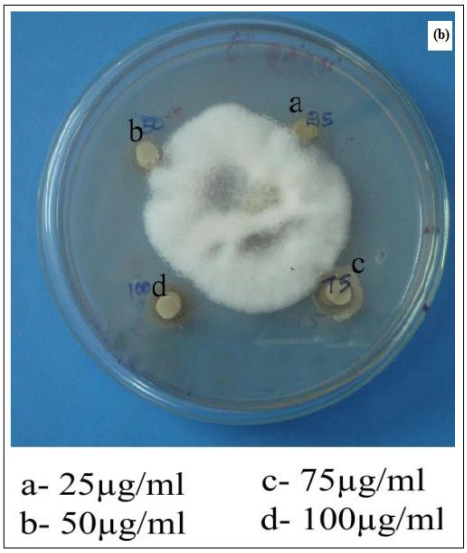

Fig-5(b): Growth inhibiton of (b) *Pyricularia oryzae* in the presence of *Solanum trilobatum* protein

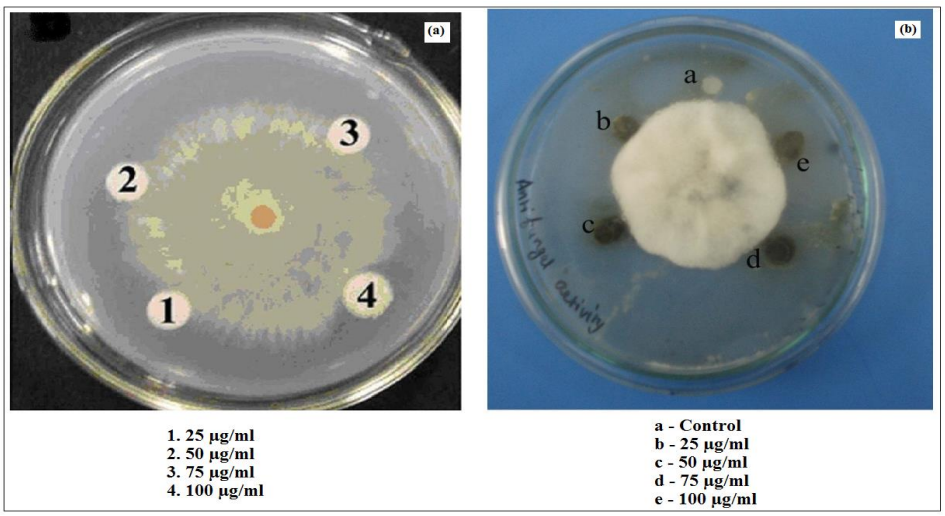

Fig-6: Growth inhibition of (a) *Alternaria brassicae* (b) *Pyricularia oryzae* in the presence of *Lepidagathis cristata protein*

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Morphological difference in inhibited hyphae

The fungal growth inhibition was routinely checked microscopically. A striking difference in the morphology of inhibited hyphae was apparent between fungi, treated with higher to lower concentration of purified anti-fungal protein. The 100 µg/ml purified protein of *Solanum trilobatum* caused severely delayed growth of hyphae of *Alternaria brassicae*, otherwise normal morphology comparatively concentration of purified protein. The 100 µg/ml purified protein of *Lepidagathis cristata* on *Pyricularia oryzae* revealed different type of inhibition occurred. At higher concentrations of anti-fungal protein, no spore formation occurs but occurs at low (Fig-7).

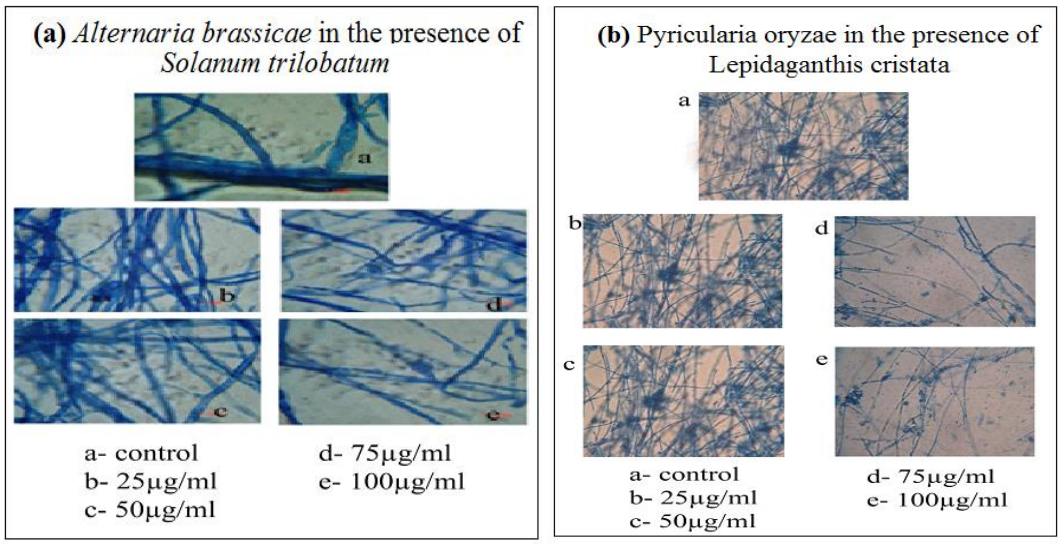
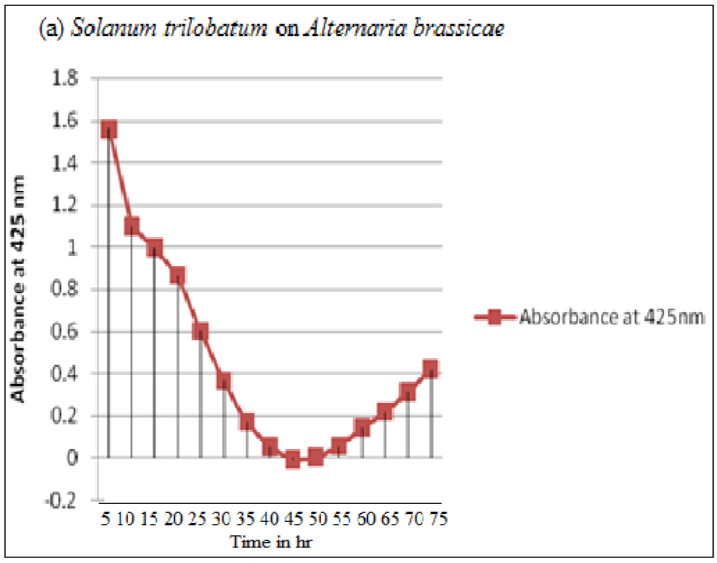


Fig-7: Morphological differences in inhibited hyphae



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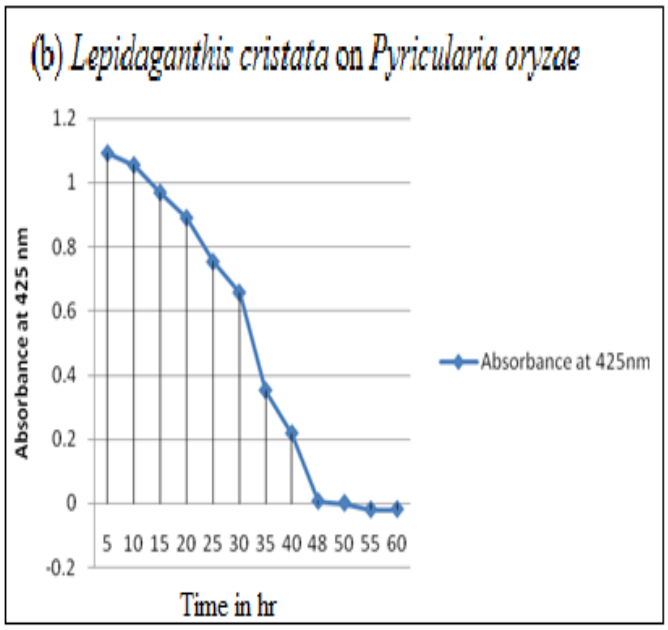

Fig-8: Non-Lethal effect of protein

Lethal effect of protein

The non-lethal effect of protein isolated from *Solanum trilobatum* on *Alternaria brassicae* was confined in this experiment, were the protein added to the fungal culture showed loss of viable cells. After 48hrs, the medium replaced with protein by fresh PD broth, growth of mycelium was resumed, whereas the culture still containing protein remained inhibited.

The protein from *Lepidagathis cristata* on *Pyricularia oryzae* was confirmed by the loss of viable cells in the presence of antifungal protein shows the lethal effect of protein. The replacement of medium does not show any change in the growth of mycelium (Fig-8).

The non-lethal effect of protein from *Solanum trilobatum* on *Alternaria brassicae* was confirmed by the growth of mycelium resumed, after replacement of fresh media. But the protein from *Lepidagathis cristae* on *Pyricularia oryzae* was confirmed for its lethal effect and the replacement of medium didn't resume the growth of mycelium. Hence the antifungal activity of protein from *Solanum trilobatum* must be qualified as fungi static, antifungal protein from *Lepidagathis cristae* and qualified as fungicidal.

Antifungal activity of protein in the presence of metal ions

The % zone of inhibition of hyphae by both proteins on *Alternaria brassica* and *Pyricularia oryzae* are summarized in Table-3. Antifungal activities of protein in the presence of metal ions were compared with protein in hyphal extension inhibition assay to calculate the reduction of activity. The results are summarized in Table-4. From the result addition of 5 Mm $CaCl_2$ to the media containing antifungal protein from *Solanum trilobatum* with 100 µg/ml concentration caused only 3% reduction of antifungal activity against *Alternaria brassicae,* but the same protein on another species *Pyricularia oryzae* caused 6% reduction of protein activity against. It seems likely that antagonistic effect of cations is not the result of a hypothetical conformation change of protein by direct interaction with the cations. Rather an interaction occurs between the fungus and the cations, where by the fungus acquires protection against the action of the protein.

Table-3: Antifungal activity of protein in the presence of metal ions

Cations	Concentration	*Solanum trilobatum*		*Lepidagathis cristata*	
		A.brassicae	*P.oryzae*	*A.brassicae*	*P.oryzae*
KCl	50 mM	10%	23%	14%	28%
KCl	5 M	10%	21%	13%	27%
$CaCl_2$	5 mM	7%	18%	11%	18%
$CaCl_2$	50 mM	5%	13%	8%	10%

Table-4: Reduction of activity in the presence of metal ions

Cations	Concentration	*Solanum trilobatum*		*Lepidagathis cristata*	
		A.brassicae	*P.oryzae*	*A.brassicae*	*P.oryzae*
KCl	50 mM	-	1%	-	-
KCl	5 M	-	3%	1%	1%
$CaCl_2$	5 mM	3%	6%	3%	10%
$CaCl_2$	50 mM	5%	11%	6%	18%

***IN-VIVO* STUDIES**

Clearance of zone of infection

After one week of incubation with antifungal protein, the zone of clearance of infection with *Alternaria brassicae*, for *Solanum trilobatum* protein was applied on the test plant was observed to be very less when compared with that of control plant. But the zone of clearance of infection with *Alternaria brassica,* for *Lepidagathis cristata* was applied on test plant was observed to be less when compared with that of control plant. Anyway in *in-vivo* condition antifungal protein took more than five days to act on phytopathogens. But in case of *in-vitro* conditions it took only 48 hrs to form an inhibition zone.

These results may be due to the concentrations of K^+, the most abundant cellular and apoplastic cation, reach about 100mM in the cytosol and vary from 10-100mM in vacuoles and from 2-100 mM in the apoplast. The most abundant divalent cations in plant tissues are Ca^{2+} and Mg^{2+}. In the cytosol, the free Ca^{2+} concentration is very low, whereas free Mg^{2+} reaches about 1mM. Free Ca^{2+} concentrations in plant vacuoles are about 0.06-1mM, and apoplastic free Ca^{2+} ranges between 0.02 and 1.3 mM. It appears that relatively high ionic strength conditions occur in all cellular compartments (Franky R.G.Terras, 1991).

However, in many of the cases fungal infection leads to the disruption of intact cells and the exposure of cellular contents with the environment (e.g. the external imbibing soil water in the case of seeds). This makes it very difficult to predict the exact ionic conditions under which the antifungal proteins interact with the invading hyphae. Undoubtedly, this condition will influence the activity of cation-sensitive antifungal proteins.

Antifungal activity of protein

Antifungal activity of proteins from *Solanum trilobatum* and *Lepidagathis cristata* in *in-vivo* conditions is summarized in Table-5 in the form of absorbance value.

Table-5: Antifungal activity of protein *in-vivo*

S.NO	ANTIFUNGAL PROTEIN	TEST SAMPLE ABSORBANCE AT 425nm	CONTROL ABSORBANCE AT 425nm
1	*Solanum trilobatum*	0.072	0.091
2	*Lepidagathis cristata*	0.054	0.088

From the above results, we infer that the difference in absorbance for the protein *Lepidagathis cristata* was greater than that for the protein from *Solanum trilobatum*. It showed that the presence of viable

cells present in the *Lepidagathis cristata* treated leaves was found to be less than that of *Solanum trilobatm* treated leaves. Therefore it can be concluded that the antifungal activity of *Lepidagathis cristata* was higher than that of *Solanum trilobatum* in *in-vivo* condition.

CONCLUSION

Thus the antifungal proteins have been studied extensively using the fungal strain of *Alternari brassicae& pyricularia oryzae*. From the present study it was clear that *Solanum trilobatum & Lepidagathis cristata* was suitable and can function as a feasible antifungal agent. The protein extraction was done using the cheapest raw materials and still the plant showed admirable fungal resistance. From the study, it was estimated that the protein has maximum activity at high concentration and has a good activity at a pH range 5 to 8.5 and at a temperature range 45 to 55^{o}C. Morphological analysis showed that the protein from *Lepidagathis cristata* inhibits the spore formation and has fungistatic action. Protein from *Solanum trilobatum* delays the hyphal extension and has the fungicidal action. *In-vivo* analysis showed that the antifungal activity of protein was reduced when compared to *in-vitro* condition, due to the presence of different concentration of cations present in the plant cells. By *in-vitro* and *in-vivo* studies it was concluded that the protein from *Solanum trilobatum* and *Lepidagathis cristata* were effective in inhibiting economically important crops infecting fungi *Alternari brassicae & pyricularia oryzae***.**

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