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Isolation and partial characterization of proteins from *Tinospora cordifolia* against *Pseudomonas aeruginosa PAO1*

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ABSTRACT

Tinospora cordifolia is a deciduous shrub. It is a well recognized for its medicinal properties in Indian Ayurveda system. It is one of the most versatile shrub commonly known as "giloy". The plant gained attention due to its various biological activities such as anti-diabetic, anti-microbial, anti-allergic, anti-oxidant and anti-cancer activities. The plant parts contain various chemical constituents such as alkaloids, steroids, glycosides and polysaccharides. The presence of high levels of polyphenols, mucilage, and other components often leads to protein precipitation hence reduced solubility. Therefore, the present study was undertaken for isolation and purification of the antibacterial protein of T. cordifolia collected from Hamirpur district (Neri), Himachal Pradesh. Analysis was done by methanolic, methanolic, extract of samples taken from Neri village. It was found that methanol act as better solvent for extraction of proteins. Ammonium sulphate precipitation of methanolic crude extract showed that 60-70% precipitation was found best for precipitation of proteins. Further, 60-70% precipitates were subjected for dialysis followed by ion exchange chromatography. The fractions obtained after ion exchange chromatograph were assayed by antibacterial activity against test organism. The active fractions were pooled together and carried out for SDS-PAGE. The total protein content present in methanol in the crude extract was 2.056mg/ml. The ammonium sulphate precipitation showed 10.833±0.928mm zone of inhibition and after purification the antibacterial activity increased to 15±0.289 mm. The partial characterization of proteins revealed that the antibacterial proteins was stable at optimal pH 8, heat stable at 40 °C temperature and stable at low salt concentration. For future aspects, the stem extract and protein can be further exploited for its other properties such as it sequencing.

Keywords: *Tinospora cordifolia, Antibacterial, Ammonium sulphate precipitation, Ion-Exchange Chromatography, SDS-PAGE, Purification, Medicinal plant.*

INTRODUCTION

Herbal medicines have been used for thousands of years in many part of the world. According to the World Health Organization, traditional medicines involving plant extracts or their active ingredients are used by 80% of the world's population. India's mega-biodiversity and knowledge of rich historic traditional medical systems (Ayurveda, Siddha, Unani, Amchi and local health traditions) give a strong foundation for the use of a wide range of plants in general healthcare and alleviation of common diseases [1]

Among the vast library of important medicinal plants *T. cordifolia* Miers of the family Menispermeaceae is immensely valuable in terms of chemical constituents and in pharmacology. This plant is also known as Rasayana in Ayurveda and very well known for building immunity against microorganisms [2]. In India it is known as Tippa-tegga (Telugu), Shindilakodi (Tamil), Amrutha balli (Kannada), Gurcha (Hindi), Garo (Gujrati), and Guluchi (Gujrati) [3]. The plant is quite stiff and can be cultivated in virtually any temperature, though it prefers warm ones. The rainy season (July-August) is the best time to plant [4].

T. cordifolia's roots, stem, and leaves have yielded a variety of components such as steroids, polysaccharides, alkaloids, glycosides and diterpenoid lactones. Tinosporic acid, cordifolisides A to E, syringen, berberine, giloin, tinosporone crude giloininand, gilenin, tinosporide, arabinogalactan polysaccharide, picrotene, gilosterol, tinosporol, tinosporidine, sitosterol, heptacosanol, octacosanol columbin, bergenin, chasmanthin, palmarin, palmatosides C and F, tetrahydropalmaitine, isocolumbin, amritosides are some of the plant compounds that have been identified [5]. *T. cordifolia* methanolic extract effective against microbial infection [6]. *T. cordifolia* extract has been bio-assessed for its antibacterial activity against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Proteus vulgaris,Salmonella typhi, Shigella flexneri, Salmonella paratyphi, Salmonella typhimurium, Pseudomonas aeruginosa* and *Enterobacter aeruginosa* [7]. Keeping in view the wide application of *T. cordifolia*, it has been made to carry out isolation and purification of proteins from *T. cordifolia* against opportunistic *Pseudomonas aeruginosa PAO1*.

Materials and Methods

Materials

Fresh stem of *T. cordifolia* was collected from Neri village of Hamirpur district and dried under shade. The dried stem was grinded by a mechanical grinder and powdered stem of plant was stored at -80 °C for further use.

Crude extract preparation

The powdered sample of *T. cordifolia* was homogenized at room temperature in 10 ml volumes of methanol, stirred at 4 °C overnight, filtered through muslin cloth and centrifuged at

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10000 rpm for 10 minutes. The supernatant was collected and kept for further use at 4 °C.

Ammonium sulphate precipitation

The supernatant was subjected to precipitation using 0-50% and 50-100% saturated ammonium sulphate at 4 °C and the supernatant was discarded, while the precipitate obtained was resuspended in Tris.HCl buffer. The antibacterial activity of each fraction was determined using indicator bacteria *Pseudomonas aeruginosa* and desired fraction was further subjected to precipitation using 50-60%, 60-70%, 70-80%, 80-90% and 90-100%. Again, the antibacterial activity of each was checked and desired fraction was resuspended in Tris.HCl buffer pH (7). The precipitate containing protein fraction was dialyzed extensively in Tris.HCl buffer for 24 hrs to remove ammonium sulphate. The procedure proposed by [8] with slight modifications was followed for this purpose and conditions were standardised. The concentrations of proteins present in precipitated samples was estimated by Lowry assay.

Purification by ion-exchange chromatography

The dialysate was loaded onto DEAE-cellulose anion exchange column pre-equilibrated with distilled water and 1.5 mmol/l Tris.HCl (pH 7). The column was then washed with the same buffer to remove unbound proteins. Adsorbed proteins were desorbed using stepwise elution with increasing Tris.HCl concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mol/l. The flow rate was 0.5 ml/min and the eluate was monitored at 280 nm. Elution fractions were pooled, and antibacterial activity was tested against *P. aeruginosa* [8].

Concentration measurement and homogeneity analysis of purified protein

During the purification process, protein concentrations were measured by Lowry assay. Bovine serum albumin was used as standard. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was used to detect the apparent molecular mass and homogeneity of purified antibacterial proteins. All samples were heated for 5 min in boiling water prior to SDS-PAGE. The protein bands were visualised by staining with R-250 (Lammeli, 1970) with slight modifications.

Antibacterial activity determination

Agar well method was utilized to detect the antibacterial activity of crude extract, protein fractions of ammonium sulphate precipitation, dialyzed fraction and ion-exchange chromatography fractions. A suspension of the indicator strain was spread on LB agar plates. Wells were created by using microtips, and 50 µl protein fractions were filled into wells and methanol used as negative control. The incubation was done at 37°C for 24 h in incubator.

Effect of pH

Stability of antibacterial proteins was checked at different pH 4, 6, 8 and 10. Antibacterial activity was assayed as described in earlier section 3.1.8. 50 µl of purified proteins was treated with 10 µl of Tris-HCl buffer consist of pH 4, 6, 8 and 10 [8].

Thermostability

The thermostability of antibacterial proteins was observed at different temperatures 30°C, 40 °C and 50 °C, 55 °C, for a period of 3 hrs. The purified proteins were incubated at 30 °C, 40 °C, 50 °C and 55 °C for 3 hrs [8].

Effect of salt concentration

Stability of antibacterial proteins was checked at different salt concentrations 0.1mM, 0.2 mM, 0.4 mM and 0.5 mM. Antibacterial activity was assayed as described in earlier section 3.1.8. 50 µl of purified protein was treated with 10 µl NaCl salt consist of 200 mM, 300 mM, 400 mM and 500 mM [8].

Results and Discussion

Purification of antibacterial protein

The protein was purified from *Tinospora cordifolia* using ammonium sulphate precipitation, ion-exchange chromatography using DEAE-Cellulose 52 (Table 1). The protein in the crude extract was 205.6mg in 100 ml. The crude extract was precipitated with ammonium sulphate (50-60%, 60-70%, 70-80%, 80-90% and 90-100% saturation). The results indicated that the 60-70% achieved highest yield of crude extract i.e. 81.3%. The 60-70% ammonium sulphate fraction was separated using DEAE-cellulose 52 into 50 fractions and fractions 33, 34, 35, 36, 37 and 38 exhibited antibacterial activity against *P. aeruginosa*. These fractions were further pooled together for SDS-PAGE analysis. Correspondingly, Chandrashekar *et al* 2009 also reported that 90% ammonium sulphate saturation of aged garlic was found best for precipitation of proteins. It was found that 80% ammonium sulphate saturation of *T. cordifolia* stem extract was observed best for precipitation of proteins [9].

Antibacterial activity

The antibacterial activity of the protein extracted from *T. cordifolia* was examined by agar well diffusion method against the indicator strain shown in (Table 2). The crude extract, dialysed fraction and ion-exchange fractions displayed clear antibacterial activity.

Table 1: Purification profile of antibacterial protein from T. cordifolia

Purification step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Yield (%)	Purification fold
Methanolic extract	100	2.056	205.6	100	1
Ammonium sulphate precipitation	50	0.335	1.673	81.3	6.47
Dialysis	10	0.1126	1.126	54.7	11.4
DEAE Cellulose	10	0.054	0.54	26.2	27.7

Table 2: Diameter zone of inhibition of protein isolated from T.cordifolia against P.aeruginosa

S.No	Test material	Zone of inhibition(mm)
1	Crude Methanolic Extract	7.333±0.167
2	Ammonium sulphate precipitation (50-100%)	7.5±0.236
3	Ammonium sulphate precipitation (60-70%)	10.833±0.928
4	Dialysis	12.833±1.093
5	DEAEcellulose-52	15±0.289
	CD0.05	0.702

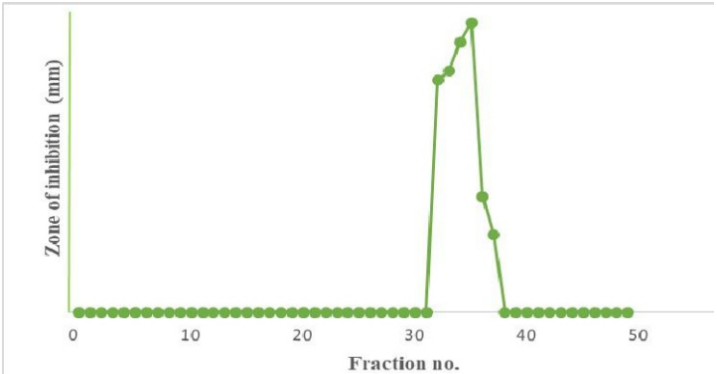

Fig. 1 Activity profile of fractions 33, 34, 35, 36, 37 and 38 obtained after ion exchange chromatography

Molecular mass determination of protein through SDS-PAGE analysis

SDS-PAGE method was used for the determination of molecular weight of protein. Prestained molecular weight marker (Hi-Media) of 6.5-115kDa was used for the molecular weight determination. The molecular weight of protein was found to be approximately 30 kDa (Fig 2).

Similarly, it was reported that molecular weight of purified novel protein from *Aloe vera* leaf gel was 14 kDa [10]. It was observed that the molecular weight of trypsin inhibitor isolated from *Albizia lebbeck* seeds was 12 kDa [11].

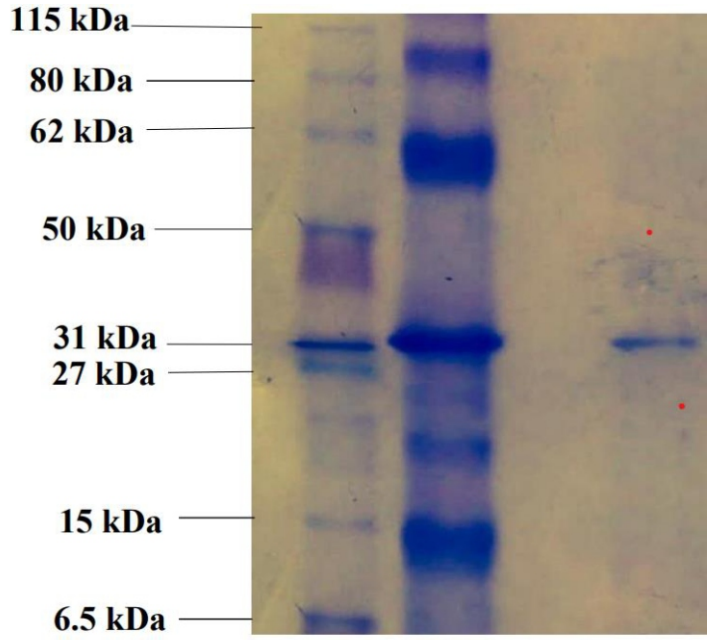

Fig.2 Gel plate showing single band of isolated purified antibacterial protein

Lane 1: Low weight molecular marker (6.5- 115kDa)
Lane 2: Crude methanolic extract
Lane 3: Purified protein

Partial characterization of purified protein

Effect of pH

To investigate the optimal pH range for the antimicrobial protein of interest, the protein was exposed to different range of pH values. Different pH conditions had a significant effect on the antibacterial activity of protein of interest. The protein was highly stable at alkaline conditions at pH 8(11.50±0.177mm) zone of inhibition rather than in acidic conditions. Chen *et al.,* 2018 observed that *Gastrodia elata* protein displayed higher antimicrobial activity in pH 6. Similarly, Chandran *et al.,* 2018 gave their findings that *Ferula asaefoetida* root exudate protein was stable at pH 8.

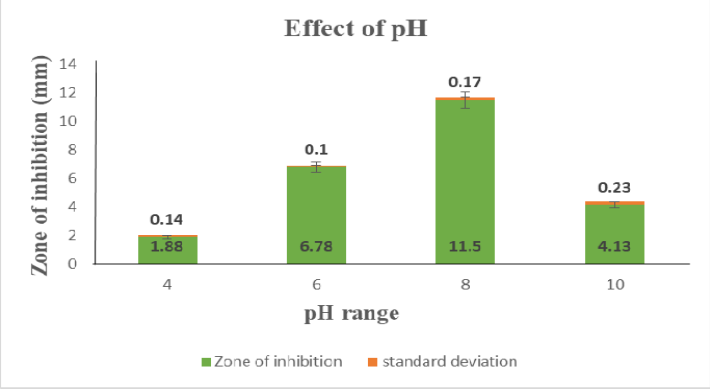

Fig.3 Effect of pH on purified antibacterial protein from T. cordifolia

Thermo stability

To investigate the thermal stability of protein of interest, the antibacterial protein was exposed to different temperature for 3 hrs. The temperature ranges from 30°C to 55°C. The antibacterial protein was found to be thermally stable at 40°C signifying (13.313±0.188mm) zone of inhibition after 3 hrs and minimum activity of (2±0.204mm) zone of inhibition observed after 3 hrs. Correspondingly, Sharma *et al* 2012 reported that protease inhibitor isolated from *Albizia lebbeck* seeds was heat stable at 60°C. Similarly, Chen *et al.,* 2018 observed that GEP was heat stable below 70°C for 30 minutes.

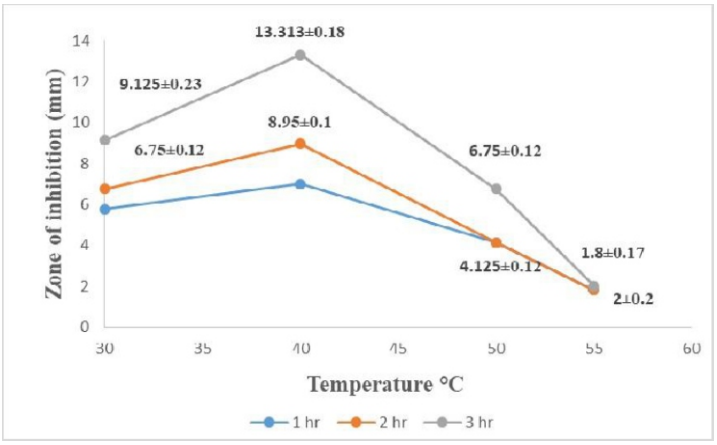

Fig.4 Effect of temperature on purified antibacterial protein from T. cordifolia

Effect of salt concentration

Todeterminethestabilityofantibacterialproteinatdifferentsaltconcentrationprotein was treated with different concentration of salt (NaCl) ranging from 200 mM to 500mM. The protein was found to be stable at low salt concentration and possessed 12.01±0.128mm) zone of inhibition. The activity of protein at high salt concentration was minimum i.e. (1.988±0.194mm) zone of inhibition. Chen *et al.,* 2018 observed that *Gastrodia elata* protein was stable at low salt concentration.

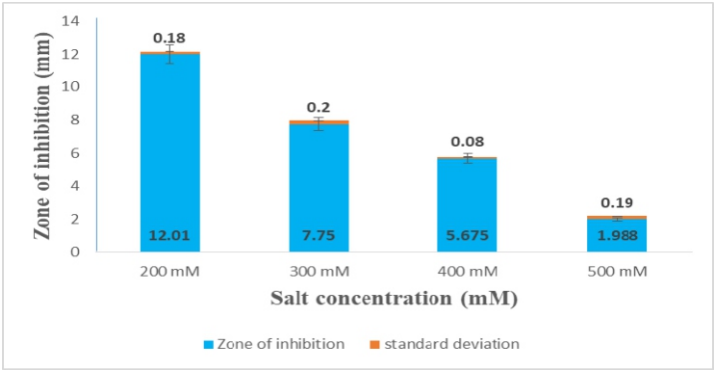

Fig.5 Effect of salt on stability of purified antibacterial protein from T. cordifolia

Conclusion

In the present study protocol for isolation, purification and partial characterization of protein from stem of *T.cordifolia* having antibacterial activity against *Pseudomonas aeruginosa,* was standardized. Various ammonium sulphate precipitation, dialysis and ion exchange chromatography were performed for purification of protein from *T.cordifolia*. The antibacterial activity was mainly shown by 60%-70%ammonium sulphate precipitation fractions. The purification and characterization of antibacterial protein was preceded with thisfraction inwhich the presence of antibacterialprotein was identified. It was previously reported that the plant extract possess antimicrobial activity against pathogens.

This work is of immense importance as the antibacterial activity was analyzed against clinical pathogen *P.aeruginosa*. SDS PAGE analysis has revealed that the protein had a molecular weight of approximately 30kDa.The present investigations exposed that protein isolated from *Tinospora cordifolia* is suitable for pharmacological expenses. Furthermore, It could be further explored for its other properties such as sequencing.

Conflict of interest: Authors declare that there is no conflict of interest.

Ethical Approval: Not applicable

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Availability of data and materials: Not applicable.

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