

Original Research Article

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Purification and Characterizations of Anti-Microbial Protein from *Calotropis procera*

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ABSTRACT

Calotropis procera is strongly recommended in leprosy, hepatic and splenic enlargement, dropsy and worms. The latex is applied to painful joints and swelling fresh leaves are also used for same purpose. Oil which the leaves have been boiled is applied to paralyzed part. The milky juice is used in India as purgative, while flowers are considered as digestive, stomachic, tonic and useful in cough, asthma, catarrh, loss of appetite and hepatitis. The root bark is used to promote secretion and to be useful in treating skin disease, enlargement of abdominal viscera, intestinal worms, ascites and anasarca. The leaves of Aak are warmed and tied around any body organ in pain. It is practically useful in backache and I joint pains. *Calotropis procera* latex contain cardioids such as calotropin, calotoxin, uscherin, uschardin, glycoside calotropagin, choline, o-pyrocatechuric acid, benzoyllineolone, benzoylisoloneolane, uzariganin and syriogenis proteins such as osmotin, calotropin etc. Osmotropin and calotropin shows the antimicrobial activity against microbe. Here we were isolated a novel protein from latex using ionexchange and gel filter Traction chromatography that shows the antimicrobial activity. Antimicrobial activity assay done against bacterial and fungi. The bacterial and fungi strains are collected from Gautam Buddha University Greater Noida (U.P). *E. coli*, *S. enterica*, *Vibrio sp.*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Fusarium sp.* and *saccharomyces sp.* Microbe were used for antimicrobial assay at different pH, temp, & concentration. Protein are being crystalized for structural and functional application.

Keywords

Calotropis procera,
Soft-wooded,
Evergreen

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Introduction

Calotropis procera plant is a soft-wooded evergreen, perennial shrub. Common names of *Calotropis procera* is Akund, apple of sadow, auricular tree, gaint milkweed, mudra, rooster tree, rubber bush, small crown flower, sadow milkweed, swallow wort [English] Aak, akada [Hindi].

The medicinal value of *Calotropis* has been described in older pharmacopeias, it strongly

recommended in leprosy, hepatic and splenic enlargement, dropsy and worms. The latex is applied to painful joints and swelling fresh leaves are also used for same purpose. The milky juice is also used in India as purgative.

Chemical screening of latex of *Calotropis procera* the latex revealed that the plant contain cardioids such as calotropin, calotoxin, uscherin, uschardin, glycoside calotropagin, choline, o-pyrocatechuric acid, benzoyllineolone, benzoylisoloneolane,

uzariganin and syriogenis etc. The latex in *Calotropis procera* show the antidysenteric, antirheumatic, antinociceptive and anti-inflammatory activity. The leaves of *calotropis* shows antioxidant and anti-microbial activity. The flowers show antisialogous activity. Basic objective of this study was Extraction, Purification, and functional characterization of antimicrobial proteins from *Calotropis procera* latex.

Materials and Methods

Sample collection

Calotropis procera latex was obtained from the industrial area of Kasna Greater Noida (U.P). The latex contains protein and many other proteases and pigments. Latex is also rich compound such as salt, minerals, lipids, carbohydrates, and in particular complex membrane systems, which can be co-extracted with proteins and interfere with protein purification. So removal of these compounds was necessary for this purpose we made the modified buffer that also provide protein stability.

The modified buffer composition was 2mM PMSF (phenylmethanesulfonyl fluoride), 100mM EDTA, 100mM Tris base (pH-8.0), 50mM Vitamin C & 30% sucrose. The every component of modified buffer have specific roles PMSF is a serine protease inhibitor that inhibit the chymotrypsin, trypsin, thrombin and cysteine protease papain. PMSF does not inhibits metallo-protease such as most cysteine protease or aspartic protease these proteases inhibited by the metallo-protease inhibitor such as EDTA. Tris provide the stability of the target protein with respect to pH and the buffering compound. Vitamin C is an antioxidant. The roles of sucrose reduce the "Nonspecific interaction" between proteins and stabilize the protein and help in prevent protein aggregation.

Fresh latex was collected from healthy plants by small incisions near the youngest leaves and left to flow off into modified buffer in order to obtain a mixture 1:1 (v/v). The mixture was gently handled to maintain homogeneity during transport to the laboratory.

Protein extraction

The mixture of latex and buffer vigorously vortexes for 30 min at room temperature. Then sample was sonicated for 5 min at temperature 37, amplitude 40%, pulse 2sec on and 2sec off on ice. Then sample was centrifuged for 1 hour at 14500 rpm and 4⁰ c.

The middle phase collected as total purified latex. The rubber particle and pallet centrifuged again. The upper phase transfer into new centrifuged tube and pallet and rubber particle was discarded. Sodium Azide was added as antifungal agent. The extract was left for 2 hours at 4 C.

The protein from the crude extract was precipitated by a gradient of saturated ammonium sulphate solution. The solution was centrifuged at 12000 rpm for 20 minutes to get the protein pellet. This pellet was re-suspended in minimum volume of buffer and was set on dialysis in a 3kDa membrane for 24 hours against buffer. The dialyzed protein was stored at 4°C for further purification.

Protein was purified with the help ion exchange and gel filtration chromatography. CMC 50 (GE healthcare) was used as an ion exchanger matrix for protein purification.

Protein quantification and estimation

The purified protein sample was quantified using Lowry method for protein quantification (Lowry, 1951) and the samples of the fraction were run on the SDS PAGE.

Antimicrobial activity

Various microorganisms were used for the investigation which included bacterial and fungal strains. All the strains were obtained from Gautam Buddha University, Greater Noida, and Uttar Pradesh, India. Lurea Broth and YPD were used for Prokaryotic and Eukaryotic systems respectively.

Sterile Filter paper (What man No. 1) discs with 4mm diameter were impregnated with different concentrations of the purified protein (10ul) and were incubated for 24 hours in case of bacterium and 72 hours in case of fungal strains. Amp and autoclaved distil water were used as positive and negative control during the investigation, respectively. Only few microorganisms showed activity against the protein and they were further carried for further characterization of the various property of the purified protein on various parameters as temperature and pH. Activity was determined by radial diffusion assay again and zone of inhibition was measured by HI media Zonal scale.

Results and Discussion

Quantification of purified protein

Purified protein was quantified by using Lowry method. The absorbance measured at 650 nm determined for BSA protein concentrations ranging from 0.0 to 05 mg/ml. A polynomial non linear equation describing the standard curve generated. Protein was tested against standard protein curve that shows the 1.6-2.0mg/ml (Fig. 4).

Antimicrobial activity of the purified protein

Antimicrobial activity of the purified protein performed against many bacteria shows response to the purified protein. Some bacteria were sensitive to purified protein, some

bacteria not sensitive to purified protein some bacteria killed by the proteins. For the stability of protein many variations was applied such as pH, temperature and concentration of the proteins. As the antimicrobial activity of the protein was judged against various microorganisms, but only bacterial strains responded to the various concentrations of the protein. Fungal strain did not respond to the protein. The inhibitory activity was measured as zone of inhibition on the bacteria showed a zone of inhibition while fungal pathogens were not affected by the protein (Fig. 5).

Antimicrobial activity of protein was tested against some common microorganisms and further characterization of protein was done at different temperature and pH. Antimicrobial protein showed maximum activity at pH7 and minimum at pH9and moderate at pH4.

It means that the protein is most active at pH7. The effectiveness of the protein was also studied at different temperatures and minimum activity was found at 45⁰C and maximum at 30⁰Cand moderate at 37⁰ c. It means that protein is most active at 30⁰ C.

While conclusion and future aspects of this study includes, more than 12,000 plant species contain latex, a milky fluid in which occur a wide range of proteins. As the latex is abundant in the green parts of the plant, it is thought to be produced and accumulated as a defence strategy against viruses, fungi and insects. Indeed, besides proteins involved in rubber biosynthesis, latex fluids have been shown to contain proteins implicated in plant defence and oxidative metabolism. Today, latex is considered as a promising source of pharmacologically active molecules that might be chemically modified to improve their effectiveness With regards to *C. procera*, although the pharmacological potential of its latex has well been proven, only few active latex molecules have been identified until today (Fig. 1–5 and Table 1).

Fig.1 Chromatogram showing various fractions after IEC

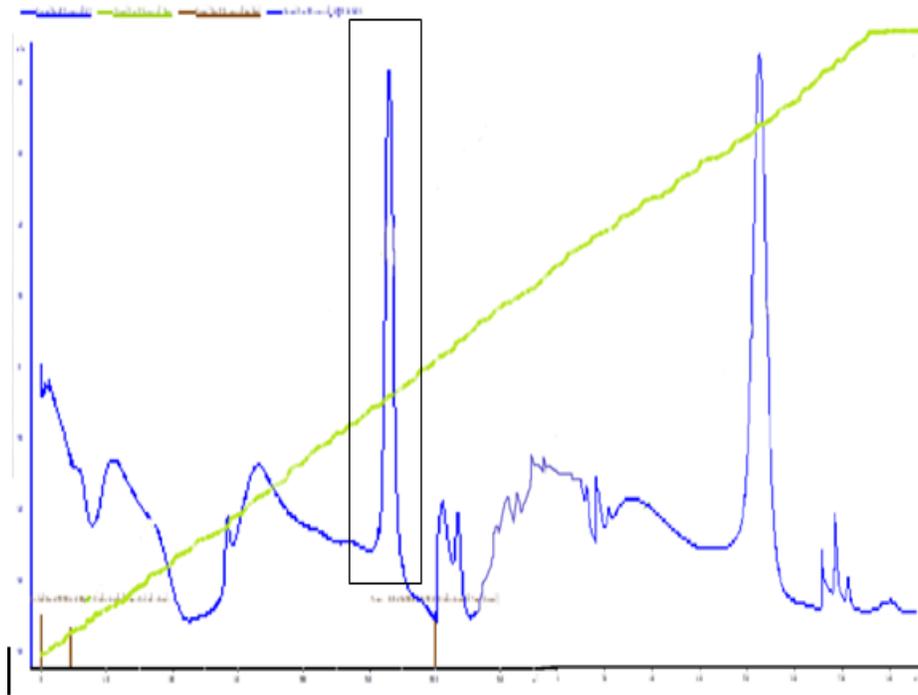


Fig.2 Chromatogram showing various O.D various fractions after IEC at $\lambda 280$ (series1) & $\lambda 215$ (series2) on spectrophotometer

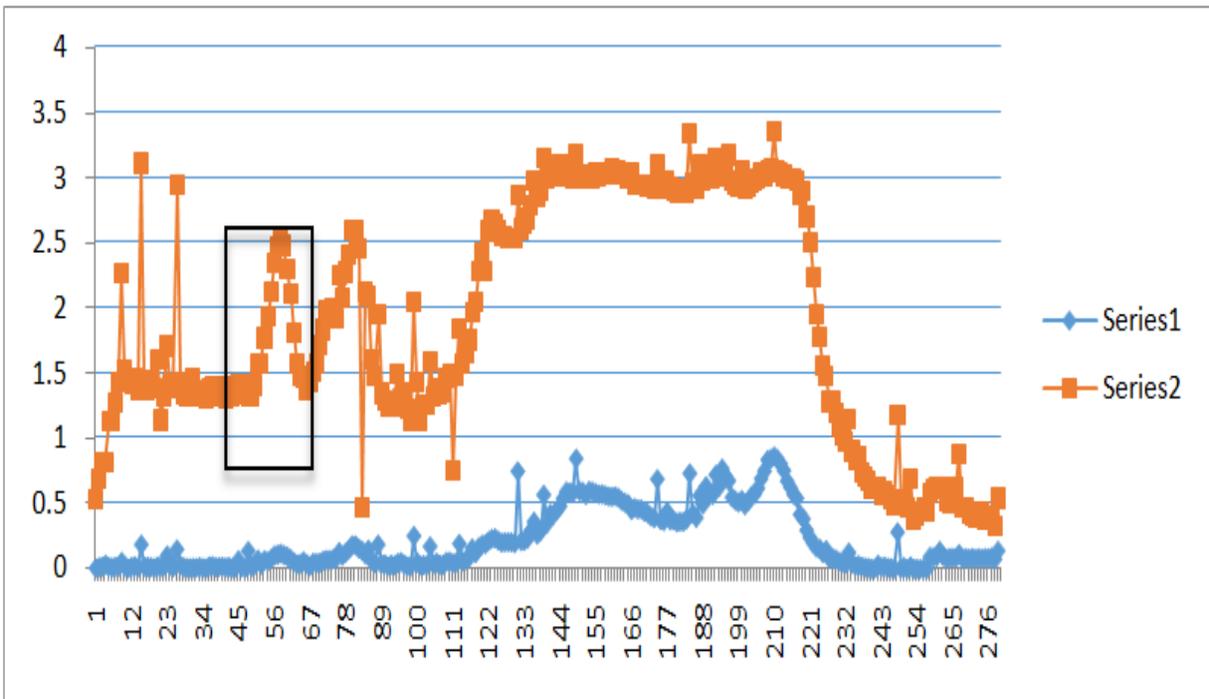


Fig.3 SDS PAGE of selected peaks of ion exchange chromatography

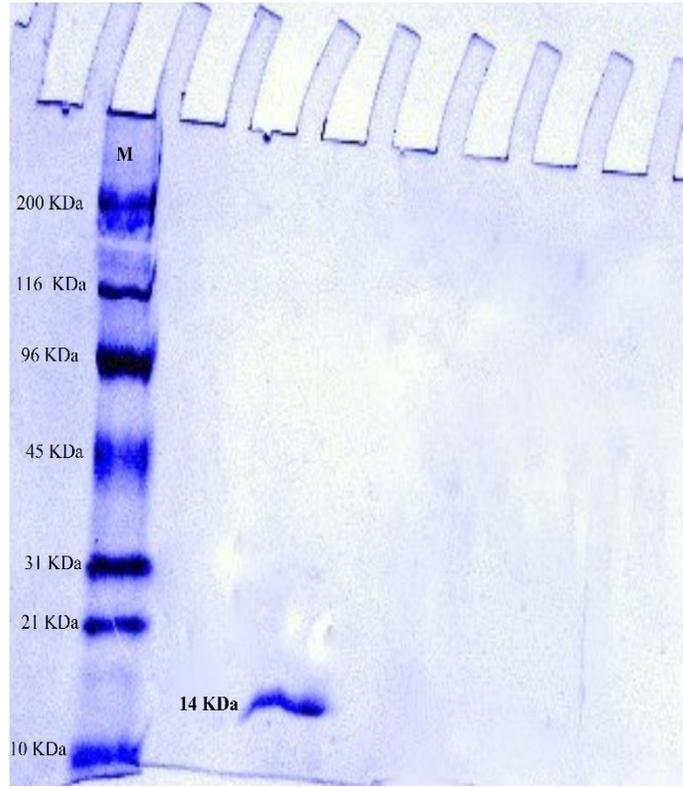
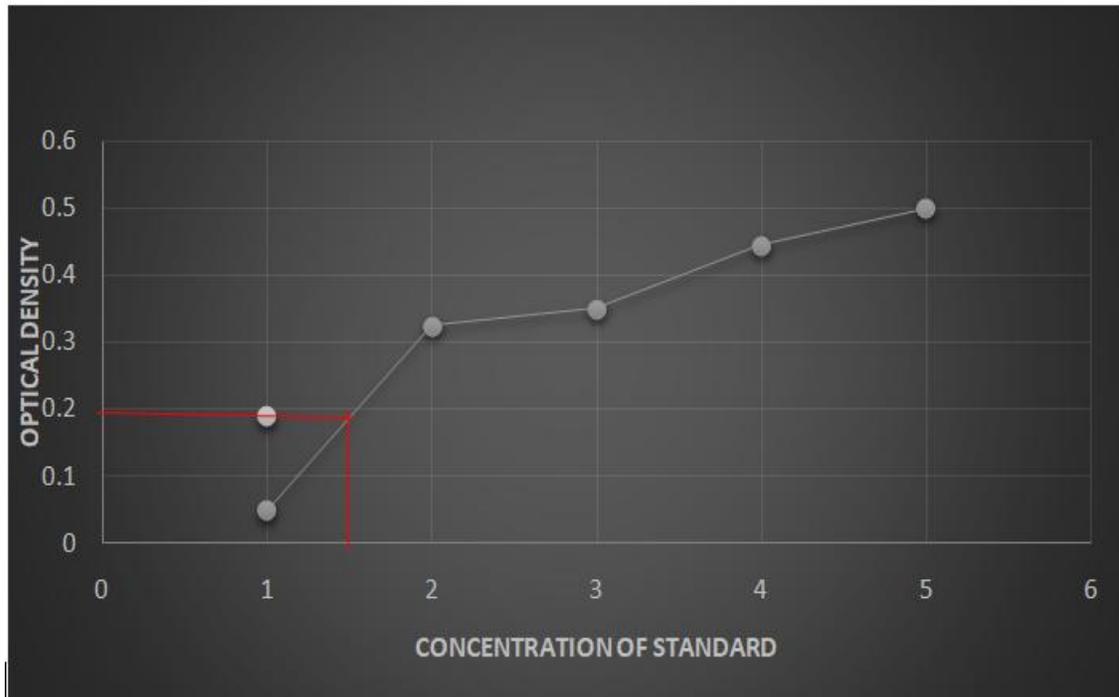


Fig.4 Standard curve for quantification of purified protein by lowry method



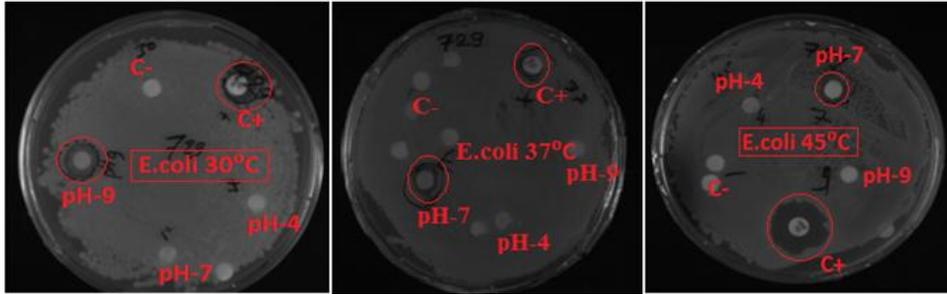


Figure - 5A. Antimicrobial activity of protein against *E.coli* at different pH (4, 7&9) and (A)30°C (B)37°C and (C) 45°C temperature.

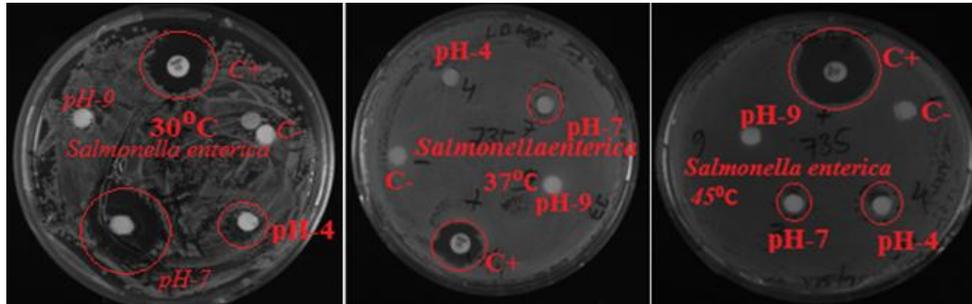


Figure - 5B. Antimicrobial activity of protein against *Salmonella enterica* at different pH(4,7&9) and (A) 30°C,(B) 37°Cand (C)45°C temperature.

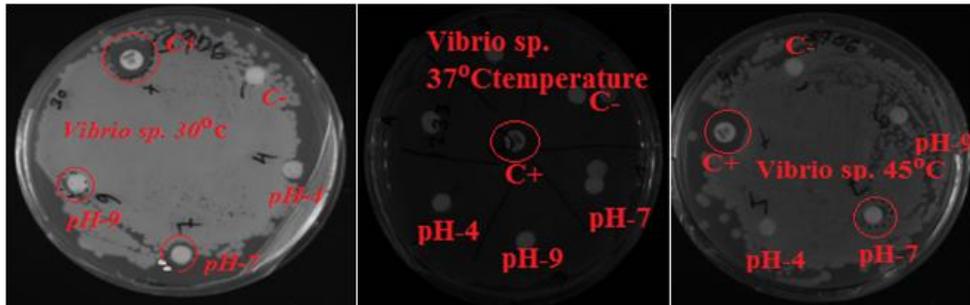


Figure - 5C. Antimicrobial activity of protein against *Vibrio sp.* at different pH (4, 7& 9) and (A) 30°C and (B) 45°Ctemperature.

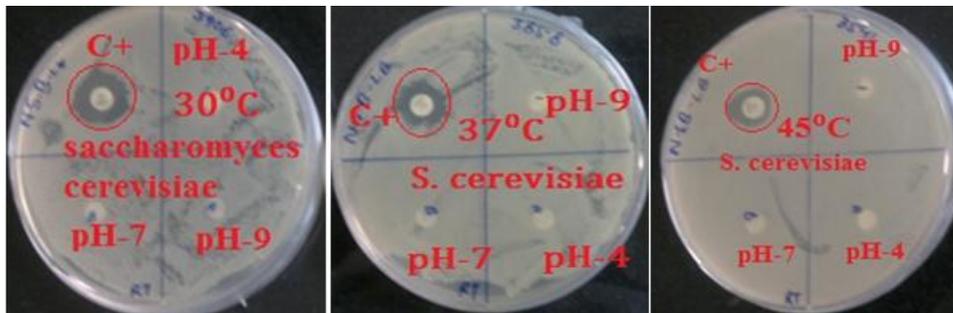


Figure - 5D. Antimicrobial activity of protein against *saccharomyces cerevisiae* at different pH (4, 7& 9) and (A) 30°C and (B) 45°Ctemperature.

Table.1 Antimicrobial activity of protein against different microbes at different pH and temperature in terms of zone of inhibition (in mm)

Microrganisms	Condition→ Temperature ↓	POSITIVE CONTROL (mm)	NEGATIVE CONTROL (mm)	pH 4 (mm)	pH 7 (mm)	pH 9 (mm)
<i>E.coli</i> (MTCC No.729)	30 ^o C	10	0	0	0	11
	37 ^o C	10	0	0	12	0
	45 ^o C	17	0	0	10	0
<i>Salmonella</i> <i>enterica</i> (MTCC No.735)	30 ^o C	18	0	12	23	0
	37 ^o C	14	0	0	10	0
	45 ^o C	12	0	12	10	0
<i>Vibrio sp.</i> (MTCC No.3906)	30 ^o C	14	0	0	9	8
	37 ^o C	12	0	0	0	0
	45 ^o C	15	0	8	0	0
<i>Saccharomyces</i> <i>species</i>	30 ^o C	0	0	0	0	0
	37 ^o C	0	0	0	0	0
	45 ^o C	0	0	0	0	0

The antimicrobial protein has been purified to homogeneity using chromatographic methods. The protein has been found inhibiting some bacterial species in vitro. The protein will be identified using sequencing approach and the will be expressed for further characterization and production in larger amount. This protein can be used as antibacterial agents as whole. The structural characterization of this protein will provide details about it antimicrobial function, which can be used for designing new antimicrobial agents to combat microbial infections.

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