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Characterization of Trypsin-Like Serine Protease from *Lethocerus indicus* Salivary Venom and its Cytotoxic Effect against Human Epidermoid Carcinoma Cell, A431

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Authors' contributions

This work was carried out in collaboration between all authors. Author HD performed practical work, wrote the first draft of the manuscript. Author TS designed the study, performed the statistical analysis, wrote the protocol. Author RKL managed the analyses of the study. Author SG critically analyzed the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Objective: *Lethocerus indicus* salivary venom characterization and evaluation of extracellular degradation activity and cytotoxic effect against native human collagen type 1 and epidermoid carcinoma cell, A431.

Method: Salivary venom extract was collected from adult insects by injecting 2% pilocarpine of 50 µml. Enzyme presence was detected by the apiZYM assay. The proteolytic activity was tested by the photometric and zymogram methods using specific fluorescent substrates and inhibitors. The cytotoxic activity was determined by the MTT assay and Trypan blue exclusion method. Apoptosis induction was observed using AO/EB staining solution. Digestion of extracellular matrix protein was detected against native human type I collagen.

Result: *L. indicus* salivary venom presents amylases, proteases, carbohydrases, phosphatases and lipases. Among them, protease enzyme showed highest composition. The highest rate of proteolytic activity observed at pH 8 in 35ºC (100 %). Serine proteases present predominantly in salivary venom. Cysteine and metalloproteases are also detected. The activation energy of salivary venom is 49.86 kJ. Use of serine inhibitor, PMSF inhibited 92.77% which indicated that the maximum activity was due to serine protease. Detection of trypsin-like protease was confirmed by using PMSF and TLCK with specific substrate, BApNA. It shows significant inhibitions, 82% and 78% respectively suggesting maximum influence in salivary venom. Degradation of the fibrillar native state collagen Type I into 8 smaller peptide bands showed it importance in medical application. IC50 concentration of venom that induces cytotoxicity in epidermoid carcinoma cells, A431was 2.3 µg/ml only. It gives prominent apoptotic features such as cytoplasmic membrane blebbing, nuclear contraction, nuclear fragmentation and contact inhibition.

Conclusion: We suggest that further investigation of the venom will lead to identification of active compound in *L. indicus* salivary venom for its potential use in therapeutic application.

Keywords: Human epidermoid carcinoma cell; Lethocerus indicus; proteolytic activity; salivary venom.

1. INTRODUCTION

Hemipteran insect, *Lethocerus indicus* of the family, Belostomatidae produces toxic saliva capable of provoking intense pain and paralysis in vertebrates including human [1-2] The venom is a secretion produced in a specialized gland in one animal and delivered to a target animal through inflicting a wound that causes disruption in normal physiological processes [3]. The salivary venom of *L. indicus* is used as an anti tumour medicine by the traditional healers since long back [4]. The salivary venom of the poisonous predators is interested as a potential source of bioactive substances. Insect venom is a complex mixture of certain proteins, enzymes, small peptides, inorganic elements and acids. These venom components are responsible for multiple pharmacological effects in different organisms [5]. The complex mixture of toxic saliva of the predatory bugs are used for diverse purposes such as immobilizing and pre-digesting their prey and defence against competitors and predators [6]. The venoms isolated from various poisonous predators such as snakes, scorpions, marine cone snails, spider, other animals and predatory insects have novel peptides of medicinal importance [7]. For instance, bee venom, which is well known as a traditional medicine, exhibited anti-arthritic and anti-carcinogenic effects [8]. Insect toxins are reported to elevate

the level of blood sugar, lactate, glucagon and cortisol. It also causes massive destruction of erythrocytes and nerve cells [9]. Among the predatory hemipterans, venoms of reduviid predators are known to have long-term, non-lethal paralytic effects on their prey. The immobilized or partially digested prey is used as food by the reduviid predator [10-11]. Paralytic activity in the prey was due to novel neurotoxic compounds present in the venom of predators [6]. The giant water bug, *L. indicus* induces a paralytic effect on its prey by inflecting the salivary venom and suck out the partially digested liquefied tissue as food. The majority of predaceous arthropods performed extra-oral digestion. These predators obtain prey extraction and nutrient concentration by refluxing or non refluxing application during injection of hydrolytic enzymes [12]. This phenomenon of *L. indicus* was used by traditional healers in the treatment of outgrowth tumour of skin by inducing salivary venom. In order to understand the nature of the salivary venom, characterization of the salivary venom and in-vitro testing were conducted. Salivary enzymes of the true bug are reported to have predominantly composed of groups of protease enzymes [13]. Proteases have long been associated with cancer progression because of their ability to degrade extracellular matrices. Intracellular and extracellular proteases can function as signalling molecules in various cellular processes that are essential for cancer biology. Extracellular proteases of all major catalytic classes might act to suppress tumour progression. All classes of the proteolytic enzymes known from vertebrates are also present in insects [14]. Hence, the salivary venom of the giant water bug was studied to determine its basic properties and pharmaceutical nature in two different aspects:

1) Characterization of the enzymatic nature of the salivary venom and its protease enzymes
2) Extracellular degradation activity and cytotoxic effect of salivary venom against native human collagen type 1 and epidermoid carcinoma cell, A431.

2. MATERIALS AND METHODS

2.1 Collection of Salivary Venom Extract

Salivary venom extract was collected from adult insects in two ways, by giving electrical shock (12 V) at coxa of the forelegs and by injecting 2% pilocarpine of 50µl with 300mOsm NaCl [15]. After 1-3 minutes, the bugs started releasing salivary venom through their proboscis. The secretion was collected in capillary tubes and transferred to micro centrifuge tubes containing 150mMTris–HCl (pH 7.5) and kept at -80ºC for further analysis. The protein content of the enzyme was determined using the Bradford assay. Bovine serum albumin was used as standard [16].

2.2 Qualitative Analysis of Enzymes

Salivary venom extract from *L. indicus* was screened for a variety of enzymes using a qualitative and colorimetric enzyme assay system (apiZY MBio Mérieux, Lyon, France). The apiZYM assay has a test strip consisting of 20 cupules. Each cupule contains a synthetic substrate specific for a particular enzyme. This test detected 19 enzymes belonging to the amylases, proteases, carbohydrates and phosphatases enzyme groups. The first cupule was used as a negative control. 50µl of the salivary gland extract was added to each cupule of the test strip. After inoculation, the test strip was incubated in the dark at 37ºC for 4 hours. Then, one drop of reagent A (Tris-hydroxymethyl-aminomethane, HCl, sodium lauryl sulfate and water) and one drop of reagent B (Fast Blue BB, 2-methoxyethanol) was added to each

cupule and incubated at room temperature for 5 min to allow staining of the product. Colour developed in the test strip couples were compared to a standard color chart. Colour development was graded with a qualitative scale of 0 to 5+ on the basis of the intensity of the colour.

2.3 Proteolytic Activity Assays

The total proteolytic activity of the salivary venom extract was tested at different temperature and pH by the photometric and zymogram methods. In photometric assay, the total proteolytic activity of venom was determined according to Garcia-Carreno and Haard [17] with some modifications. It was evaluated using the protein substrates, Azocasein. Digestion of azocasien with venom extract was tested at different temperatures (25ºC, 30ºC, 35ºC, 40ºC and 45ºC) and pH (6, 6.5, 7, 7.5, 8, 8.5 and 9). 10µl of venom extract was added to 40µl of 150mM TrisHCl. The reaction mixture added with 50µl of substrate solution (2% azocasein) was incubated for 5 hours. The reaction was stopped by adding 100µl of 30% TCA, held at 4^{o}C for 30 min and centrifuge at 10,000rpm for 15 min. The resulting supernatant was dissolved in an equal volume of 1M NaOH before recording the absorbance at 440nm. TCA mixed with the substrate was used as blank. One unit of activity was defined as the amount of enzyme required to produce 1.0 units of absorbance increase in one minute.

The protease activity was further characterized through zymogram study [18] with some modifications. The sample was dissolved in non-reducing buffer and loaded in 12% polyacrylamide gel incorporated with 0.1% casein for separation. The gel was run with a constant voltage of 110V. The gel was washed for 45 min in 50mM TrisHCl (pH8) containing 2% Triton X 100 and kept in incubation buffer (50mMTrisHCl containing 0.2 M NaCl and 5 mM $CaCl_2$) for overnight at 37ºC. Different sets of pH treatment were evaluated (6.5–8.5). A similar set of experiment was also performed for different temperature (25ºC to 45ºC) at constant pH 8. The gels were stained with coomassie blue and destained with a mixture of 20% acetic acid and 40% ethanol.

2.4 Specific Proteolytic Activity

In order to determine the specific protease activity in the salivary extract, five different specific fluorescent substrates viz., BApNA (Nα-benzoyl-arginine 4-nitroanilide-hydrochloride) (for trypsin-like proteinases activity), BTEE (benzoyl-L-tyrosine ethyl ester) (for Chymotrypsin-like proteinases activity), SAAApNA (N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide) (for elastase-like proteinases activities), Z-Arg-Arg-AMC (Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride) (for capthepsin B-like proteinases activities) and Z-Gly-Gly-Leu-pNA (Z-Gly-Gly-Leu-p-nitroanilide) (for endopeptidases-like proteinases activities) were used. The chromogenic substrate solution was prepared in dimethylsulfoxide at 1mM for each substrate. The final reaction mixture was consisted of 40µl of Tris buffer (pH 8.0), 50µl of each chromogenic substrate and 10µl of the premix inhibitor enzyme solution. The reaction mixture was incubated at 37ºC for 60 minute and the reaction was stopped by adding 3% acetic acid. The absorbance of the resulting mixture was measured at 410 nm. The effect of an inhibitor measured as the percentage decrease in reaction rate.

Percent inhibition was calculated as:

$$\frac{\text{Enzyme activity of control—enzyme activity in the presence of inhibitors}}{\text{Enzyme activity of control}} \times 100$$

2.5 Inhibition Assays

Proteases present in the extract were subjected to the effect of 13 different inhibitors. Each inhibitor was tested at different concentration of 2.5, 5 and 10mM. The various inhibitors used were general serine inhibitor, PMSF (phenylmethylsulfonyl fluoride), trypsin inhibitors, TLPK (N_{α}-p-tosyl-L-lysine chloromethyl ketone) and aprotinin, chymotrypsin inhibitors, TPCK (N-tosyl-L-phenylalanine chloromethyl ketone) and chymostatin, elastrase inhibitor, SBTI (soybean trypsin inhibitor), metalloprotease inhibitor, EDTA (ethylene diamine tetra acetic acid), EGTA (ethylene glycol bis β-amin-oethyl ether) and E-64, cysteine proteases inhibitor, DTT (Dithiothreitol), Aminopeptidase inhibitor, Bestatin, Cathepsin B inhibitor, Leupeptin hemisulfate salt and cocktail (mixture of serine, cystein, aspartic, threonine and amino peptidases inhibitors). The effects of these inhibitors were determined by pre-incubating the enzyme extracts with the appropriate inhibitors for 30 minute. The assays were carried out by both spectrophotometric and zymogram methods.

2.6 Kinetic Studies

Kinetic studies were carried out for salivary venom extract using 8 different concentrations of the substrate, Azocasien ranging from 0.25 to 5 mg/ml. The Michaelis-Menten constant (K_m) was evaluated by non linear regression analysis using the software package Prism (Graph Pad Software, www.grahpad.com).

2.7 SDS-PAGE and Zymogram

SDS-PAGE of the salivary venom was performed according to Laemmli [19] by using 4% as stacking and 12% polyacrylamide as separating gel. The protein bands were visualized by coomassie brilliant blue and molecular mass was determined based on the protein standard. Characterization of proteases classes in SDS-PAGE zymogram using specific inhibitors was done (Garcia-Carreno and Haard, 1993). 10µl of the salivary extract was mixed with 5µl of inhibitor solution and incubated for 30 minute at 37ºC. The reaction was stopped by adding 2X non-reducing SDS buffer in a 1:1 ratio and 15µl of the final solution was loaded on SDS-PAGE. Extract incubated without inhibitor was used as a control. Electrophoretic and zymogram assays were done as described before.

2.8 Digestion of Extracellular Matrix Protein by Salivary Venom

Native human type I collagen was dissolved in ice cold 0.5 M acetic acid for 16 hours at 4ºC. The final concentration of the stock solution was taken as 0.1mg/ml and the frozen liquid was stored at -20ºC for further use. 10µl collagen solution was prepared by mixing with 5 µl of 0.2M Tris HCL and 83µl of buffer (10 mMTris pH 7.4, 10mM NaCl and 10mM $CaCl_2$). The salivary venom was added to type I collagen solution in the ratio of 1:50 and the reaction was carried out at 37ºC for 24 and 48 hours to assess the degradation activity. The fragments generated by salivary venom were verified by SDS-PAGE followed by the silver stain procedure.

2.9 MTT Cytotoxicity Assay

The cytotoxic effect of salivary venom was determined by the MTT (3-(4,5-dimethyl-2-thiazoyl)-2-5-diphenyl-2H-tetrazolium bromide) assay [20] using A431, human epidermoid carcinoma cells. A-431 ($1x10^5$/well) cells were dispensed in 96-well culture plates and incubated for 24 hours in a humidified atmosphere of 5% (v/v) CO_2 at 37ºC with different concentration (0.38, 0.95, 1.9, 2.87 and 5.7 µg/ml) of salivary venom. Cells without venom were used as growth control. After treatment for 24 hours, cells were washed with sterile PBS and 10µl of MTT (5 mg/ml and pH 4.7) were added to each well and incubated for 3 hours. After removing the supernatant, 100µl DMSO was added to each well and incubated for 15 minute at 37ºC. The absorbance was measured at 590nm using microplate reader (Thermo Scientific, Multi Skan, type 1500).

2.10 Cell Viability Test at IC_{50}Concentration

Cell viability was measured using Trypan blue exclusion method [21]. 100 µl of A431 cells ($1x10^5$/ well) cultured in DMEM were seeded into 96 well culture plates and incubated for 24 hours. To each well, 100µl of salivary venom having IC_{50} concentration (2.3µg/ml) was treated for 24 and 48 hours at 37ºC under humidified atmosphere (5% CO2). 10µl of 0.1% Trypan blue was added to each well and the changes in cell morphology was examined under a stereoscopic microscope (Nikon, TS 100-F; Tokyo, Japan). Results were expressed as percentage of viable cells.

2.11 Apoptosis in A431 Epidermoid Carcinoma Cells

100µl of A431 cells ($1x10^5$/ well) were seeded in a 96-well plate, 12 hours prior to treatment. A431, epidermoid carcinoma cells were treated with salivary venom at IC_{50} (2.3µg/ml) concentration at 37ºC for 24 and 48 hours to detect the apoptosis induction. 10µl of AO/EB staining solution (1µg/ml) (Sigma Chemical Co.) was added to each well. Induction of apoptosis in A431 cells were examined under a fluorescent microscope (Nikon, TS 100-F; Tokyo, Japan).

3. RESULTS

3.1 Qualitative Enzyme Analysis

The apiZYM enzyme assay gives a positive reaction for 15 enzymes in the salivary venom extracts.The enzyme groups present in the saliva were amylases, proteases, carbohydrases, phosphatases and lipases. Among these, phosphatases and proteases groups show intense colour indicating the presence of high proportion than other enzymes. Five types of protease enzymes are present in the salivary venom Table 1. The salivary venom contain less amount of carbohydrases enzymes such as Beta-galactosidase, Alpha-glucosidase and Alpha-fucosidase. Intensely coloured enzymes maintained the activity even after 72 hours.

3.2 Enzyme Activity Assay

On Azocasien substrate, proteolytic activity of the salivary venom exhibited a broad range of pH profile (6.0-9.0) Fig. 1A. Maximum activity was observed at pH 8.0 under the temperature of 35ºC. The activity decreases at 30ºC (60%) and 40ºC (42.5%) Fig.1B indicating the

denaturation of enzymes. The activation energy calculated from the Arrhenius plot of the data was 49.88k/J Table 2.

Table 1. Comparative API ZYM assay results of salivary extract of *L. indicus* and their relative strength of the reaction

Enzyme assayed	Substrate	pH	Colour	*L. indicus*	Relative strength
Control	-	-	Colourless	0	0
Alkaline phosphatase	2-napthyl phosphate	8.5	Violet	5	>2.40 10^{-4}mol/l
Esterase (C4)	2-napthyl butyrate	7.5	Violet	4	1.92 10^{-4}mol/
Esterase lipase(C8)	2-napthyl caprylate	7.5	Violet	3	1.44 10^{-4}mol/l
Leucinearylamidase	L-leucyl-2- napthylamide	7.5	Orange	1	9.60 10^{-5}mol/l
Lipase (C14)	2-napthyl myristate	7.5	Violet	1	<4.80 10^{-5}mol/l
Valinearylamidase	L-valyl-2- napthylamide	7.5	Orange	2	<4.80 10^{-5}mol/l
Cystinearylamidase	L-cystyl--2-napthylamide	7.5	Orange	1	<4.80 10^{-5}mol/l
Trypsin	N-benzoyl-DL-arginine -2- napthylamide	8.5	Orange	5	>2.40 10^{-4}mol/l
Alpha chymotrypsin	N-glutaryl-phenylalanine-2-napthylamide	8	Orange	2	9.60 10^{-5}mol/l
Acid phosphatase	2- napthylamide phosphate	6	Violet	5	>2.40 10^{-4}mol/l
Naphthol AS-BI-phosphohydrolase	Naphthol AS-BI-phosphate	6	Blue	5	>2.40 10^{-4}mol/l
Alpha galactosidase	6-Br-2-naphthyl-alphaD-galactopyranoside	6	Violet	0	0
Beta galactosidase	2-naphthyl-betaD-galactopyranoside	6	Violet	1	<4.80 10^{-5}mol/l
Beta –glucuronidase	Naphthol AS-BI-betaD-glucuronide	6	Blue	0	0
Alpha- glucosidase	2-napthyl-alphaD-glucopyranoside	6	Violet	1	4.80 10^{-5}mol/l
Beta- glucosidase	6-Br-2-napthyl-betaD glucopyranoside	6	Violet	0	0
N-acetyl-beta-glucopyranoside	1-napthy-N-acetyl betaDglucopyranoside	6	Brown	5	>2.40 10^{-4}mol/l
Alpha-mannosidase	6-Br-2-napthyl-alphaD mannopyranoside	6	Violet	0	0
Alpha –fucosidase	2-napthyl-alpha L-fucopyranoside	6	Violet	1	0

The zymographic profiling of the salivary extract was evaluated at different pH range of 6.5-8.5. The intensity of protease activity increased progressively from pH 6.0-8.0. The complete protease activity was resolved at pH 8.0 Fig. 2A. To analyze the temperature dependence of protease activity, the gels were incubated at different temperature ranging from 25 to 45ºC. The activity was increased with the increase in temperature from 30 to 40ºC. However, the activity was found reduced at 45ºC Fig. 2B.

3.3 Calculation of K_{max} and V_{max}

The reaction velocity of salivary venom increased correspondingly with the increase in azocasein substrate concentration Fig. 3. A hyperbolic response was observed. The

breakdown of azocasein by the enzymes was linear upto 60 minute of incubation. From the Lineweaver- Burke plot, the apparent value of K_{max} was obtained as 3.33mg whereas $V_{max}$was 0.08 mg/min Table 2.

Table 2. Kinetic parameters of salivary extract of *L .indicus*

Samples	V_{max} (mg/min)	K_m (mg)	Turnover number ($K_{cat}= V_{max}/E_T$)	K_{cat}/K_m	E_a(kJ)
L .indicus	0.08	3.33	40	12.01	49.88

V_{max} – maximum velocity, K_m= concentration of the substrate that gives a velocity equal to one-half the maximal velocity, K_{cat}= the number of the substrate molecules that converted into product per enzyme molecule per unit time when the enzyme is saturated with substrate, E_T = concentration of the enzyme and E_a=activation energy

3.4 Inhibition Assay

The total proteolytic activity of the salivary extract was further characterized using specific inhibitors. The extent of the inhibition of azocasein hydrolysis indicated the relative contribution of specific protease present in the salivary venom extract Table 3. The inhibitor assay indicates the protease in salivary extract was subclass of serine protease. The serine protease inhibitorsviz., PMSF, TLCK and TPCK decreased the enzymatic activity significantly with 92.77%, 83.05%, and 55.71% respectively. Other inhibitors namely aminopeptidase and metalloprotease inhibitors (i.e. Bestatin and EGTA) also inhibit the enzyme activity of the salivary extract.Cocktail, the mixture of inhibitors (serine, cysteine and aspartic proteases, amino and threonine peptidases) responded 96% inhibition. The variation in the inhibition percent among the inhibitors showed the presence of different proteases in the salivary venom.

3.5 Characterization of Salivary Protease by SDS-PAGE

A series of biochemical determination and electrophoretic studies were conducted to analyze the activities of salivary extract. The SDS-PAGE analysis of crude salivary extract showed thirteen separate protein bands in *L. indicus* Fig. 4.

Zymogram analysis of salivary proteases revealed four protease bands in the control sample. Different classes of proteases were detected with specific protease inhibitors. The intensity of protease bands disappeared or reduced compared to the control. Further, characterization of the salivary enzymes was done using specific inhibitors and synthetic substrates. Slight inhibition was observed in 54 and 28kDa bands whereas 48kDa and 33 kDa protease bands were completely inhibited in the zymogram with general serine protease inhibitor, PMSF. PMSF inhibits most protease bands and other types of serine-like proteases too. Trypsin-like protease and chymotrypsin-like protease were determined by zymogram using TLCK and TPCK inhibitors respectively. 33 and 27 kDa protein bands were slightly inhibited at similar intensity indicating the presence of mixed types of protease of similar molecular weight Fig. 5A.

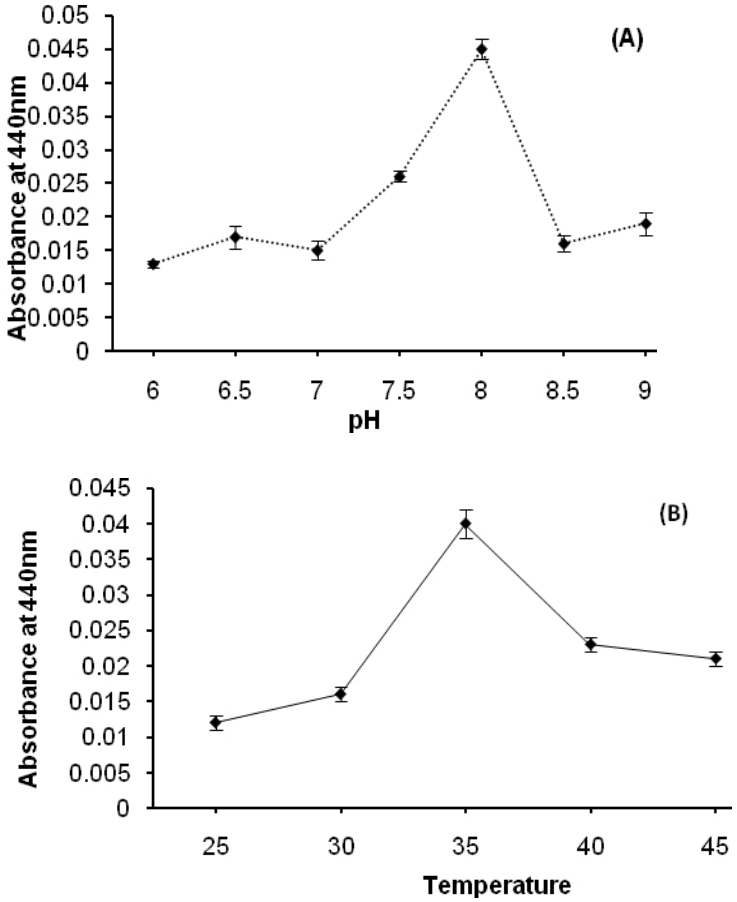

Fig. 1. Proteolytic activity of salivary venom of *L. indicus* at different pH (A) and temperature (B)

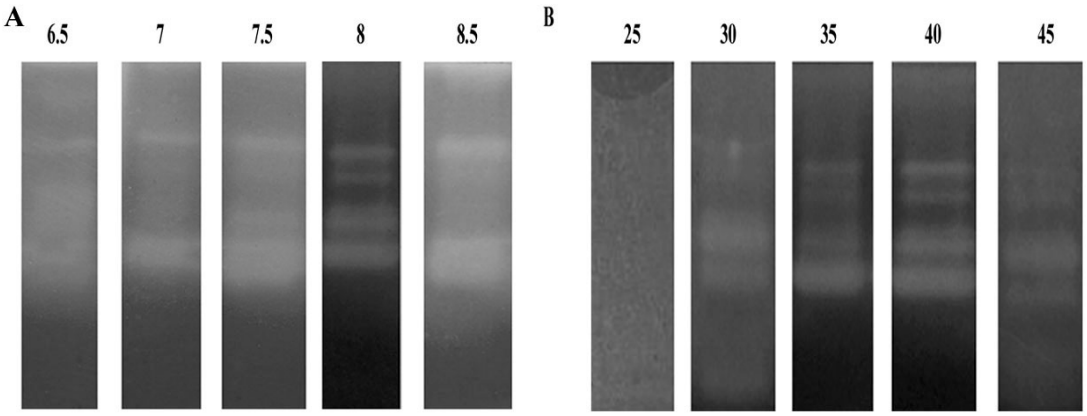

Fig. 2. Zymogram shows the influence of pH (A) and temperature (B) on the enzyme activity

Table 3. Effect of specific inhibitors on proteolytic activity of salivary extract of *L. indicus*

Inhibitors	Target protease	Concentration	Inhibition Percentage
PMSF	Serine	2.5 mM	92.77±0.73
		5 mM	85.33±0.50
		10 mM	77.77±2.02
TLCK	Trypsin	2.5 µM	54.83±0.63
		5 µM	38.10±0.34
		10 µM	26.38±0.8
Aprotinin	Trypsin	2.5 µM	37.05±2.10
		5 µM	27.05±1.20
		10 µM	13.83±0.96
TPCK	Chymotrypsin	2.5 µM	83.05±1.21
		5 µM	76.94±1.54
		10 µM	46.44±0.77
Chymostatin	Chymotrypsin	2.5 µM	55.71±0.58
		5 µM	46.77±0.98
		10 µM	11.66±0.19
Leupeptin	Cathepsin B	2.5 µM	43.77±0.86
		5 µM	28.71±1.10
		10 µM	14.05±1.01
E-64	Metalloprotease	2.5 µM	28.22±1.30
		5 µM	12.16±0.88
		10 µM	08.71±0.81
EDTA	Metallo protease	2.5 µM	44.63±0.44
		5 µM	29.93±0.34
		10 µM	25.93±0.58
EGTA	Metalloprotease	2.5 µM	87.20±0.58
		5 µM	56.10±0.58
		10 µM	26.26± 0.64
DTT	Cysteine	2.5 µM	21.20±1.86
		5 µM	40.40±0.70
		10 µM	48.40±2.27
SBTI	Elastrase	2 µg	48.65±1.54
		4 µg	67.79±0.79
		8 µg	79.18±1.20
Cocktail	Serine Cysteine Aspartic Amino peptidase Threonine peptidase	1:100	96.00±1.42

Zymogram analysis was also carried out to detect other types of protease using specific inhibitors Fig. 5B. Bestatin, specific inhibitor of amino peptidase showed the inhibition on 48, 33 and 22kDa of the protease bands. Cocktail, the mixed protease inhibitor showed the maximum number of bands inhibition. There might be other specific proteases not inhibited by these inhibitors. EGTA and DTT, specific inhibitors of Metalloprotease and Cysteine showed the inhibition at 54 and 48kDa.

To detect trypsin-like activities, photometric assay was conducted using a general serine protease inhibitor, PMSF (10mM) and trypsin specific inhibitor, TLCK (10µM) with BApNA as a substrate. The substrate reaction showed 82% and 78% inhibition, respectively compared to relative activity of the enzyme without inhibitor Fig. 6A-B. Chymotrypsin-like activity detected using chromogenic substrate, BTEE and specific inhibitors, PMSF (10mM) and

TPCK (10μM) showed 74.21% and 58.5% inhibition respectively Fig.6C-D.Elastrase-like activity was detected using chromogenic substrate, SAAPpNA and specific inhibitors, SBTI (8μg/ml) and PMSF (10mM) as a control. The chromogenic reaction was inhibited 83% and 96%, respectively Fig.6E-F. Slight activity of subtilisin, neutral endopeptides and capthepsin activity were also observed with specific substrates, ZGG-leu p-nitroanilide and ZAAAMC respectively (data not shown in the result). These activities showed the presence of a slight amount of substilisin, endopeptide and capthepsin in this salivary venom.

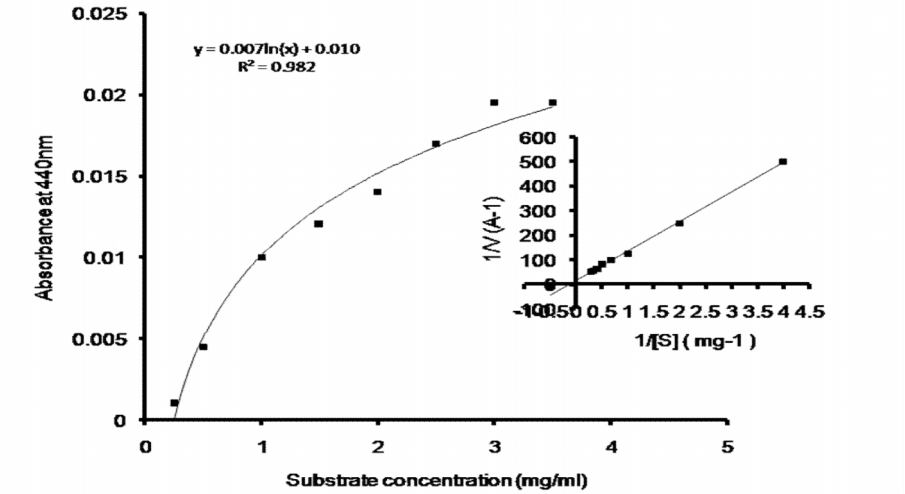

Fig. 3. Michaelis-Menten and Lineweaver-Burk plots of proteolytic activity of the salivary extract of *L. indicus* on azocasein substrate

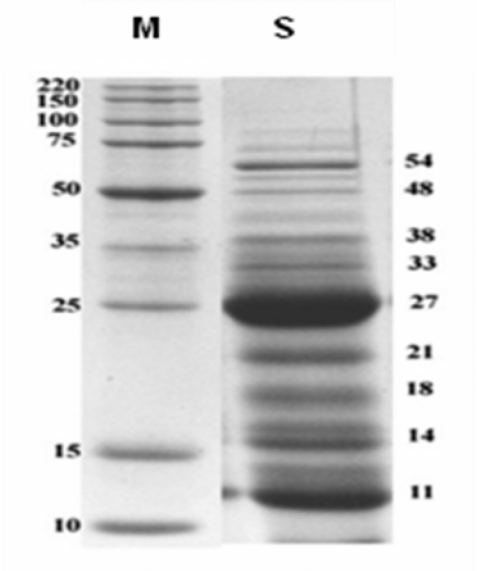

Fig. 4. SDS-PAGE electrophoresis gel shows existence of different protein bands in salivary extract of *L. indicus*. M indicates protein ladder and S indicates salivary extract

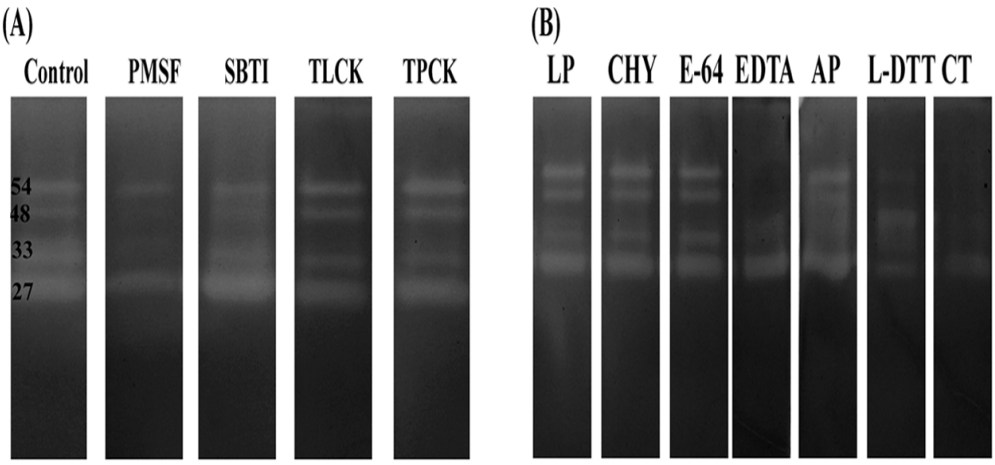

Fig. 5. Zymogram of the salivary proteases shows the detection of serine-like proteases with serine type specific inhibitors (A) and (B) other proteases enzymes with specific inhibitors Leupeptin (LP), Chynostatin (CHY), Aprotinin (AP) and cocktail (CT)

3.6 Collagen Degradation

To determine the hydrolysis properties, human type I collagen was incubated at 37ºC with the salivary venom (enzymes/substrate mass ratio of 1/50)for 24 and 48 hours.The pattern of collagen type 1 dergradation hydrolysis by salivary enzymes was found well defined and maintained until 48 hours, suggesting that salivary enzymes can hydrolysed collagen molecules of small peptide bonds. The degradation of numerous fragments by salivary venom required longer time. Incubation of collagen type 1 performed in the same condition without salivary enzymes showed intact band without fragmentation in α1 and α2 bands Fig. 7. The collagen type 1 generated more number of fragements in 48 hours (8 bands) than 24 hour (4 bands) which indicates an increase in digestion activity of the venom with the increase in exposure time as a result generated more peptide bonds correspondingly.

3.7 Growth Inhibition of Human Epidermoid Carcinoima cells (A431)

Salivary venom extract of *L. indicus* was evaluated for anti-proliferative effects in A431 (human epidermoid carcinoima cells) by the MTT assay. The crude venom treatment of A431 cells showed the dose-dependent activity in which the growth of cells decreases with the increase in concentrations of venom (0.38 to 5.7µg/ml) Fig. 8. Cytotoxicity was expressed as percent increase relative to the unexposed control (0%). The IC_{50} values for A431 cell lines determined by sigmoidal curve was found 2.3 µg/ml, in which 50% inhibition was observed in the cell growth of cell line compared to the untreated control.

3.8 Cell Viability and Induction of Apoptosis in A431 Cancer Cell Lines

The trypan blue exclusion test in A431cancer cell line with IC_{50} concentration of salivary venom determined the viable and dead cell intensity varied with exposure time (24 and 48 hours). The live cell having intact cell membrane was observed in the control cells (without extract) and unable to stain with trypan blue. It traverses the membrane of dead cell

indicating a distinctive blue colour in the treated cells. More than 60% death cells were noticed in 48 hours. The cytostatic activity of extract was demonstrated in Fig. 9. The number of viable cells was decreased significantly in 48 hour treatment, indicating the time dependent effect of the venom activity.

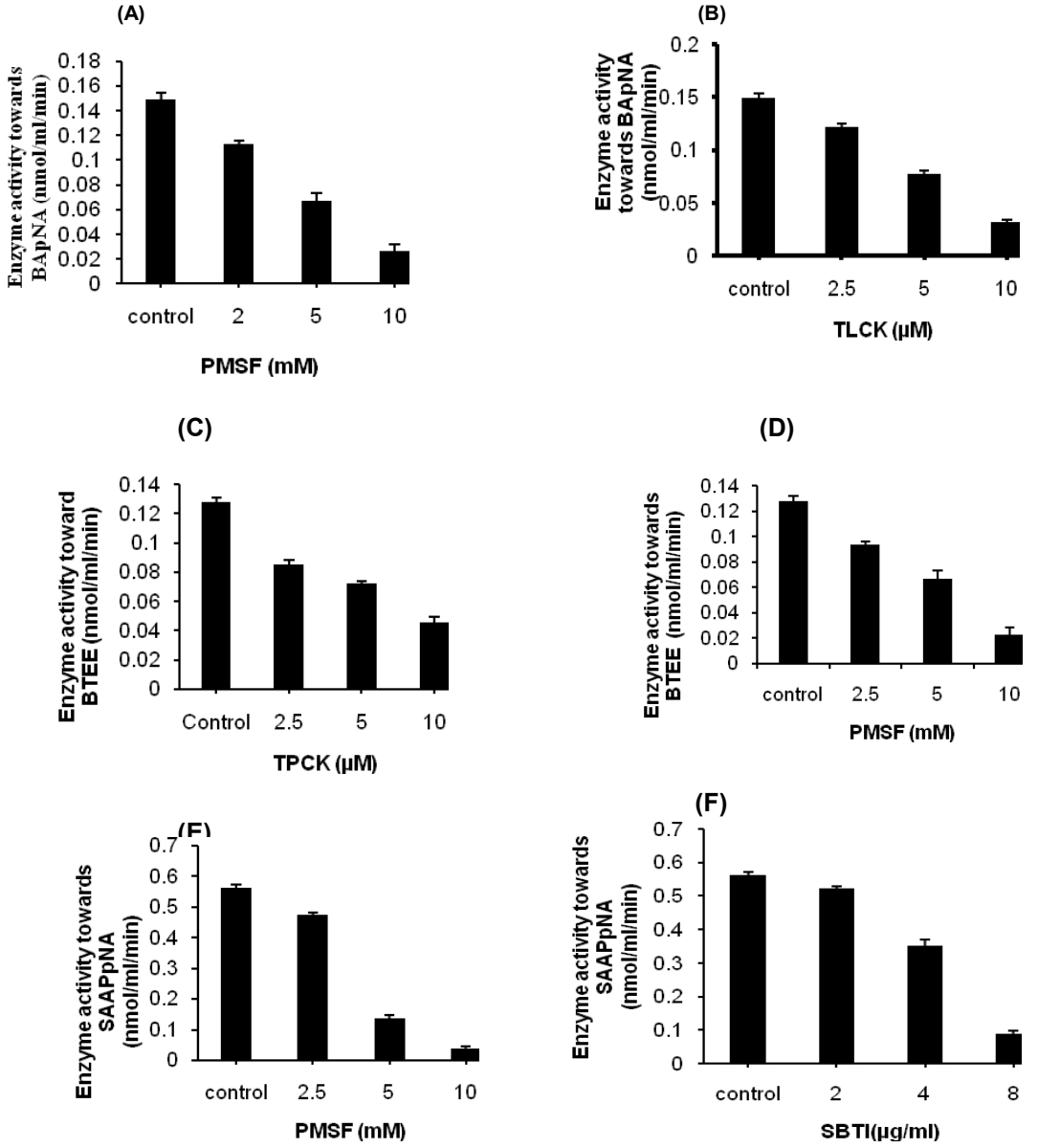

Fig. 6. Photometric assays of the enzyme extracts using BApNA with PMSF and TLCK for detection of trypsin (A-B), BTEE with PMSF and TPCK (chymotrypsin) (C-D) and SAAPpNA with PMSF and SBTI (Elastase) (E-F)

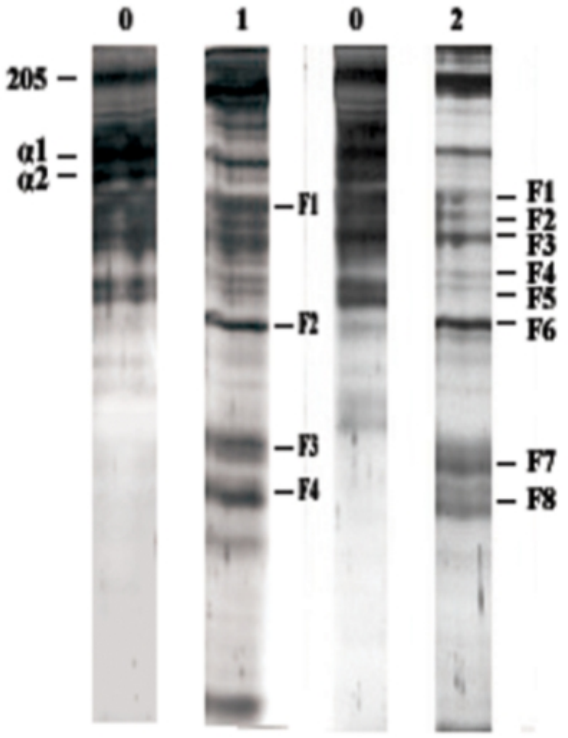

Fig. 7. SDS-PAGE time course degradation of collagen type I by salivary venom; 0) represent intact type I collagen with ∞1 and ∞2 chains; 1,2) time course of degradation of type I collagen by salivary venom 24 and 48 hours

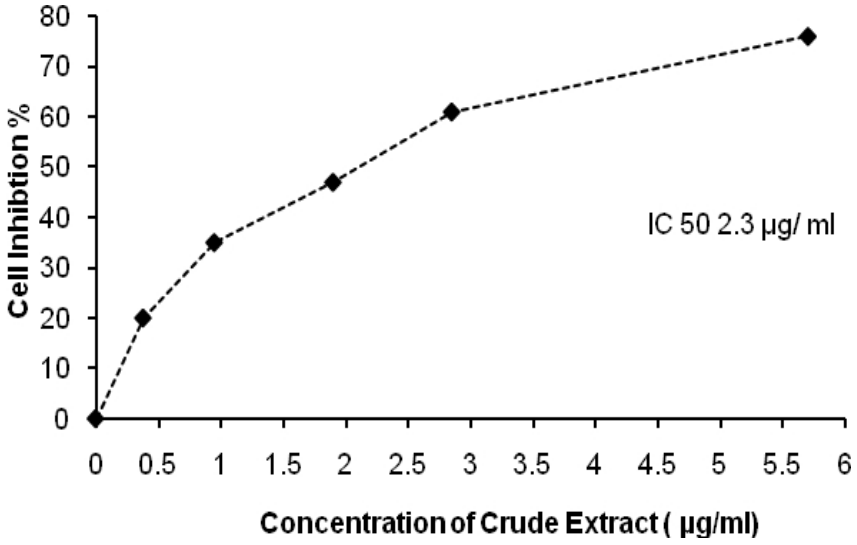

Fig. 8. MTT assay of *L. indicus* salivary venom on A 431cell

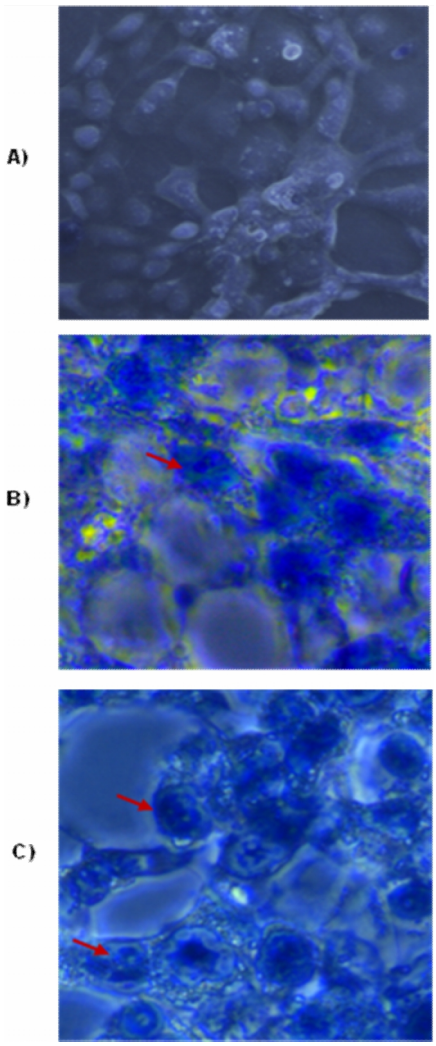

Fig. 9. Effects of the salivary venom of *L. indicus* on the morphology of A431cells. (A) Nontreated cells. (B) Cells treated with IC 50 2.5 (µg/ml) incubated for 24 hours (C) Cells treated with IC 50 2.5 (µg/ml) incubated for 48 hours. Red arrows indicate vacuole cells (Magnification X 200)

Acridine orange/ethidium bromide (AO/EB) staining visualized nuclear changes and apoptotic body formation in A431 cancer cell lines treated with salivary venom. Acridine Orange (AO) permeates cells and makes the nuclei appear green. Ethidium bromide (EB) is taken up by cells when cytoplasmic membrane integrity is lost and stains the nucleus red. EB also dominates AO. Live cells have a normal green nucleus, early apoptotic cells have bright green nucleus with condensed or fragmented chromatin and late apoptotic cells display condensed and fragmented orange chromatin, cells that have died of direct necrosis have a structurally normal orange nucleus. The figure shows the appearance of stained A431 cells under fluorescence microscopy after treatment with the IC_{50} concentration of salivary venom. Characteristic features of apoptotic cells, such as cytoplasmic membrane blebbing, nuclear contraction, nuclear fragmentation, and contact inhibition was depicted at

24 and 48 hour treatment Fig.10. Early apoptotic, late apoptotic and apoptotic body formation was more distinct at 48 hours.

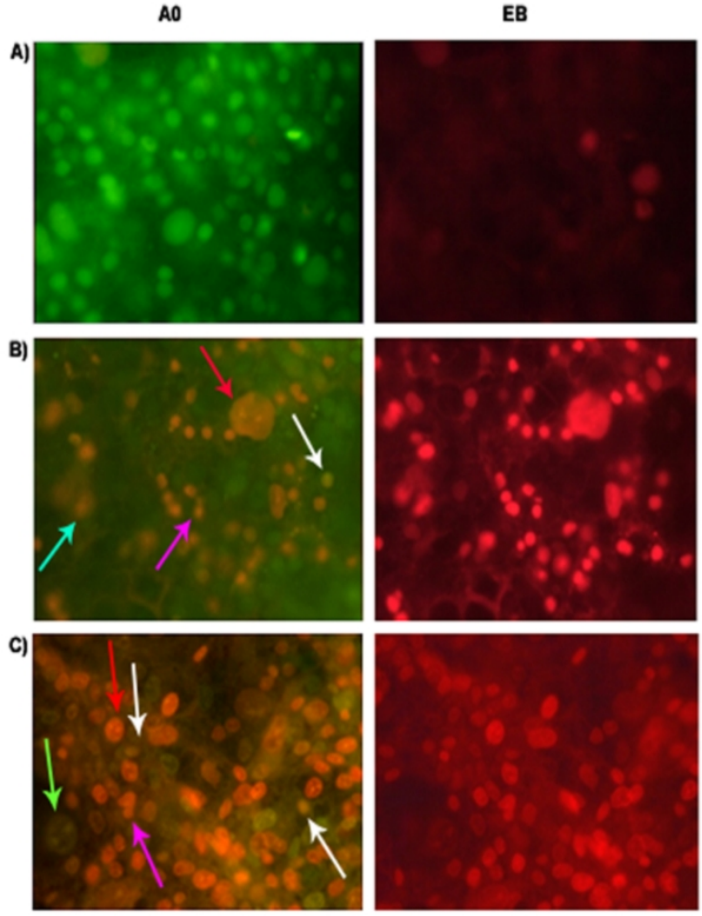

Fig. 10. A431cells stained with AO/EB and viewed under fluorescence microscope (400×) showing apoptosis. (A) Healthy control cells; (B) 24 hours venom treatment (C) 48 hours venom treatment (2.3 µg/ml) on A431 showing nuclei fragments (red arrow), nuclear fragmentation (magenta arrow), budding to form apoptotic body (white arrow) nuclei fragment to form early apoptotic (violet arrow), membrane blebbing (green arrow)

4. DISCUSSION

L. indicus is known for its edible and medicinal properties in various countries like India, Thailand, Korea, China, Zaire etc. [4,22,23]. Besides nutritional properties [24], enzymatic nature of salivary venom of *L. indicus* is less studied. Therefore, we described its enzymatic properties, extracellular matrix degradation and cytotoxic activity in epidermoid carcinoma cell, A431. The total enzymatic profile of the salivary venom showed the presence of 15 different enzymes by ApiZYM assay. The numbers of enzymes observed were more compared to aquatic bugs, *B. lutarium* and *L. uhleri*. Out of the total enzymes, composition of the proteases enzymes was more than other groups of enzymes that may be because of the

major role of proteases in the digestion of proteins into oligo- and di-peptides [24]. Two metallo proteases enzymes, leucine arylamidase and cysteine arylamidase were also present in *L. indicus* compared to *L. uhleri* [25]. The presence of amylase enzymes in the salivary extract indicated the ability to exploit the plant material already ingested by the prey. Enzymatic profiles may be modified according to environmental conditions and food availability from the host [24].

Proteolytic activity assay of salivary venom of *L. indicus* indicated the presence of all the three types of proteases, cysteine, metalloprotease and serine. The proteolytic activity in this salivary enzyme is dynamic and dependent on the interactions among tissues, pH, temperature and protease classes. The highest rate of proteolytic activity was occurred at pH 8 in 35ºC (100%). Highest activity at pH 8 suggested that the proteolytic activity in salivary venom was predominantly due to serine proteases. Slight pH optimum was also got at pH 6.5 (37.7%). It indicates the presence of cysteine protease. Serine proteases had higher activity at alkaline pH, while cysteine proteinases were more active at acidic pH. The gradient from acidic to alkaline pH and enzyme compartmentalization negates the need for structural adaptations in digestive enzymes [26]. The maximum peak activity observed at pH 8 was similar to the predatory bug, *Andrallus spinidens* [27]. Serine proteases were the prevailing proteolytic enzymes in this salivary venom was evidence from the optimum pH, specific substrate and inhibition assays [28-30]. Proteolytic activity in the digestive system of Hemiptera was due to serine and cysteine proteases [31]. At different pH and temperature, the activity of the protease was affected. A higher activity of the enzymes in a specific temperature generally reflects the temperature of the environment where the organism fed on the hosts. Extreme temperature can also disrupt the hydrogen bonds that hold the enzyme in its three-dimensional structure leading to the denaturation of the protein [32]. Protease activity increased with increasing pH and temperature [25]. The activation energy calculated from the Arrhenius plot was 49.88 k/J. The K_{max} value has an inverse relationship with the increase in the substrate concentration, saturating the active site of the enzymes is required [27]. V_{max} value showed the breakdown rate of the enzymes-substrate complex. The higher V_{max} value of azocasein substrate observed in the present investigation showed the higher enzymatic velocity of the salivary enzymes.

Zymogram study gives four protease bands in *L. indicus* approximately ranging from 21 to 48 kDa. The predatory insect of same genus, *L. uhleri* and *B. lutarium* exhibited proteases in the range of molecular masses, 30 to 40kDa [25]. Molecular weights of these enzymes were significantly different from its counterpart.

Thirteen different specific protease inhibitors viz., PMSF, TLCK, aprotmin, TPCK, chymostatin, SBTI, EDTA, EGTA, E-64, DTT, Bestatin, Leupeptin and cocktail were used to confirm the specific proteases in the salivary venom of *L. indicus*. The effects of 11 specific inhibitors were studied on the proteolytic activity of *L. lineolaris* saliva [33]. Among protease inhibitors, PMSF inhibited (95.5%) maximum activity than other inhibitors. Bell et al. [34] also reported that the serine protease inhibitor (PSMF) caused the most remarkable reduction in the rate of proteolysis, with a residual activity of 53% in the saliva of *Podisus maculiventris*. Proteolytic activity in the saliva of *B. lutarium* and *L. uhleri* were reduced by the protease inhibitors, PMSF, TLCK and TPCK [25].

PMSF and DTT showed significant inhibition percentage in the enzymatic activities of salivary venom of L. indicus. These results demonstrated the presence of serine protease and metalloprotease in the salivary venom. The effect of TLCK (trypsin-like inhibitor) and

TPCK (chymotrypsin-like inhibitor) that decreased the enzymatic activity in *L. indicus* showed a similar trend in activities with the salivary extracts of *L. lineaolaris* [33].

The protease inhibitor, PMSF (serine inhibitor) showed the highest inhibition (92.5 %) against the azocasein activity of the salivary venom. The cysteine protease inhibitor, DTT caused no significant effect on the proteolytic activity in the salivary venom of *L. indicus*. The less effect of DTT was also reported in the predatory bug, *A. spinidens* [27]. Serine proteases were the major proteases found in the salivary gland of many heteroptera insects [11]. It takes a longer time (four to five hours) to utilize prey before satiation suggesting that the enzymes injected into the prey waits to act than to recover the digested product.

Photometric assays using BApNA and SAApNA substrates revealed the significant activity of trypsin and elastase serine proteases in the enzyme extract. Trypsin and chymotrypsin serine proteinases are the main endopeptidases in most of Heteroptera insects [30,31]. Predominant activities of trypsin-like and cymotrypsin-like proteases, based on hydrolysis of trypsin-specific and chymotrypsin-specific substrate inhibition by TLCK and TPCK inhibitors was observed in other insects, *E. integriceps* and *L. hespresus* [29,30].

Several extracellular matrix molecules have been described as targets for degradation evoked by proteases present in snake venom that cause hemorrhage, necrosis and edema [35,36]. However, presence of venom proteases of *L. indicus* that degrade extracellular matrix molecules was not known. *L. indicus* salivary venom could hydrolysed Collagen type I. More fragments were degenerated in 48 hours of exposure time than 24 hours. An increase in exposure time increases the enzyme activity. Cardosin A extracted from *Cynara cardunculus* have reported to hydrolyzed fibrillar collagen within the α-chain, hence proposed for an eventual medical or technical procedures assisting ECM remodeling [37]. The native state collagen is resists to most endogenous and exogenous proteolytic enzymes. Generally, bacterial proteinases have weak degradative activity against collagen [38,39]. Therefore, the property of fibrillar collagen degradation by salivary enzymes has been the subject of considerable interest for its subjacent medical application.

An automated fluorescence method with sequential acridine orange (AO) and Ethidium bromide (EB) staining were used to detect death cell by necrosis and apoptosis due to salivary venom treatment. Death cell by apoptosis due to salivary venom treatment such as cytoplasmic membrane blebbing, nuclear contraction, nuclear fragmentation, and contact inhibition were depicted in A431 cancer cells. It was also reported that the venom elevates NO and RNI production, causes depolarization of the mitochondria, releases pro-apoptotic factors, and activates the intrinsic apoptosis pathway in human breast cancer cells [40].

5. CONCLUSION

The salivary venom of *L. indicus* contains multiple enzymes such as amylases, proteases, carbohydrases, phosphatases and lipases. Among them, protease enzymes showed maximum composition than other enzymes. The proteolytic activity at optimum pH, temperature, specific inhibitors and substrates supported the strong presence of trypsine like-serine protease. It also indicates the presence of metalloprotease. Salivary venom have the capacity to degrade the fibrillar native state collagen Type I. The endogenous and exogenous proteolytic enzymes of *L. indicus* are important in subjacent medical application. The cytotoxic activity of salivary enzyme may be because of serine proteases presence in it. We are working on the mechanism of these cytotoxic and proteolytic effects of the venom using different assays and different cell types to identify the active fractions. We suggest that

this study will lead to the identification of active compound in salivary venom of *L. indicus* and its potential use in therapeutic application.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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