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ISOLATION AND CHARACTERISATION OF *Clostridium histolyticum* AND IT'S TOXIN

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ABSTRACT

A gram positive, motile, strictly anaerobic bacterium was isolated from the sediment samples of Vellar estuary, India and was identified as *Clostridium sp.* by biochemical methods. The organism was found to be resistant only to erythromycin among the various antibiotics tested. The culture supernatant was checked for its protein concentration by lowry method and optimum conditions for maximum toxin production was found out as 42hrs incubation , pH 8, temperature of 35˚C and 1.5% salinity. Extraction of the toxin was carried out under optimum conditions and were partially purified by ammonium sulphate precipitation and dialysed against acetate buffer. From the 500 ml culture broth used for mass scale production, it was possible to get 1.6 g of toxin from 3g of biomass . When the antibacterial activity of the toxin was tested it was found to be high against *Proteus vulgaris* and *Klebsiella pneumoniae* with a maximum inhibition zone of 48mm and 27mm respectively. Antifungal activity was absent. Antioxidant activity of the toxin was found to moderate. The toxin extract was found to be highly β-hemolytic. Molecular weight of the toxin was found by SDS PAGE and it was found to contain 3 bands with the molecular weight of 56KDa, 47 KDa, 39KDa. Brine shrimp acute toxicity assay confirmed that the LC_{50} value of the toxin was 1x.5 mg/ml.

Keywords: *Clostridium histolyticum*, Toxin, lethal toxicity, antibacterial activity, SDS PAGE.

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INTRODUCTION

Anaerobic spore-forming bacilli of the genus Clostridium are ubiquitous in the environment, existing in the form of exo-spores that can remain viable indefinitely[2]. Clostridia are widely distributed in nature and are found in soil as well as in freshwater and marine sediments throughout the world. *Clostridium* species vary considerably in their oxygen tolerance [11]. The genus *Clostridium* contains several species of known potential pathogenicity for man and animals. They are normally found in the mouth, gastrointestinal tract, and vagina, and on the skin. The pathogenic clostridia include the gas gangrene group – *C. perfringens, C. septicum*, *C. sordellii, C. novyi* and *C. histolyticum* – and the causal organisms of *tetanus (C. tetani), botulism* (*C. botulinum*), clostridial food poisoning (*C. perfringens*) and antibiotic- associated diarrhoea and colitis (*C. difficile*) [3]. Some species such as *Clostridium hemolyticus* are among the strictest of obligate anaerobes and may require extended incubation on pre reduced or freshly prepared plates and total handling in an anaerobic chamber[1]. Conversely *Clostridium tertium,Clostridium histolyticum* and *Clostridium carnis* are aerotolerant and form colonies on blood agar plates incubated in an atmosphere of air with 5-10% added CO_2 [12]. Virtually all of the members of the genes except *Clostridium perfringens* are motile with petrichous flagellae and form oval or

spherical endospores that may distend the cell. They may be saccharolytic or proteolytic and are usually catalase-negative [10].

Clostridium histolyticum is an anaerobic, motile, gram-positive bacterium that thrives in feces and soil[5] . The ammonia and proteases it produces, including several collagenases, digest proteins in the environment. When *Clostridium histolyticum* infects an open wound, it can also necrotize tissue by secreting an exotoxin that induces cytolysis [23]. In the mid-1910s, Michel Weinberg and Pierre Séguin found this species to be the primary pathogen in cases of gas gangrene. Weinberg and Séguin originally classified *Clostridium histolyticum* as a member of the genus *Bacillus* after isolating the bacteria from patients suffering from gas gangrene. Heller renamed the species *Weinbergillus histolyticus* [25] in 1922, the following year, Bergey, Harrison, et al. reclassified it as a *Clostridium* species. A collagenase produced by this species, is applied for the medical treatment of chronic conditions characterized by excessive collagen deposition [21].

Toxigenesis or the ability to produce toxin, is an underlying mechanism by which many bacterial pathogens produce disease[7]. Endotoxins generally act in the vicinity of bacterial growth or presence. Exotoxins are usually secreted by bacteria and act at a site removed from bacterial growth. However, in some cases, exotoxins are only released by lysis of the bacterial cells. Exotoxins are usually proteins, minimally polypeptides that act enzymatically or through direct action with host cells and stimulate a variety of host responses [17]. Most exotoxins act at tissue sites remote from the original point of bacterial invasion or growth. However, some bacterial exotoxins act at the site of pathogen colonization and may play a role in invasion [26].

This protein family represents the most potently acute lethal toxic substances known[8]. Reports on the nature of proteolytic enzymes obtained from *Clostridium histolyticum* have been contradictory. Maschmann 1938 described two extracellular and one intracellular enzyme besides peptidases [16]. One of the challenges in working with potently toxic proteins is to prepare variants that harness the biological functions of the toxin whilst reducing the toxic effect to a minimum [18].

In the present study, a *Clostridium* spp. was isolated and identified from the Vellar estuary. The toxin produced by the bacteria was extracted and partially purified and characterized for its toxic properties and its benefits.

Materials and Methods

Collection of samples

Sediment samples were collected from Vellar estuary indifferent stations using PVC pipe corer. The inner core of the sample was used for isolation of anaerobe. All the procedures were carried out inside an anaerobic glove bag where carbondioxide , nitrogen and hydrogen gases were used to maintain anaerobic environment.

Isolation of anaerobic microbes

The soil samples were enriched in anaerobic media containing Casein Enzymatic hydrolysate-20g/L, Sodium Chloride-5g/L, Sodium thioglycolate- 2g/L, Sodium formaldehyde sulphoxylate-1g/L, Agar-15g/L for the growth of anaerobic bacteria .

Serially diluted enriched media were plated on selective media for Clostridial species containing Beef extract-10.0g/L, Peptone- 10.0g/L, Sodium Chloride-5g/L, Glucose Monohydrate- 5g/L, Sodium acetate-3g/L, Yeast Extract- 3g/L, Starch Soluble- 1.0g/L, L-cysteine hydrochloride-0.5g/L, Agar-0.5g/L.

Identification of Clostridial species

Biochemical identification

The Clostridial species were identified to the species level by some biochemical methods as given in the identification flowchart [9].

Evaluation of antibiotic resistance pattern

Antibiotic sensitivity of *Clostridium histolyticum* was tested using anaerobic agar. Anaerobic agar plates were prepared by using 10-15 ml of the medium into each sterilized petriplates and are allowed to set at room temperature. The bacterial suspension was inoculated over the surface of agar medium using sterile cotton swab in the anaerobic chamber. This test was performed using 16 antibiotics by placing two different sets of octa discs over the swabbed plates. The zone of inhibition was observed after 24 hr of incubation at 37˚C under strict anaerobic conditions.

Optimization for Clostridial toxin production

The conditions (incubation period, pH, salinity and temperature) were optimized for maximum toxin production using Reinforced Clostridial broth medium. Toxin production was studied at different incubation periods (24-54 hrs with 6 hrs interval), temperature (25-50^{0}C with 5^{0}C interval), pH (6 to 10 with 1 interval) and Salinity (1 to 3% with 0.5% interval).

Extraction of toxin

500ml of culture broth of the Clostridial species was centrifuged at 12,000 rpm at 4˚C and the supernatant was collected in sterile bottles[23].

Partial purification of toxin

Ammonium sulphate precipitation

The culture supernatant of 500ml was divided into two equal parts and added with 50% $(NH_4)_2SO_4$. Saturation of solutions was carried out under 4°C over night. The precipitated proteins were collected through centrifugation at 3000 rpm for 30 min. the non residue matters were discarded and the resulted pellets were dissolved in 50 mmol/L Tris-HCl buffer (pH7.5).

Dialysis using acetate buffer

The obtained residues, collected from the ammonium sulphate precipitation were introduced into a regenerated cellulose-dialysis tube (this membrane is partially permeable) for dialysis under 4^{0}C against acetate buffer at pH 5.5. Dialysis was done for 24 hrs. Finally samples were lyophilized. The lyophilized, powdered sample of partially purified *Clostridium histolyticum* toxin was preserved under 4^{0}C for further analysis.

Characterization of toxin produced

Antibacterial activity

Antibacterial assay was carried out by a modified agar well diffusion method [27]. Muller Hinton agar was used to check the antibacterial activity of the partially purified protein suspension produced by *Clostridium histolyticum* against six different bacterial pathogens. The sterile media containing agar was poured into the sterilized petriplates and allowed to solidify at room temperature. Wells were made in the medium using cork borer. Different bacterial pathogens (*Staphylococus aureus, Salmonella typhi, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris* and *Escherischia coli*) were swabbed over the sterile plates. 100µL of protein suspension was poured into the wells and incubated for 24hrs at 37ºC. The zone of inhibition was measured using a millimeter ruler after incubation.

Hemolytic Activity

100 µl of partially purified *Clostridium histolyticum* toxin was added to the well in blood agar medium under anaerobic conditions and incubated for 24 hrs to check its hemolytic property.

Antioxidant Activity

Free radical scavenging properties of the toxin was assessed using total antioxidant method by using ascorbic acid as standard. The activity increased with increase in concentration of the sample (Fig.10). When compared with standard ascorbic acid (1.64 µg/ml) the toxin showed moderate activity (0.67µg/ml).

Determination of protein concentration

The protein concentration of the partially purified lyophilized protein was determined by the method of Lowry [15] using bovine serum albumin (BSA) as standard.

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Determination of molecular weight by electrophoresis (SDS- PAGE)

Electrophoresis of protein samples in the polyacrylamide gels is an indispensable analytical and in some cases, preparative tool for the protein scientist. SDS PAGE was done for the partially purified potein to determine the molecular weight of the toxic protein [14]. The migration rate of protein is determined by the gel pore size and the protein charge, size and shape.

Acute Toxicity Test- 24-hrs LC_{50} Bioassay

An LC_{50} is defined as the percent by volume that kills 50% of the test organisms in 24hrs. In the present study toxicological analysis was conducted using the brine shrimp (*Artemia salina*) lethal assay (BSLA). Biological activity using BSLA was recorded as the median lethal concentration (LC_{50}) that kills 50% of the larvae within 24 hrs of contact with the *Clostridium histolyticum* toxin. The shrimps were divided into 5 groups, five different toxin concentration test groups, one positive control and one negative control group. The five nominal concentrations were 0.5ml, 1.0ml, 1.5ml, 2.0ml, 2.5ml of dissolved *Clostridium histolyticum* toxin. One container was used exclusively for the positive control *Artemia* with highly concentrated toxin and another container with negative group, only animal without toxin and the other 5 test containers contained different concentrations of the toxin solutions. 10 shrimps were randomly placed in each container, they were not fed prior to experimentation and during toxin exposure for 24hrs. Two replicate experiments were performed. The mortalities were recorded after 0, 6, 12, 18 and 24h of exposure at 29±2°C and the dead shrimps were removed from the test containers. *Artemia* nauplii have branch-like appendages that also serve as gills and continually flutter when the organism is healthy. The shrimps were considered dead when they displayed total lack of movement. The LC_{50} and 95% confident interval limits were computed using the Probit analysis.

RESULTS AND DISCUSSION

The main objective of the study was to isolate and to evaluate a toxin producing marine *Clostridium* spp. *Clostridium* strains were isolated from Vellar estuary. The obtained anaerobic bacteria were grown in *Clostridium* selective media. Biochemical tests confirmed that the cultured bacteria is *Clostridium histolyticum.* 16s rRNA also revealed the genus as *Clostridium.* Antibiotic resistance pattern was also found out using 16 different antibiotics.The toxin produced was extracted, partially purified and its molecular weight was determined by SDS PAGE. Optimization of incubation period, pH, temperature, salinity was carried out for maximum toxin production. Toxin was characterized by antimicrobial activity, Hemolytic activity and protein estimation[19].

Isolation of Anaerobic Clostridial species

Properly diluted sediment samples were plated on selective media for Clostridial species and the resulting colonies were pure cultured and used for the further assays. Sediment samples were found to harbour anaerobic clostridial forms around $1.9x\ 10^6$ CFU/g.

Identification of the Clostridial species

Species level identification of *Clostridium* spp. was done using biochemical tests given in Table 1. Conventional biochemical tests included acid production from sugars, reaction in milk, meat and gelatin, lecithinase and lipase production,nitrate reduction, indole production and esculin hydrolysis[13]. Sagua et al [22] used the above to identify the volatile acids produced in peptone-yeast extract-glucose medium. The biochemical tests were performed with tubes of anaerobic differential media. Strains were inoculated in CMM and incubated for 24h at 34 °C, in anaerobic conditions. These cultures were used to inoculate the tubes with the differential media. However in the present study whole work was done inside an anaerobic glove bag.

Biochemical identification

This *Clostridium* strain was further identified biochemically and it confirmed as *Clostridium histolyticum* (Table 1).

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Table. 1. Biochemical Characteristics of *Clostridium histolyticum*.

Biochemical characters	Results
Gram staining	Positive
Spore	Positive
Shape	Rod
Pigment	Negative
Vancomycin	Sensitive
Kanamycin	Sensitive
Co-Trimoxazole	Sensitive
H_2S production	Positive
Nitrate reduction	Negative
Indole test	Negative
Urease production	Negative
Lecithinase	Negative
Lipase	Negative

Evaluation of Antibiotic resistance pattern

Antibiotic sensitivity (Fig 1) of the bacteria was checked for 16 different antibiotics and *Clostridium histolyticum* was found to be resistant to all antibiotics except Erythromycin (Table 2), thus proving it to be a potential pathogen.

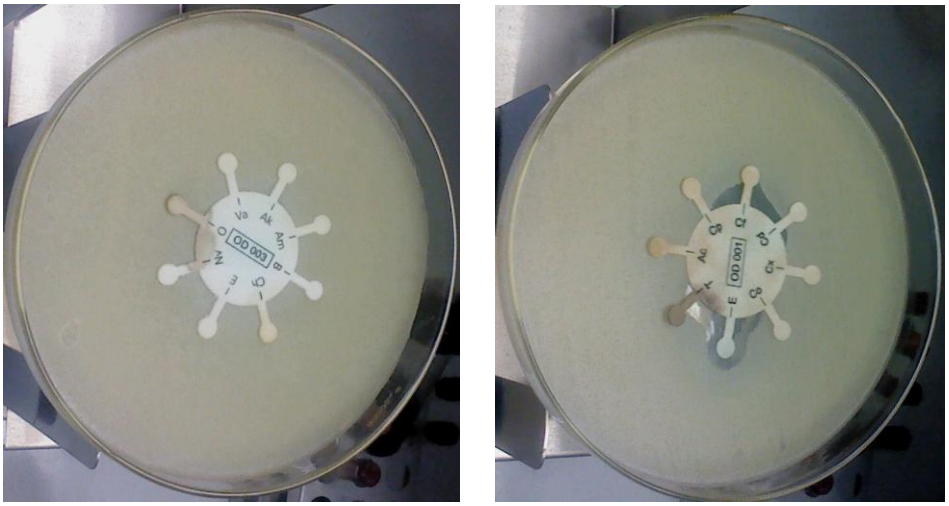

Fig.1. Antibiotic resistance of *Clostridium histolyticum*.

Table.2. Antibiotic resistance of *Clostridium histolyticum*.

Antibiotics	Activity
Amikacin (Ak)	Resistant
Amoxyllin(Am)	Resistant
Bacitracin(B)	Resistant
Cephalothin(Ch)	Resistant
Erythromycin(E)	Sensitive(5mm)
Novobiocin(Nv)	Resistant
Oxytetracycline(O)	Resistant
Vancomycin(Va)	Resistant
Amoxyclav(Ac)	Resistant

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Cephalexin(Cp)	Resistant
Ciprofloxacin(Cf)	Resistant
Clindamycin(Cd)	Resistant
Cloxacillin(Cx)	Resistant
Co-Trimoxazole(Co)	Resistant
Tetracycline (T)	Resistant
Enoxacin(En)	Resistant

In the present study, *Clostridium histolyticum* was found to be resistant against 15 out of the 16 antibiotics tested. Only Erythromycin inhibited the growth of the bacteria with the inhibition zone of 5mm. Sagua *et al*., [22] described that Ampicillin and ampicillin-sulbactam MICs for 46 *C.botulinum* strains tested were between 0.06 and 2 µg/ml, and50% MIC was 0.5 µg/ml. Clindamycin MICs for those strains were between 0.5 and 64 µg/ml, and the 50% MIC was 32 µg/ml. Metronidazole MICs for the 46 strains were between 0.25 and 64 µg/ml, and the 50% MIC was 8 µg/ml.The MICs for *C. botulinum* type A isolated from infant *botulism* cases were compared with MICs for type A strains isolated from other sources. There were no statistically significant differences between the two groups of strains[27]. Compared to these strains the *Clostridium histolyticum* isolated from Vellar estuary was highly resistant and it might have serious implication from public health point of view.

Optimization of culture conditions for *Clostridial histolyticum* toxin production

The maximum toxin production observed in incubaction period (42 hrs), pH (8), Temperature (35°C) and Salinity (1.5 %) (Fig. 2 to 5).

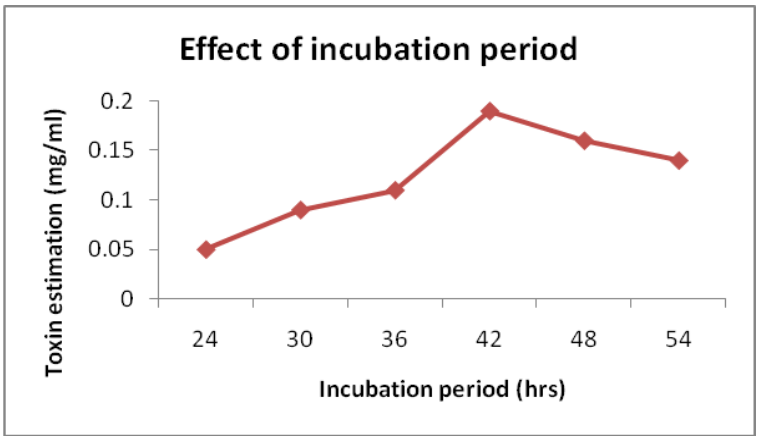

Fig.2. Effect of incubation period on Toxin production

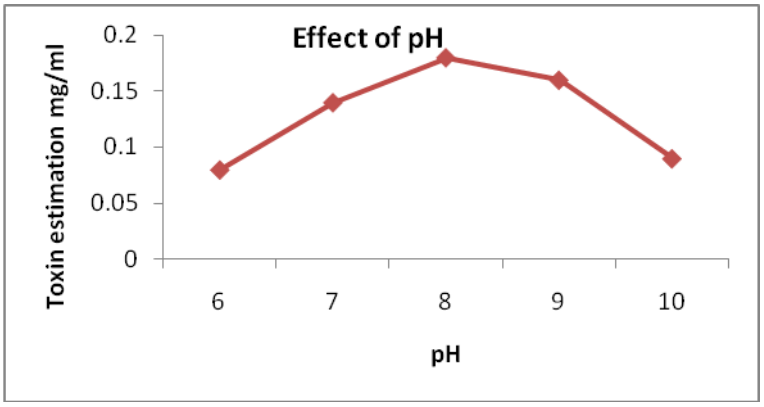

Fig.3. Effect of pH on Toxin production

Scott Moncrief et al [20],further purified toxin A and B of *Clostridium difficle* by acid precipitation in dialysis tubing with acetate buffer at pH 5.6.Other classical methods of protein purification have employed to purify

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the toxins [18]. In general, the methods require careful attention to separation of toxins A and B owing to the similar size of the toxins.

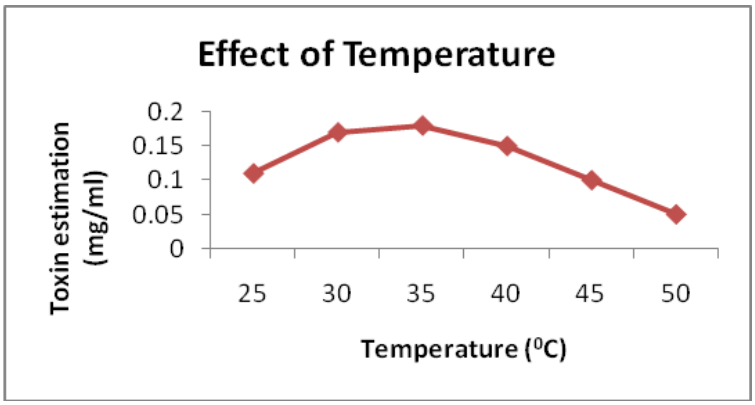

Fig.4. Effect of Temperature on Toxin production

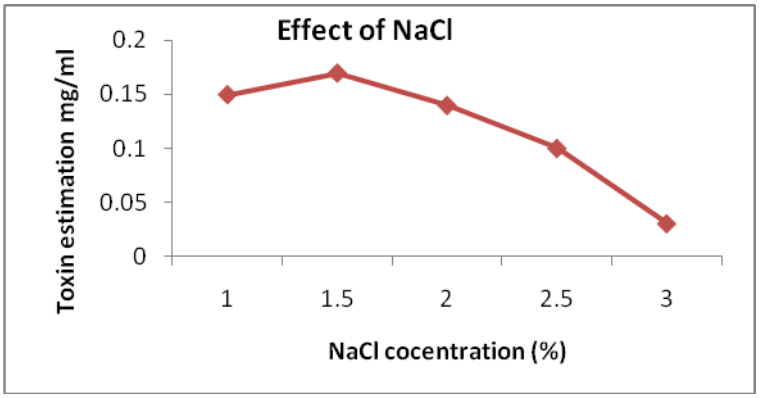

Fig.5. Effect of Salinity on Toxin production

Optimization conditions for maximum toxin production was found to be, 48 hrs of incubation period, pH of 9 , temperature of 40˚C and NaCl concentration of 2.5%, Whereas in contrast, Stewart as said by Harry Bowen[4], did a detailed investigation of the production of toxin by *Clostridium histolyticum* and found that potent toxins could be produced in a peptone meat infusion broth at pH 7.6 at 37˚C with an incubation period of 13-15hrs.

Toxin extraction and partial purification

500ml of *Clostridium histolyticum* culture broth were maintained in optimum condition in an anaerobic glove bag and after 42 hrs, culture was centrifuged at 12,000 rpm at 4˚C and the supernatant was collected in sterile bottles. Ammoniuum sulphate precipitation was done by adding 65g ammonium sulphate in 500 ml of supernatant and the resultant pellet after dialysis and centrifugation was lyophilized to get partially purified toxin powder [23].

Characterization of Toxin produced

Antibacterial activity

The partially purified toxin obtained from *Clostridium histolyticum* was used for testing antibacterial activity. Five bacterial pathogens were used (Table 3 and Fig 6), among which *Proteus vulgaris* (48 mm) and *Klebsiella pneumoniae* (27 mm) showed the maximum zone of inhibition compared to other pathogens.

Table.3. Antibacterial activity of *Clostridium histolytium* toxin

Pathogens	**Diameter for zone of inhibition** (mm)
Proteus vulgaris	48
Staphylococcus aureus	15

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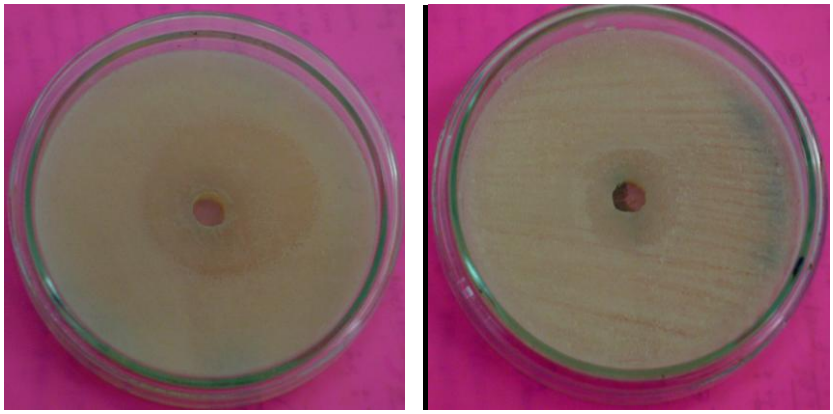
Salmonella typhi	10
Escherischia coli	17
Klebsiella pneumonia	27



(a) (b)

Fig.6. Maximum Zone was formed against (a) *Proteus vulgaris* (b) *Klebsiella pneumoniae*

Hemolytic Assay

The β-hemolysis property of the *Clostridium histolyticum* toxin shown in sheep blood agar medium (Fig 7).

Fig. 7. β-hemolytic activity of *Clostridium histolyticum* toxin.

Sigler as described by Harry E. Bowen [4], confirmed Stewart's findings regarding the production of toxin and hemolysin. On the basis of this similar conditions required for the production of lethal toxin and hemolysin , he suggested that the two activities are due to single substance. It is the purpose of this investigation to examine his suggestion in greater detail using SDS PAGE.

Antioxidant activity

The antioxidant activity was found to be moderate on comparison with the standard.

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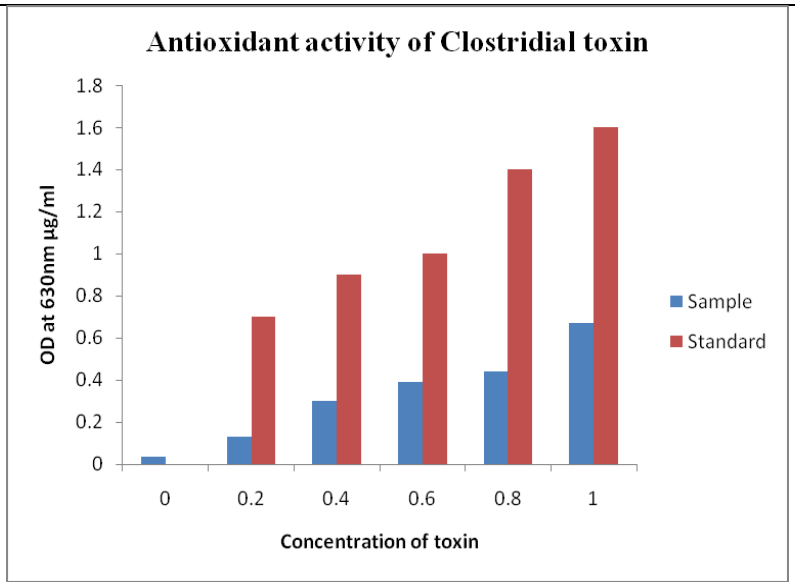

Fig .8. Antioxidant activity of clostridial toxin

Protein Estimation

Protein concentration was estimated at different steps of purification by lowry et al, method and is given in table 4.

Table.4. Protein Concentration of the sample at different steps of purification

Analysis	Protein Concentration (mg/ml)
In supernatant	0.18
After ammonium sulphate	0.28
After dialysis	0.32

SDS PAGE

The SDS PAGE of the *Clostridium histolyticum* toxin showed three different molecular weights (Fig 9). The molecular weights of the toxin produced were 56KDa, 47KDa and 39 KDa.

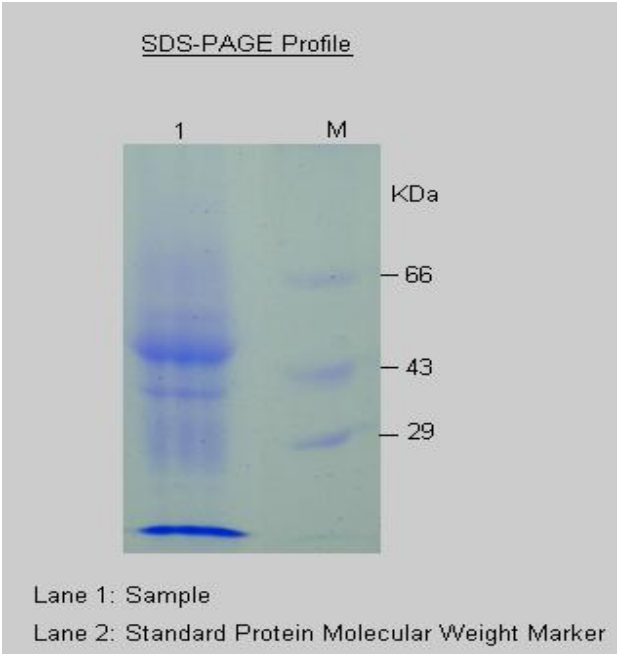

Fig.9.Protein profile of *Clostridium histolyticum* toxin

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SDS PAGE was done to determine the molecular weight of the toxic protein and was obtained as 56Kda, 47KDa and 39KDa. Kamiya et al. [11] described about the possible sub-unit structures of toxin A, and they examined it under reducing and no reducing conditions by SDS-PAGE followed by silver staining. The size of the major band was estimated to be 240 KDa, which is similar to that reported by other investigators [24] and [6] in addition ten minor and 27 faint bands under non-reducing conditions, and four minor and 31 faint bands under reducing conditions, of 38-440 KDa, were detected. However in the present study the molecular weight of the proteins were comparatively low. Further research is needed to confirm whether they are subunits of the same proteins (or) entirely different one.

Acute toxicity

Acute toxicity refers to the immediate effects of exposure to a toxin. Clostridial toxins can be lethal at very low doses. The acute toxicity range of Clostridial toxin was examined using brine shrimp *Artemia salina.* The 50% of *Artemia* deceased in1.5mg/ml of partially purified toxin with two repeated toxicological analysis after 24 hrs of incubation was observed (Fig 10).

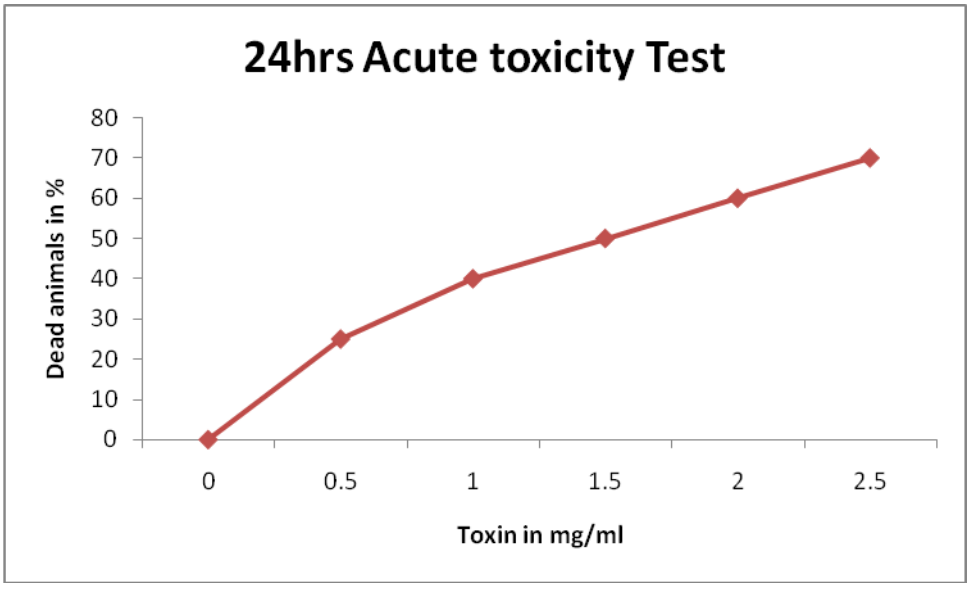

Fig.10. Brine shrimp assay of *Clostridium histolyticum* toxin

The Acute toxicity assay using brine shrimp larvae showed that at a concentration 1.5mg/ml, it could kill 50% of them. On further purification the LC_{50} value may be further reduced. Antioxidant activity indicates the potential of the toxin from *Clostridium histolyticum* to be developed into an anticancer therapeutic.

This preliminary study on *Clostridium histolyticum* which included isolation, identification, antibiotic resistance analysis, separation of toxin, its antibacterial activity and LC_{50} value in brine shrimp assay can be considered as a good piece of work as no information is available on anaerobic bacteria in the study area.

Conclusions

The clostridium histolyticum toxin isolated is of high medicinal importance. Its ability as a tissue lyser is under serious research and has the potential to act as an efficient anaesthetic. Hence this piece of work is a preliminary study showing the presence of the organism in the vellar estuary and its toxic effects. Further animal model studies can be done in order to identify the level of toxicity in tissues and also for the development of a local anesthetic.

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