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Purification, Characterization of Protein from the Ventral Portion of Puffer Fish (Tetraodon patoca Hamilton) and Comparison of Their Characteristics with Tetrodotoxin

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Purification, Characterization of Protein from the Ventral Portion of Puffer Fish (*Tetraodon patoca Hamilton*) and Comparison of Their Characteristics with Tetrodotoxin

M. Phil Thesis

A dissertation submitted to the University of Rajshahi, in partial fulfillment of the requirements for the degree of Master of Philosophy in Biochemistry and Molecular Biology.

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Dedicated

To

My Beloved Heavenly Father

Certificate

This is to certify that the materials included in this thesis are the original research works conducted by **Md. Sohel Hasan.** The thesis contains no materials previously published or written by another person except when due reference is made in the text of the thesis.

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ABSTRACT

A lectin was isolated and purified from the ventral portion of the Potca fish, *Tetraodon patoca*.The method was accomplished by gel filtration of crude protein extract on Sephadex G-50 followed by Ion exchange chromatography on DEAE- cellulose and finally by affinity chromatography on ConA-Sepharose matrix. The molecular weight of the lectin as determined by the gel filtration and SDS-PAGE was about 82,000 and 80,000 respectively, but 42,000 and 38,000 were indicated by SDS-PAGE in the presence of 2-mercaptoethanol. The lectin agglutinated rat red blood cells and in a hapteinhibition test, the lectin was inhibited specifically by the D-mannose and mannose containing saccharides. The lectin is glycoprotein with neutral sugar content of about 0.35%.

Like tetrodotoxin, the purified lectin also showed strong cytotoxic effects, which was confirmed by brine shrimp lethality bioassay and histopathological examinations.

The N- terminal amino acid sequences of both the subunits of the lectin were also identified and used a blast search on N- terminal amino acid sequences of the subunits revealed that the lectin showed significant homology with the homologous proteins in database.

Among the marine toxins, relevant for human intoxication, tetrodotoxin has been known as one of the most prejudicial. Potca fish is originally thought to be the only animal from which tetrodotoxin could be isolated. Five different parts of potca fish were analyzed for their toxicity using brine-shrimp lethality bioassay. Among the parts examined, the ventral portion and egg contained comparatively the highest amount of toxicity.

Tetrodotoxin(TTX) was isolated and purified from the ventral portion including liver by thin layer chromatography (TLC) using the solvent system 1-butanol : acetic acid: water (8:1:1): Two spots with R_f values of 0.76 and 0.65 were showed under UV light and after spraying with 10% potassium hydroxide/ninhydrin reagent The toxicity of both the compound were further reconfirmed by brine-shrimp lethality bioassay.

The TTX and Potca Fish Protein(PFP) have showed no activities against gram positive as well as gram negative bacteria. Subacute toxicity study showed that the TTX and PFP had pronounced effects on the haematological profiles, such as total RBC, total WBC, platelet, % of hemoglobin and ESR.. The other biochemical parameters such as SGPT, SGOT, SALP, serum bilirubin, creatinine and urea were also affected by the toxins. The histopathological examinations showed that the tissues such as liver, lung, kidney and heart of rats were severely affected after treated with both TTX and PFP as compared to those of the control group rats. Again although TTX and PFP showed also similar effects but PFP is slightly less effective than TTX.

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INTRODUCTION

INTRODUCTION

1.1 General Introduction

Lectins, which are sugar binding protein or glycoprotein of non-immune origin, have been identified in various species of all taxa from viruses and bacteria to vertibrate. By virtue of their sugar binding property, they are useful candidates for detection of cell surface carbohydrates (L.G. Yu, J.D. Milton, D.G. Fernig, J.M. Rhodes, 2001), biomedical applications (F. Pryme, 2002 and J. Grant, 2001) and purification of glycoconjugates(K.Yamamoto ,T. Tsuji, O. Tarutami, T. Oswa, 1984). It was known that potca fish stores higher concentrations of Tetrodotoxin(TTX) in various organ, which causes very much intoxication for human. TTX is one of the best-known notorious marine toxins that occasionally cause human intoxication, including fatality. Poisoning from TTX has been almost exclusively associated with the ingestion of toxic patca, especially from the waters of Indo-Pacific Ocean region (Nakamura, 1985).

Lectins were also isolated from fish, which showed cytotoxic activity as well as other biological properties. Potca fish is a nutririch food because it contains a higher amount of protein (24%) and minerals such as Ca, Fe, P etc. Our interest focuses on the nature of the protein those present in the puffer fish which could provide information whether the protein have any activities similar to the tetrodotoxin (TTX).

Bangladesh is a land of river and is rich in marine resources. It is generally accepted that tens of thousands species of fish and shell fish inhabit the sea. Many of them are used as an important food source for us. However, atleast several hundred species are regarded as toxic and many causes human poisoning, when ingested beyond a safety level. Some example of marine toxins are paralytic shellfish toxin, diarretic shellfish poison, ciguateric toxin as well as tetrodotoxin will be the subject of this review.

In potca fish, two large plates in each jaw with interior suture and forming a powerful beak. By means of a cavity connected with the pharynx and provided with a valve the fish is able to inflate itself enormously like a balloon. There are three local species under two genera, such as :

a. *Tetraodon cutcutia* Hamilton *(Tetraodon patoca)*
b. *Chelonodon fluviatilis* (Hamilton)
c. *Chelonodon patoca* (Hamilton)

Among the marine toxins, relevant for human intoxication, tetrodotoxin (TTX) has been known as one of the most prejudicial. Potca fish (*Tetraodon patoca*) is originally thought to be the only source from which TTX could be isolated. It is widely known as the blowfish or the puffer fish, because they can swell up their bellies until they resemble a ball. It is also used more narrowly as the name of the genus fugu of the family tetraodontidae living only in water surrounding Japan.

The people can eat this fish as a source of protein, but as it contains serious toxins, the people can not have potca fish as usually as a good source of protein.

Potca fish store high concentration of toxins in various organs. TTX is a violent neurotoxin present in potca fish. The liver, gonads, intestine, egg, and skin of ventral portion mainly contain PFT (puffer fish toxin), a powerful neurotoxin that can cause death who ingest it. Every year many people died in the world including Bangladesh by the puffer fish poisoning. In 1963, eight people died in Japan by this poisoning. In our country, six people of a family died by potca fish poisoning at Potuakhali in 2001. On 16 november,1998, eight people died among 15 victims in Khulna [3]. The people eat potca fish and affect by it's toxins due to lack of knowledge. Three of ten fatalities, occured between 1950 and 1990 in USA [7] and four in Hawaii between 1903 and 1925 [8, 9]. In Japan , from 1974 to 1983, there were 646 reported cases of puffer fish poisoning , with 179 fatalities .

1.2 Sign and symptoms of puffer fish toxicity

TTX, one of the most potent molecule is known to selectively block off the voltage-sensitive sodium channels of excitable tissues and neural transmission in skeletal muscle0 (Agnew et. al. 1984 and Catterall et al. 1980). Tetrodotoxin is complex in structure by small molecule standards and contain guanidium moiety. The guanidium ion is able to enter the cells via the voltage sensitive Na^+ channel in human. Paresthesias begin 10-45 minutes after ingestion, usually as tingling of the tongue and inner surface of the mouth. Other common symptoms include vomiting, light headedness, dizziness, felling of doom and weakness. An ascending paralysis develops and death can occur within 6-24 hours. Secondary to respiratory muscle paralysis. Other manifestations include salvation, muscle twitching, diaphoresis, pleuritic chest-pain, sysphagia, aphonia and convulsions. Severe poisoning is indicated by hypertension, bradycardia, depressed corneal reflexes and fixed dilated pupils.

1.3 Potca fish resistant to tetrodotoxin

The potca fish resistance to TTX because it has a mutation in the protein sequence of the sodium channel pump found on the cell membranes. This sodium channel is critical for cellular signalling pathways i.e transmission of impulse and the mediation of many cell function. This point mutation in the amino acid sequence compared to the sequence in man makes this fish highly resistant to TTX poisoning, as a result, TTX does not recognise the channel in potca fish and therefore does not bind to it and block it.

1.4 Systematic position of potca fish

Kingdom : Animalia

Phylum : Chordata

Sub-phylum : Vertibrate

Class : Osteichthyes

Order : Tetraodontiformes

Family : Tetraodontidae

Genus : Tetraodon

Species : Patoca

Full Sc. name: *Tetraodon patoca*

Local name : Potca, Fotca, Tepa, Cutcutia

Japanies name : Fugu / puffer fish

Figure-1: Photograph of different varieties of Potca Fish (Top view) :
A- *Chelonodon fluviatilis* (Hamilton)
B- *Chelonodon patoca* (Hamilton)
C- *Tetraodon patoca*

Figure-2: Photograph of *Tetraodon patoca* (potca fish ,side view)

1.5 Physical characteristics of potca fish

Broad head and back tapering abruptly to tail. Mouth opening a little inferior with two large teeth on each jaw. Gill-openings very reduced and restricted in front of pectoral base. Each nostril forms single orifice situated at end of a very simple short tube. Nostril nearer to angle of mouth than to anterior margin of eye. Eyes large, situated slightly behind middle of head. Interorbital flat and broad.

The length of fishes including it's head are as follows: Head 3.2-3.5 cm in total length, height 2.5-3.5 cm standard, the total length of fish is 11-13.2 cm and Interorbital 3.0-4.0 cm.

Two lateral lines, the upper not reaching end of tail but meeting the lower above anal. Lower lateral line widely interrupted at middle. Spines entirely absent.

Pelvics absent, dorsal placed well back above origin of anal. Distance between dorsal origin and anal base equals half the distance between dorsal origin and posterior edge of mouth. All fins are rounded.

Greenish yellow above, white in the abdomen, light band between eyes. A large black ocellus surrounded by a light edge, on the side anterior to the origin of anal fin. Whole of back marked with dark greenish reticulations enclosing lighter spaces.

1.6 Habitat and distribution of potca fish

Potca fish is mainly found in Indo-pacific ocean. In Bangladesh, it is mainly found in the region of Sundarban sea water and in the Bay-of Bengal. Some times potca fish comes to the many chanels and river connecting to the Bay-of-Bengal. It is also found in Japan, Australia, Philippine,Thailand and north of America. The Japanese has been successfully produced artificial cultivation of

fugu. Fisherman caught fugu in spring as it is the spawning season. They consume about 20,000 tons of blowfish per year of which 6,800 tons are imported [11].

1.7 Aim of the present study

Potca fish stores serious toxic compounds in various organs, which cause very much intoxication for human.The present study has been undertaken in order to obtain detailed information about the toxic compounds as well as protein present in the puffer fish .Firstly, based on the reports available, we select the mostly toxic type of potca fish i.e *Tetraodon patoca* in the study. The experimental designs were as follows:

(1) A protein was isolated, purified and characterized from the ventral portion of the fish.

(2) The purified protein was sequenced by amino acid sequencer and the Homology Allignment was determined

(3) A comparison between the tetrodotoxin(TTX) and purified Potca fish protein (PFP) was performed.

CHAPTER-2

CHEMICALS AND EQUIPMENTS

CHEMICALS AND EQUIPMENTS

2.1 Chemicals

The chemicals used in this study are mentioned below with their manufactures:

Acetone

BDH Chemicals Ltd., Poole England.

Acrylamide

Sigma Chemicals co., U.S.A.

Bovine Serum Albumin (BSA)

Sigma Chemicals co., U.S.A.

Ammonium persulfate

Bio-Rad Laboratories, Richmond, U.S.A.

Ammonium sulfate

Merck, Germany.

Silica gel (TLC grade) Merck, Germany

Silica gel (TLC grade) Fluca, Switzerland

Borate (Natrium tetraborate)

BDH Chemicals Ltd., Poole England.

Bromophenol blue

Bio-Rad Laboratories, Richmond, U.S.A.

1-butanol

BDH Chemicals Ltd., Poole England.

β-D-Galactosidase

BDH Chemicals Ltd., Poole England.

Egg albumin

BDH Chemicals Ltd., Poole England.

Lysozyme

BDH Chemicals Ltd., Poole England.

Carboxymethyl Cellulose

BDH Chemicals Ltd., Poole England.

Copper sulfate

BDH Chemicals Ltd., Poole England.

Coomassie brilliant blue

Bio-Rad Laboratories, Richmond, U.S.A.

DEAE cellulose

Fluka Bio Chemika, Switzerland.

Con-A Sepharose

Amersham Pharmacia Biotech, Upsala, Sweden.

Diethyl ether

Sigma Chemicals co., U.S.A.

Ethyl alcohol

BDH Chemicals Ltd., Poole England.

Glucose

Sigma Chemicals co., U.S.A.

Glycerol

Bio-Rad Laboratories, Richmond, U.S.A.

Hydrochloric acid

BDH Chemicals Ltd., Poole England.

Glycine

Bio-Rad Laboratories, Richmond, U.S.A.

N, N- Methylene-bis-Acrylamide

Sigma Chemicals co., U.S.A.

Orthophosphoric Acid

BDH Chemicals Ltd., Poole England.

Petroleum ether (40-60° C)

BDH Chemicals Ltd., Poole England.

Phenol

Aldrich Chemicals Company Inc.,U.S.A.

Potassium Sodium Tartrate

BDH Chemicals Ltd., Poole England.

Phthalic Acid

BDH Chemicals Ltd., Poole England.

Riboflavin

BDH Chemicals Ltd., Poole England.

Ribose

Sigma Chemicals co., U.S.A.

Sephadex G-50

Sigma Chemicals co., U.S.A.

Sephadex G-150

Sigma Chemicals co., U.S.A.

Trypsin inhibitor

Sigma Chemicals co., U.S.A.

Thiamin

BDH Chemicals Ltd., Poole England.

Sodium Dihydrogen orthophosphate

BDH Chemicals Ltd., Poole England.

Sodium chloride

Merck, India Ltd.

Sodium hydroxide

Merck, Germany.

Sodium Azide

Sigma Chemicals co., U.S.A.

Sodium carbonate

Hopkin & Williams, Essex, England.

Sulfuric acid

BDH Chemicals Ltd., Poole England.

TEMED (N, N, N′, N′-tetraethylene diamine)

Sigma Chemicals co., U.S.A.

Trichloro acetic acid (TCA)

BDH Chemicals Ltd., Poole England.

Methanol

BDH Chemicals Ltd., Poole England.

n-hexane

Merck, Germany.

Chloroform

Merck Germany.

Ethyl acetate

BDH Chemicals Ltd., Poole England.

Potassium hydroxide

BDH Chemicals Ltd., Poole England.

Ninhydrin

BDH Chemicals Ltd., Poole England.

Tris

BDH Chemicals Ltd., Poole England.

2.2 Equipments

The important equipments used throughout this study are listed below:

1. Centrifuge (refrigerate) Model-H95.
2. SDS-PAGE electrophoresis apparatus-SJ 1060.
3. Electric balance-Metter H18.
4. Electrophoresis power supply.
5. pH-Meter(Corning) 215.
6. Volac pipette controller-model 958241.U.S.A.
7. Temperature controlled Water Bath.

8. Cold Chamber –Pioneer.
9. Homogenizer-Model Am-5.
10. Incubator-Gallenkamp, England.
11. Double beam Spectrophotometer UV-280.
12. Fraction collector-SF-160 (Advantec, Japan).
13. Rota-diffuser (Local Made).
14. Stirrer/Hot plate MR-2000.
15. Laboratory glass wares, "pyrex"/Jena/Duran Brand.
16. Muffle Furnace.
17. Micro kjeldahl apparatus, Gallenkamp. England.
18. Spectronic 21, Milton Ray Company, England.
19. Separating funnel, Merck England
20. TLC chamber, Osaka, Japan.
21. Aqua pump.
22. Freeze dryer, Taitec corporation, Japan.
23. Amino Acid Sequencer, Shimadzu Company, Japan.
24. Higher Magnification Microscope with photographic camera.
25. UV light short range and long range, England.
26. Iodine chamber, Osaka, Japan.
27. Desiccator, Osaka, Japan.
28. Freezer (Ultra low), Sanyo, Japan.

CHAPTER-3

PURIFICATION AND CHARACTERIZATION OF PROTEIN FROM POTCA FISH

Purification and characterization of Potca Fish Protein

Introduction

Potca fish is a good source of protein which is found largely in the river connected with the Indo-Paccific occean. Among the five parts of Potca fish examined in the present study, the ventral portion contained the highest amount of protein (23.95g %). This chapter describes the purification and characterization of a glycoprotein from ventral portion of potca fish.

3.1 Materials and methods

3.1.1 Preparation of Acetone Powder

The ventral portion of potca fish (200 g) is homogenized well with twice the volume of ice cold acetone. The suspension is filtered through double layer of muslin cloth and quickly washed with successive portion of acetone, acetone-ether (1:1), ether and then air dried. The dry powder is called the acetone powder, which can be stored in a refrigerator for a long time (J. Jayaraman 1981).

3.1.2 Preparation of Crude Extract

Immediately before use, the dry acetone powder (200 g) was suspended in 1000 ml of ice cold water. After occasional gentle stirring for 3 hours at 4°C, the suspension was filtered through double layer of muslin cloth. The filtrate was collected and centrifuged at 5000g for 20 minutes at 4°C. The supernatant was collected and saturated to 85% by addition of solid $(NH_4)_2SO_4$ with gentle stirring at 4°C. The resulting precipitate was collected by centrifugation, dissolved in minimum volume of distilled water and dialyzed against distilled water for 24 hours at 4°C. The dialyzed solution was then centrifuged at 8000 g for 5 minutes to remove the insoluble materials (if any) and the clear supernatant obtained was designated as "crude protein solution."

3.2 Purification of protein

3.2.1 DEAE-cellulose chromatography

Procedure:

i) **Activation of DEAE-cellulose powder:** The DEAE-cellulose powder was suspended in 0.2M HCl in a beaker and left to swell for a few hours. During swelling it was stirred at short intervals to prevent formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water for several times until its pH reached near to neutrality. The gel suspension was then transferred to another beaker containing 0.2M NaOH and left for a few hours with slow stirring. It was again washed with distilled water to neutralized its pH.

Packing of the column:

A column of desired length was packed in a proper way. If the column is not packed properly, accurate results can never be expected, because a poorly packed column gives rise to uneven flow rates and the resolution is also lost.

The activated DEAE-cellulose suspension was taken in a filtering flask and deaerated by vacuum pump, otherwise it would affect the flow rate of the column after packing. The gel suspension was adjusted, so that it was fairly thick slurry, but not thick enouh to trap bubbles. The column was mounted on a laboratory stand and its narrow end was fitted with an outlet tube. It was ensured that there was no bubble in the dead space of bed support. This was easily achieved by filling approximately ¼ th of the column, including the outlet tube with distilled water. When dead space was properly filled, the outlet tube was closed with pinch cork and the gel suspension from a gel reservoir was added gently to the column. In order to avoid trapping of any bubble, the gel suspension was poured to the inner wall of the column. In this way, a column of desired length was packed uniformly with the gel suspension.

ii) **Equilibration of the column:** After completion of the column packing, it was equilibrated with 10 mM Tris-HCl buffer, pH 8.4.

iii) **Preparation and application of sample:** The crude protein solution was dialyzed against 10 mM Tris-HCl buffer, pH 8.4 for 24 hours and the dialyzed sample was loaded on to the DEAE-cellulose column at 4°C. The proteins were eluted from the column with the same buffer containing NaCl by gradient and stepwise elution.

3.2.2 Gel filtration

Procedure:

i) **Activation of the gel powder:** Sephadex G-50 powder was suspended in 10% acetic acid containing 1M sodium chloride (1 mole of NaCl was dissolved in one litre of 10% acetic acid) in a beaker and left it to swell for overnight. Then it was transferred to a filtering funnel and washed with distilled water for several times until its pH reached near to neutrality.

ii) **Packing of the column:** This is very critical step in all types of column chromatographic experiment. The column was packed following the procedure as described earlier.

iii) **Equilibration of the column:** After completion of the column packing it was equilibrated with eluent buffer (10 mM Tris-HCl buffer, pH 8.4). The buffer was continued to run through the column until the pH of the eluate became same of the eluant buffer.

iv) **Application of sample:** Before loading of the sample(F-1 fraction obtained from DEAE- cellulose chromatography), the outlet tube of the column was opened and the eluent buffer from the top of the gel bed was allowed to diffuse into the gel. The protein fraction was loaded on the top of the bed. After diffusion of the sample, about 1 ml of eluant

buffer was poured on the top of the gel bed and was allowed to diffuse. Then an additional amount of buffer was added, so that the space about 3-4 cm above the gel bed was filled with eluant. The buffer was then allowed to flow continuously through the column and 3 ml fractions of the eluate were collected by an automatic fraction collector. Absorbance at 280 nm and protein conc. by Lowry method of each fraction was measured.

3.3 Affinity Chromatography

The active fraction of protein obtained from Gel Filtration was applied to ConA-Sepharose column, which was previously equilibrated with the buffer, 20mM Tris-HCl containing 0.5M NaCl, pH-7.6 at 4°C. After washing the column with the buffer the protein absorbed on the column was eluted by the buffer containing 0.1M α-D-mannopyranoside.

The purity of the active protein fraction was determined at room temperature using Native-PAGE (7.5% gel) according to the method of Davis (1964).

3.4 Test of purity

3.4.1 SDS-PAGE slab gel electrophoresis

Principle:

Sodium dodecyl sulfate(SDS) polyacrylamide gel electrophoresis method is commonly used for checking the purity of proteins as well as their molecular weight determination.SDS is an anionic detergent, which binds to most proteins in amounts roughly proportional to molecular weight of protein, about one molecule of SDS for every two molecules of amino acid residues. The bound SDS contributes large negative charge, rendering the intrinsic charge of protein insignificant. In addition, native conformation of the protein is altered when

SDS is bound and most protein assumes similar shape and thus similar ratio change to mass. Slab gel electrophoresis in the presence of SDS therefore separates proteins almost exclusively on the basis of mass, with smaller polypeptides migrating more rapidly. Protein-SDS complexes will therefore all move towards the anode during electrophoresis and their movement is inversely proportional to the log of their molecular weights. If standard proteins of known molecular weights are also run, the molecular weights of the sample proteins can be determined by comparing them with proteins of known molecular weights. The protein pattern of the selected fractions was determined by 10% SDS-PAGE according to the method of Laemmli (1970) as modified by Smith (1995).

A) Reagents and solutions:

i) Preparation of 30% acrylamide solution:

33.3 g of acrylamide and 0.9 g N, N-methylene-bis-acrylamide were dissolved in 70 ml of distilled water in a 100 ml of volumetric flask by heating in a hot waterbath and the final volume was made upto the mark by adding distilled water. The solution was filtered and stored in a dark bottle at room temperature.

ii) Preparation of 1.5M Tris-HCl buffer (pH 8.8):

18.7 g of Tris base was dissolved in 90 ml of distilled water in a conical flask and mixed well. The pH of the solution was adjusted to 8.8 by adding concentrated HCl. The final volume was made upto 100 ml with distilled water.

iii) Preparation of 0.5M Tris-HCl buffer (pH 6.8):

6 g of Tris base was dissolved in 90 ml of distilled water in a conical flask and mixed well. The pH of the solution was adjusted to 6.8 by adding concentrated HCl. The final volume was made upto 100 ml with distilled water.

iv) Preparation of 10% SDS (Sodium dodecyl sulphate) solution:

10% SDS solution was prepared by dissolving 5 g of SDS in 40 ml distilled water in a 50 ml volumetric flask. After dissolving, the final volume was made upto 50 ml with distilled water.

v) Preparation of 10% ammonium per sulphate (APS) solution:

10% APS solution was prepared by dissolving 0.5 g APS in 4 ml of distilled water. The final volume was made upto 5 ml with distilled water. The solution was stored in eppendorf tubes (500 µl in each tube) at 20°C.

vi) TEMED:

The commercially available preparation from Sigma Chemicals Co., U.S.A. was used without modification.

vii) Preparation of 4% SDS solution:

4% SDS solution was prepared by dissolving 2 g of SDS in 40 ml distilled water in a 50 ml volumetric flask. After dissolving, the final volume was made upto 50 ml with distilled water.

viii) Preparation of Bromophenol Blue (BPB) solution:

Bromophenol Blue solution was prepared by mixing the components as given below and was stored at 4°C.

Components	Amounts
Bromophenol Blue	10 g
Glycerol	2 ml
0.5M Tris-HCl buffer, pH 6.8	0.2 ml
Distilled Water	10 ml

ix) Preparation of sample buffer:

The sample buffer was prepared by mixing the following components and was stored at 4°C.

Components	Amount
4% SDS	13 ml
Glycerol	5 ml
0.5M Tris-HCl buffer, pH 6.8	7 ml

x) Preparation of Coomassie brilliant blue (CBB) staining solution:

It was prepared by mixing the following components.

Components	Amount
CBB R 250	1 g
Glacial acetic acid	15 ml
Methanol/ Ethanol	100 ml
Distilled Water	85 ml

xi) Preparation of CBB destaining solution-1:

The CBB destaining solution-1 was prepared by mixing the components as given below.

Components	Amount
Staining solution	10 ml
Glacial acetic acid	10 ml
Methanol/ Ethanol	10 ml
Distilled water	10 ml

xii) Preparation of destaining solution-2:

Destaining solution-2 was prepared by mixing the following components.

Components	Amount
Glacial acetic acid	45 ml
Methanol/ Ethanol	30 ml
Distilled water	125 ml

xiii) Preparation of electrophoretic buffer (Chamber buffer):

Electrophoretic buffer was prepared by the following components.

Components	Amount
Tris base	9.09 g
Glycine	43.2 g
SDS	3 g

These components were dissolved in distilled water and the final volume was made upto 3 liters with distilled water.

xiv) Preparation of sample:

1 ml aliquot of the protein sample was mixed with sample buffer (1:1, v/v) in an eppendorf tube and heated for 2-3 minutes at 100°C. The sample was then used for SDS-PAGE.

B) Procedure for SDS-PAGE:

Clean and dry plates (7 cm x 10 cm) were assembled with a spacer (1.5 cm thick) and were hold together on a gel casting stand. The assembly was checked for leakage.

(i) Preparation of separating or running gel for slab gel- electrophoresis:

The following solutions were taken in a conical flask and the flask was swirled gently to mix. To avoid instantaneous polymerization, the flask containing the solution was kept in an ice bath. The solution was used immediately.

Components	Amount
30% acrylamide solution	6.5 ml
1.5M Tris-HCl buffer, pH 8.8	4.05 ml
Deionized water	2.50 ml
10% SDS solution	75 µl
TEMED	6.25 µl
10% APS solution	75 µl

(ii) The separating gel solution was applied to the sandwich.

(iii) The top of the gel was covered slowly with a layer of water. It was then allowed to polymerize the gel solution for about one hour at room temperature.

(iv) The layer of water was poured.

v) Preparation of stacking gel:

The following solutions were taken in a conical flask and the flask was swirled gently to mix. To avoid instantaneous polymerization, the flask containing the solution was kept in an ice bath. The solution was used immediately.

Compound	**Amount**
30% acrylamide solution	450 µl
0.5M Tris-HCl buffer, pH 6.8	375 µl
Deionized water	2.11 ml
10% SDS solution	30 µl
TEMED	5 µl
10% APS solution	30 µl

(vi) The stacking gel was poured on the separating gel. Then the Teflon comb was inserted immediately into the layer of the stacking gel solution. Additional stacking gel was added to fill completely the space in the comb. It was taken not to trap air bubbles. The gel solution was allowed to polymerize for about 30 minutes.

(vii) The Teflon comb was carefully removed without tearing the edges of the polyacrylamide wells. After the comb was removed, the wells were rinsed with electrophoresis buffer to remove unpolymerized monomer. The gel wells were filled with electrophoresis buffer.

(viii) The gel sandwich was then attached to upper buffer chamber and filled lower buffer chamber with the recommended amount of electrophoresis buffer. The upper buffer chamber was partially filled with the electrophoresis buffer so that the top of the gel sandwich was sunk into the electrophoresis buffer.

Electrophoresis was carried out by applying electric power supply at a current of 30 mA. The power supply was disconnected when BPB dye was reached at the mark point on bottom of the gel.

(ix) Recovery of the gel:

The gel sandwich was removed from the upper buffer chamber, and laid the sandwich on a sheet of absorbent paper or paper towels. Slide one was removed carefully. Then the gel was removed from the lower plate.

(x) Staining of the gel:

After recovery, the gel was stained with staining solution for two hours at room temperature.

(xi) Destaining of the gel

After two hours, the gel was removed from the staining solution and destainiung was done by soaking the gel in distaining solution. When the gel became transparent, it was taken out and rinsed with water.

3.5 Characterization of protein

3.5.1 Molecular weight determination

3.5.1.1 Molecular weight determination by gel filtration

The molecular weight of purified protein was determined by gel filtration following the procedure as described by Andrews.[39]

Procedure:

i) **Packing of the column:** A column of desired length was packed with Sephadex G-75 gel suspension following the procedure as described before.

ii) **Equilibration of the column:** After preparation of the column it was equilibrated with the eluant buffer, 10 mM Tris-HCl, pH 8.2. The buffer was continued to run through the column until the pH of the eluate became same as the pH of the eluant buffer.

iii) **Application of sample:** The standard proteins and the unknown protein were applied to the column. During gel filtration identical conditions were maintained in each time. The buffer was finally allowed to flow continuously through the column at a flow rate of 15 ml/hour and 3 ml fractions of the eluate were collected by an automatic fraction collector. Absorbance of each fraction was measured at 280 nm. The molecular weight of the protein was determined from a standard curve, which was constructed by plotting the elution volume against log of molecular weight of standard proteins.

3.5.1.2 Determination of molecular weight by Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) method

Reagents and solutions: Same as described before.

Preparation of marker protein: Marker protein was prepared by mixing, lysozyme (14 Kd), β-Dgalactosidase(116 Kd), Egg albumin (45 kd) and BSA

(67Kd) with equal volume of buffer solution. The mixture was heated for 2 minutes and then added 1 or 2 drops of CBB.

Methods:

20 μM of known protein mixture and sample protein were carefully applied at the bottom of the different wells. The remainder of the upper buffer chamber was filled with electrophoresis buffer.

3.5.1.3Determination of the sub-unit of the protein by SDS polyacryl amide slab gel electroforesis.

Reagents and solutions: Same as described before.

Preparation of sample:

The protein sample is prepared by mixing, 100 μL of protein with 100 μL of buffer solution and 20 μL of β-mercaptoethanol. After heating at 100°C for 5 min., one drop of CBB is added in the mixure.

Procedure: Same as described before .

3.6 Determination of optical density (O.D. at 280 nm) Vs. protein concentration by the Lowry method.

3.6.1 Lowry method

Protein concentration was determined following the method of Lowry *et. al*[41] using BSA as the standard.

Reagent:

a) 2% Na_2CO_3 solution in 0.1N NaOH

b) 0.5% copper sulphate in 1% Sodium potassium tartrate

c) Folin-Ciocalteau's reagent

d) Standard protein solution: 10 mg/ 100 ml in distilled water

Procedure:

Reagents-a and b were mixed in the ratio of 50:1 and reagent-c was diluted with twice the volume of water.

For the construction of standard curve 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 ml of the standard protein solution were taken into different test tubes and made upto 1 ml with distilled water.

The protein solution (1 ml) was also taken in duplicate in different test tubes and 5 ml of a:b mixture was added to each of the test tube. After kept for 10 minutes, 0.5 ml of FCR was added to each test tube, mixed well and kept for another 30 minutes. Then spectrophotometric reading at 650 nm was recorded. A graph was constructed by plotting concentration against absorbance (O.D.) and from the graph the concentration of protein was calculated.

3.6.2 By Drying Process

A. Materials:

i) Miniature test tubes
ii) Oven
iii) Electric balance
iv) Desiccator
v) Protein solution

B. Procedure:

The absorbance (O.D.) of purified protein solution was measured at 280 nm and 1 ml was taken in a small previously weighed test tube. The protein solution in the test tube was dried by heating at 100°C under vacuum. When the protein solution has evaporated, the test tubes were placed in a desiccator and allowed to cool. After cooling the test tubes along with dried proteins were weighed again.

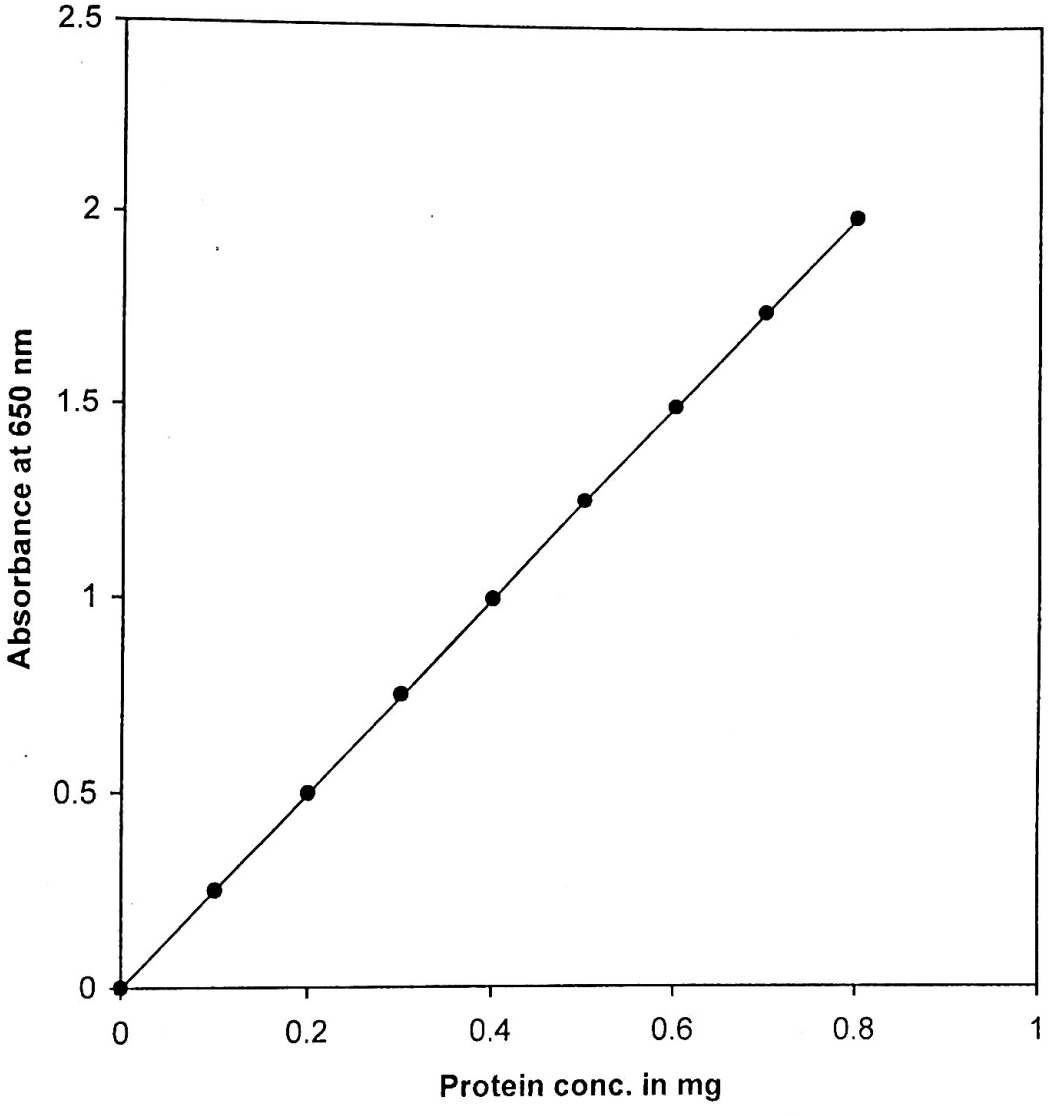


Fig. 3: Standard curve for determination of protein concentration by Lowry method.

3.7 Hemagglutination Studies

A . Materials:

(i) Phosphate buffer saline (PBS), pH-7.2.

(ii) 2% Rat red blood cells (RBC) in PBS

(iii) Protein solution.

B. Procedure:

Just before experiment, blood from albino rat was collected in centrifuge tube containing sufficient amount of pre-cooled 5 mM phosphate buffer saline pH 7.2. The blood sample was immediately centrifuged at $3X10^3$ g for 3 minutes. The supernatant was discarded and the cells were washed similarly for three times with the above buffer. Finaly a 2% suspension (W/V) of RBC was prepared and the hemagglutination was performed in siliconized test tubes (0.5 X 4 cm) as follows: 0.2 ml of 2% RBC were mixed with 0.2 ml of protein solution in PBS and mixed well by gentle stirring. The mixture was incubated at 30°C for an hour. A control containing 0.2 ml of PBS, pH 7.2, instead of protein solution and 0.2 ml of cell suspension were used as reference. After 1 hour incubation, the sedimented erythrocytes were gently mixed with the supernatant and one drop of this suspension was examined under microscope. Results were recorded as 3^+, 2^+, 1^+ and ± as reported by Read, W. P. (1981).

The agglutinating activity was expressed as the titre, the reciprocal of greatest dilution at which visible agglutination could be detected. The specific activity was expressed as titre/mg of protein.

3.8 Test for glycoprotein and Estimation of Sugar

3.8.1 Phenol-Sulfuric acid

Phenol in the presence of sulfuric acid can be used for quantitative colorimetric micro determination of sugars and their methyl derivatives, oligosaccharide and polysaccharides as described by Dubois *et al.* (1956). This method was also employed for detecting the presence of sugar in protein.

A. Materials:

(i) 5% phenol (in water)

(ii) Concentrated sulfuric acid

(iii) Protein solution

B. Procedure :

The protein solution (0.1 ml) was taken in a test tube and made upto 2 ml by distilled water. Then 1 ml of 5% phenol was added to it and finally 5 ml of conc. H_2SO_4 was added rapidly. To obtain good mixing the stream of acid being directed against the liquid surface rather than against the side of the test tube. The tube was allowed to stand for 10 minutes. Then it was shaken and kept in the dark at 25°C to 30°C for 20 minutes. It was taken out and the absorbance of the solution was measured at 490 nm.

C. Preparation of standard curve:

A standard glucose solution containing 0.1mg/ml was prepared. Then 0.0, 0.1, 0.2, 0.3, 0.4, 0.6 and 0.8 ml of this solution containing 0.0, 0.01, 0.02, 0.03, 0.04,0.06 and 0.08 mg of glucose, respectively were taken in different test tubes and made upto 2 ml with distilled water. The solution was treated similarly as described above. A standard graph of glucose was constructed by plotting concentration of glucose against their absorbance. From the graph, the concentration of sugar in protein was calculated.

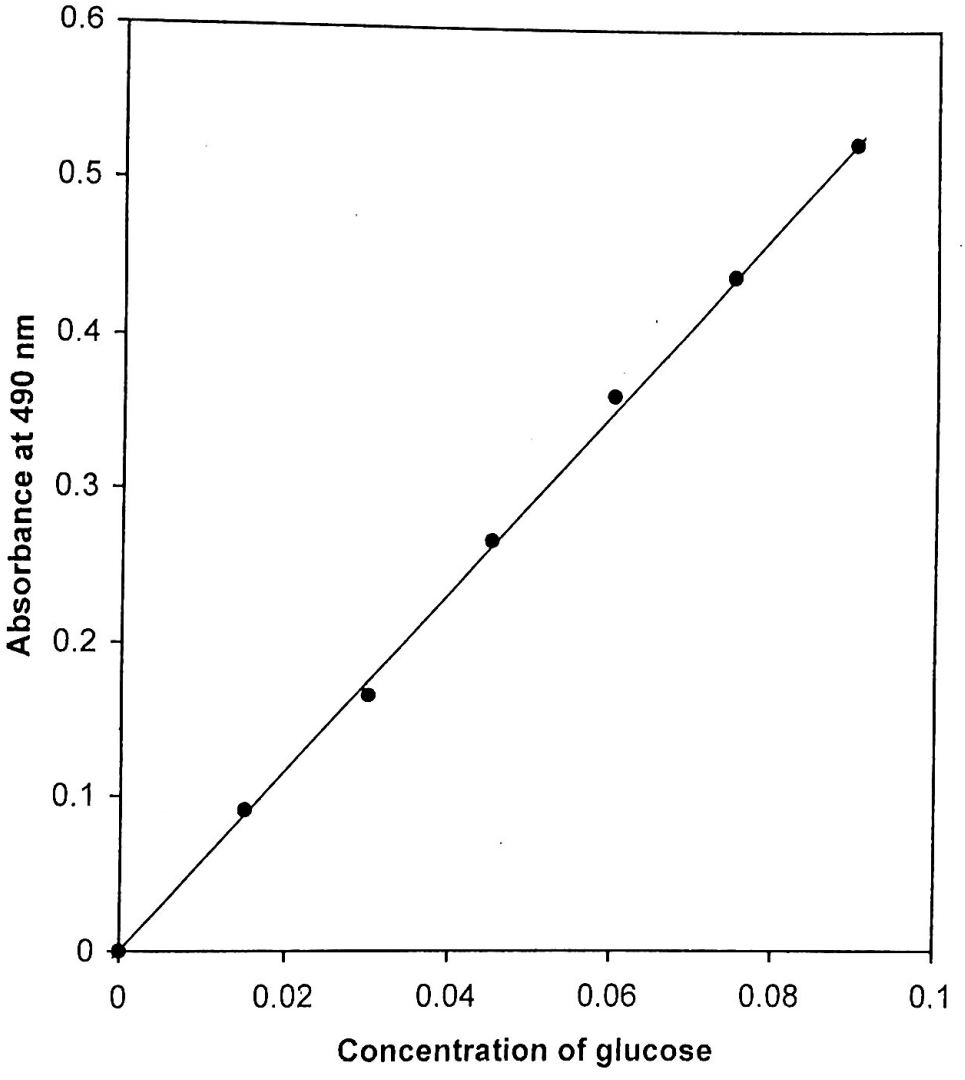


Fig. 4: Standard curve of glucose for estimation of sugar present in glycoprotein.

3.9 Sugar specificity/Hemagglutination inhibition

A. Materials:

(a) 5 mM phosphate buffer saline(PBS), P^{H} – 7.2

(b) Sugar solutions of D-glucose, D- Mannose. D-Galactose, N-Acetyl D-glucosamine, Methyl- α-D- galactopyranoside, Methyl-β -D-galactopyranoside, N-Acetyl D- galactocosamine, Methyl- α-D-manopyranoside and D-glucosamine-HCl

B. Procedure:

The hemagglutination – inhibition test was performed in the presence of different sugars as described. Protein solution (0.1 ml) containing minimum concentration of protein needed for visible agglutination were added to 0.1 ml of sugar solutions of various concentrations and mixed gently and then 0.2 ml of 2% RBC in PBS was mixed and incubated at 34° C for an hour. Reactions were compared with a positive control (0.1 ml protein + 0.1 ml buffer + 2% RBC) and a negative control (0.2 ml PBS + 0.2 ml 2% RBC) as reported by Alkinson et.el. (1980).

3.10 Assay of mitogenesis

The mitogenic activity of the protein was assayed by the stimulation of lymphocytes (Osawa & Toyoshima, 1972). Lympho nodes were minced, gently pressed through a 100-mesh Teflon screen and dispersed in RPMI-1640 medium containing 10% foetal-calf serum (Medium A). The cell suspension was passed through a loosely packed cotton pad and was then counted for viable lymphocytes by using Trypan Blue exclusion which was diluted with Medium A to give final concentration of 3.0×10^6 viable lymphocytes/ml. Solutions of all non-sterile reagents were passed through Millipore filters (0.2 μm pore size: Millipore Corp., Bedford. MA. U.S.A). Each well contained 100

μl of the lymphocyte suspension and 100 μl of PFP in Medium A at a specified concentration. Cultures-were kept at 37°C in a humid atmosphere of air/CO_2 (19:1) and then observed the changes under higher magnification microscope.

3.11 Amino acid sequence and Homology Allignment

After electrophoresis by SDS-PAGE (12.5% gel) in the presence of 2% marcaptoethanol, the protein band was then transferred to a polyvinylidene difluoride membrane (PVDF, Millipore) and protein band was visualized by Commassie brilliant blue R. The band of the protein was cut out and applied on a protein sequencer (Shimadzu Biotech protein / peptide sequencer, PPSQ-23) for sequence analysis.

RESULTS AND DISCUSSION

3.12 Purification of protein

3.12.1 DEAE-Cellulose chromatography

The crude protein solution prepared from 85% ammonium sulphate saturation of potca fish was dialyzed against 10 mM Tris-HCl buffer, pH 8.4 for 24 hrs and was applied to a DEAE-cellulose column at 4°C, which was previously equilibrated with the same buffer and eluted by a linear gradient of NaCl from 0 to 0.3 M in the same buffer. The column bound proteins were eluted as a single but broad peak, indicating the presence of more than one component (Fig. not shown). In order to separate these components, the elution was carried out stepwisely with increasing concentrations of NaCl in the same buffer.

As shown in Fig. 5, the components of crude protein solutions were separated as two major peaks, F-1 fraction and F-2 fraction. Of these fractions, F-1 was eluted by the buffer containing 0.2 M NaCl while F-2 was eluted by the buffer containing 0.3M NaCl. Only the F-1 fraction as indicated by solid bar was pooled and used for experimental purposes in the present study while F-2 fraction as indicated by solid bar was collected and stored at deep freeze for future study. The electrophoretic pattern of F-1 concluded that the fraction F-1 was not pure as it gave more than one band on the gel.

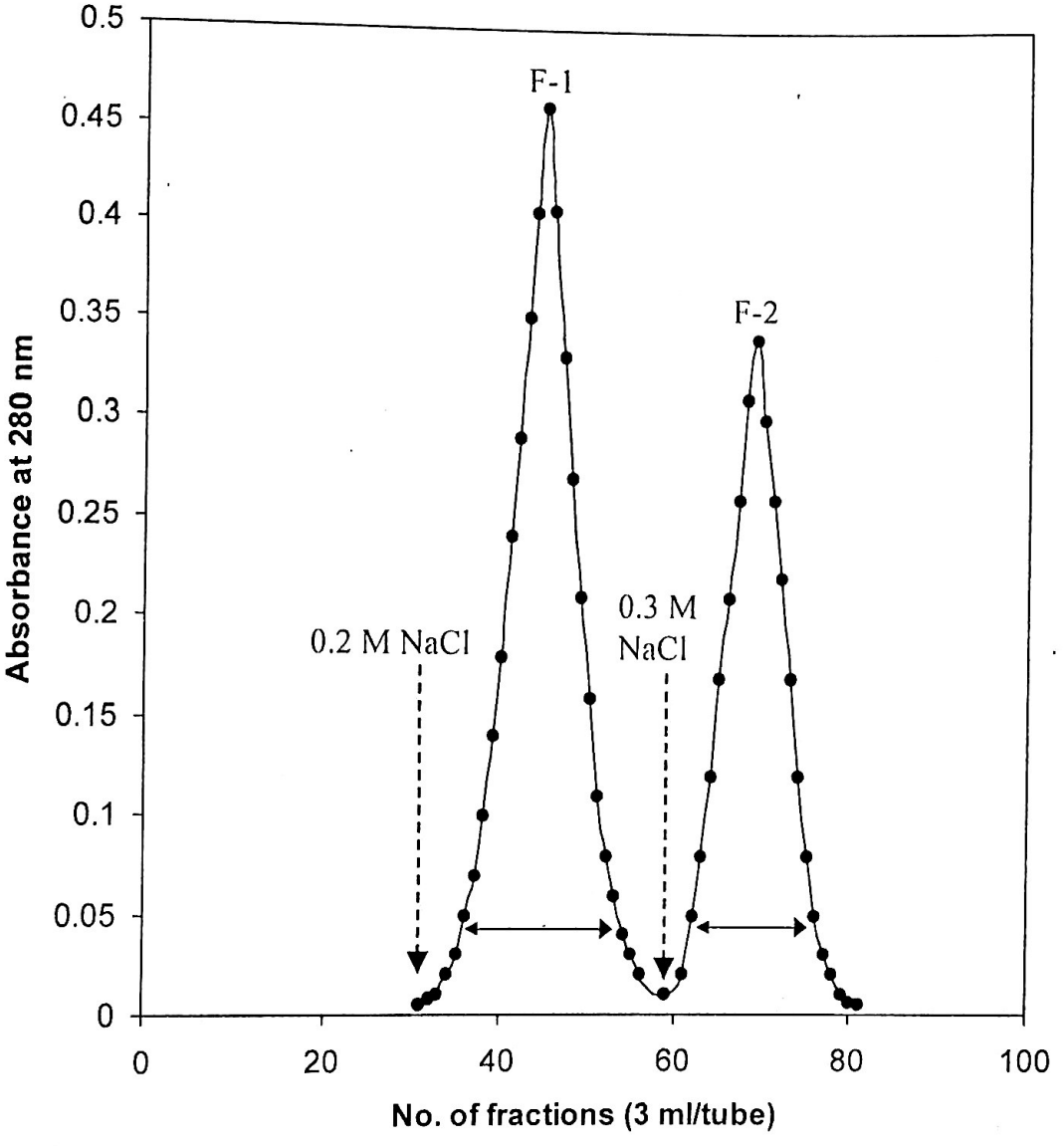


Fig. 5: Ion exchange chromatography of crude protein solution on DEAE-cellulose. The crude solution (12 mg) was applied to the column (2.1 × 25 cm) pre-equilibrated with 10mM Tris-HCl buffer, pH 8.4 at 4°C and eluted by stepwise increases of NaCl concentration in the same buffer. Flow rate: 25 ml/hour.

3.12.2 Gel filtration

The fraction, F-1 was dialyzed against distilled water for 12 hours and then against 10 mM Tris-HCl buffer, pH 8.4 for 12 hours and concentrated by sucrose. The concentrated fraction was applied to a Sephadex G-50 column at 4°C, which was previously equilibrated with the same buffer. As shown in Fig. 6 , the components of the protein solution were eluted as one major peak, F-1a and one minor peak, F-1b. The purity of F-1a was checked by polyacrylamide disc gel electrophoresis and found more than one band.

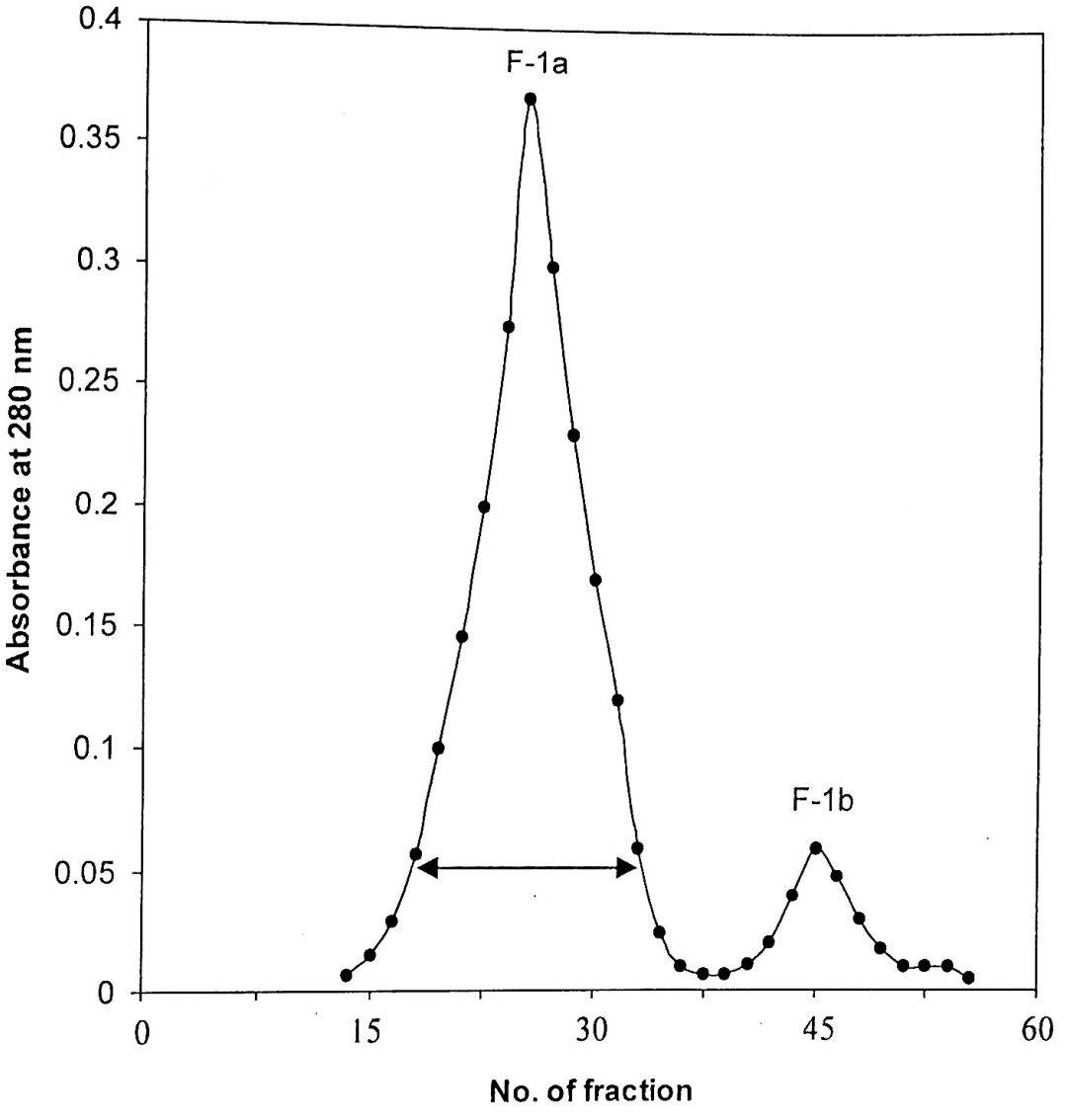


Fig. 6: Gel filtration of F-1 fraction obtained from DEAE-cellulose chromatography on Sephadex G-50. F-1 fraction (7 mg) was applied to the column (3 × 80 cm) pre washed with 10 mM Tris-HCl buffer, pH 8.4 at 4°C and eluted by in the same buffer. Flow rate : 15 ml/hour.

3.12.3 Affinity chromatography

The active fraction of protein, F-1a obtained from Gel Filtration was applied to ConA-Sepharose column, which was previously equilibrated with the buffer, 20mM Tris-HCl containing 0.5M NaCl, pH-7.6 at 4°C. After washing the column with the buffer the protein absorbed on the column was eluted by the buffer containing 0.1M α-D-mannopyranoside (fig. 7).

The purity of the active protein fraction, F-1a^2 was determined at room temperature using Native-PAGE (7.5% gel) according to the method of Davis (1964)

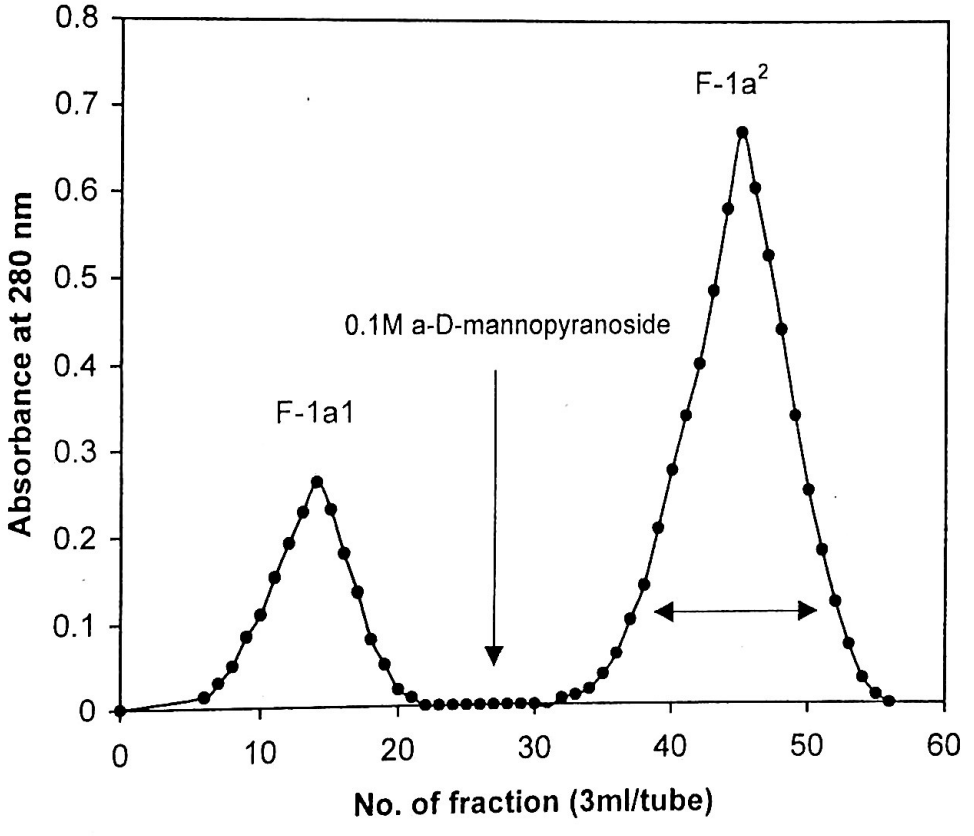


Fig.-7: Affinity chromatography of fraction F-1a (2.75mg) on ConA-Sepharose column which was previously equilibrated with the buffer, 20mM Tris-HCl buffer containing 0.5M NaCl, pH-7.6 at 4°C. After washing the column with the buffer the protein absorbed on the column was eluted by the buffer containing 0.1M α-D-mannopyranoside.

Fig. 8: Schematic representation of the purification steps of protein from Potca fish.

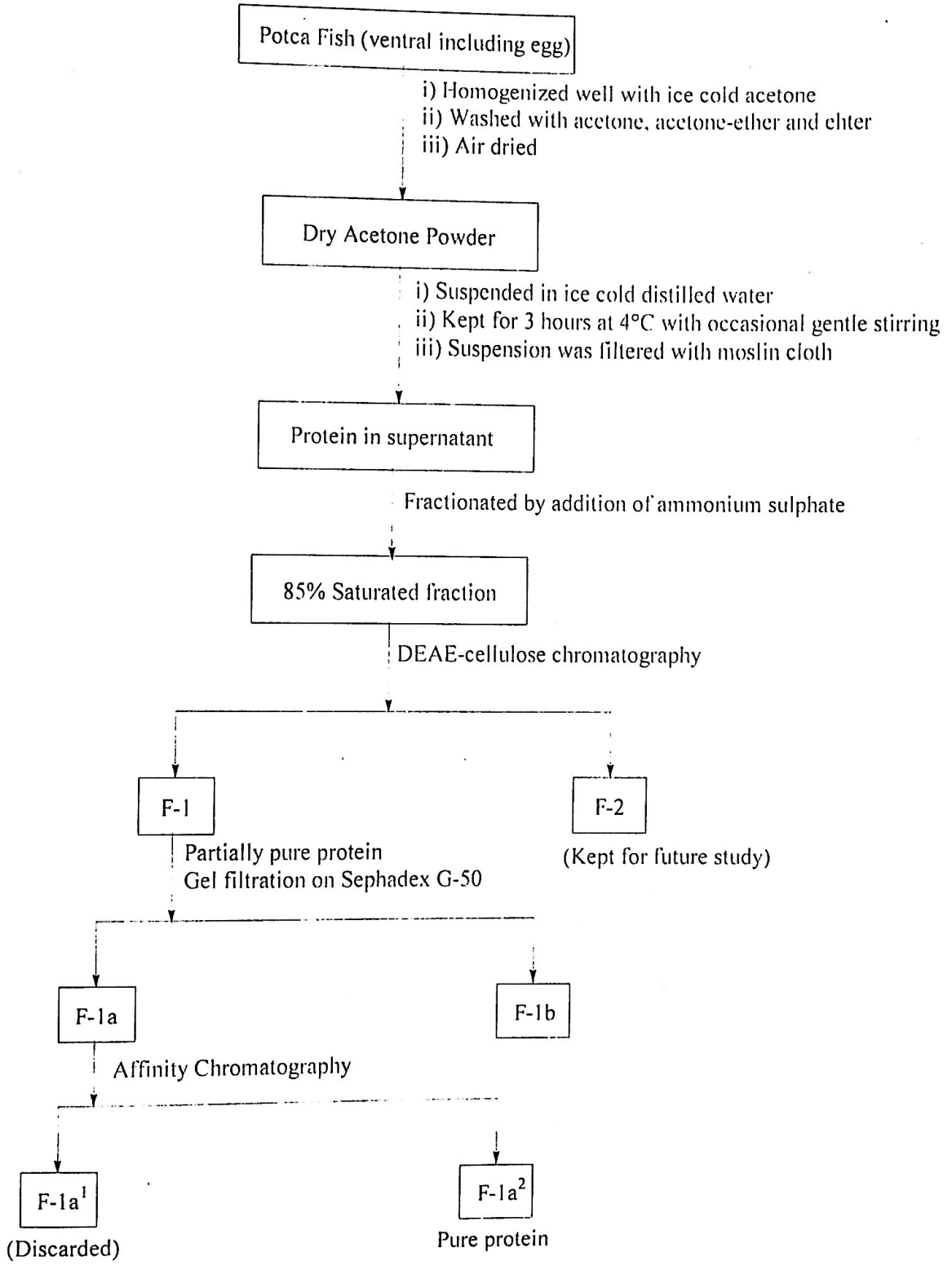


Table-1 shows the purification of potca fish protein.

Table-1: Purification of Potca fish protein.

Fraction	Total Proteins(mg)	Hemagglutination activity (titre)	Specific activity (titre/mg)	Yield %	Purification fold
Crude extract	520	1380	2.65	100	1.00
Ammonium sulfate saturated	250	843	3.37	61.08	1.27
Ion-exchange chromatography	27	220	8.14	15.94	3.07
Gel chromatography on Sephdex G-50	10	185	18.5	13.40	6.98
Affinity chromatography on ConA Sepharose	5.5	163	29.63	11.81	11.18

3.13 Characterization

3.13.1 Determination of Molecular weight by gel filtration

The molecular weight of the protein was determined by gel filtration on Sephadex G-75 using β-D-galactosidase (MW. 116 kd), Bovine serum albumin (MW. 67 kd), Egg albumin (MW. 45 kd) and Lysozyme (Mw. 14 kd) as standard proteins. The molecular weight was calculated from the standard curve of reference proteins which was constructed by plotting molecular weight against elution volume on gel filtration (Fig. 9) and the molecular weight of the purified protein was estimated to be 82 kd.

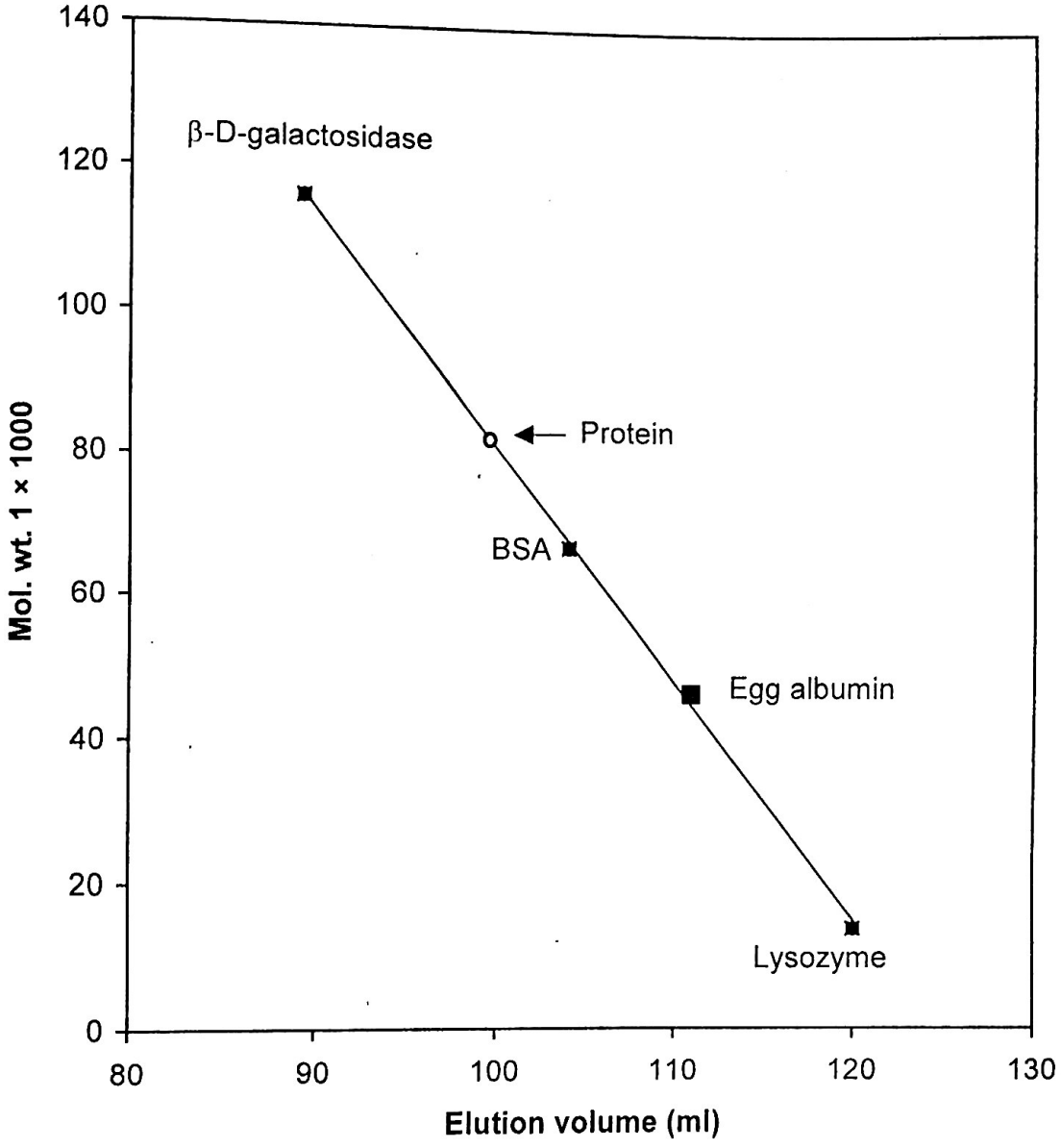


Fig. 9: Standard curve for the determination of molecular weight by gel filtration on **Sephadex G-75.**

Size of column : 1.8 × 90 cm

Buffer : 10 mM Tris-HCl, pH 8.2

Flow rate : 15 ml/hour

3.13.2 Determination of molecular weight by SDS-polyacrylamide slab gel electrophoresis

The molecular weight of the protein was also determined by SDS-polyacrylamide slab gel electrophoresis using β-D-galactosidases (MW. 116 kd), bovine serum albumin (MW. 67 kd), Egg albumin (MW. 45 kd) and lysozyme (14 kd) as standard proteins. The molecular weight was calculated from the standard curve, which was constructed by plotting log molecular weight against relative mobility of the reference proteins (Figure .10).

The molecular weight of the protein as determined by SDS-PAGE was estimated to be 80 kd (Figure. 11a and 11b).

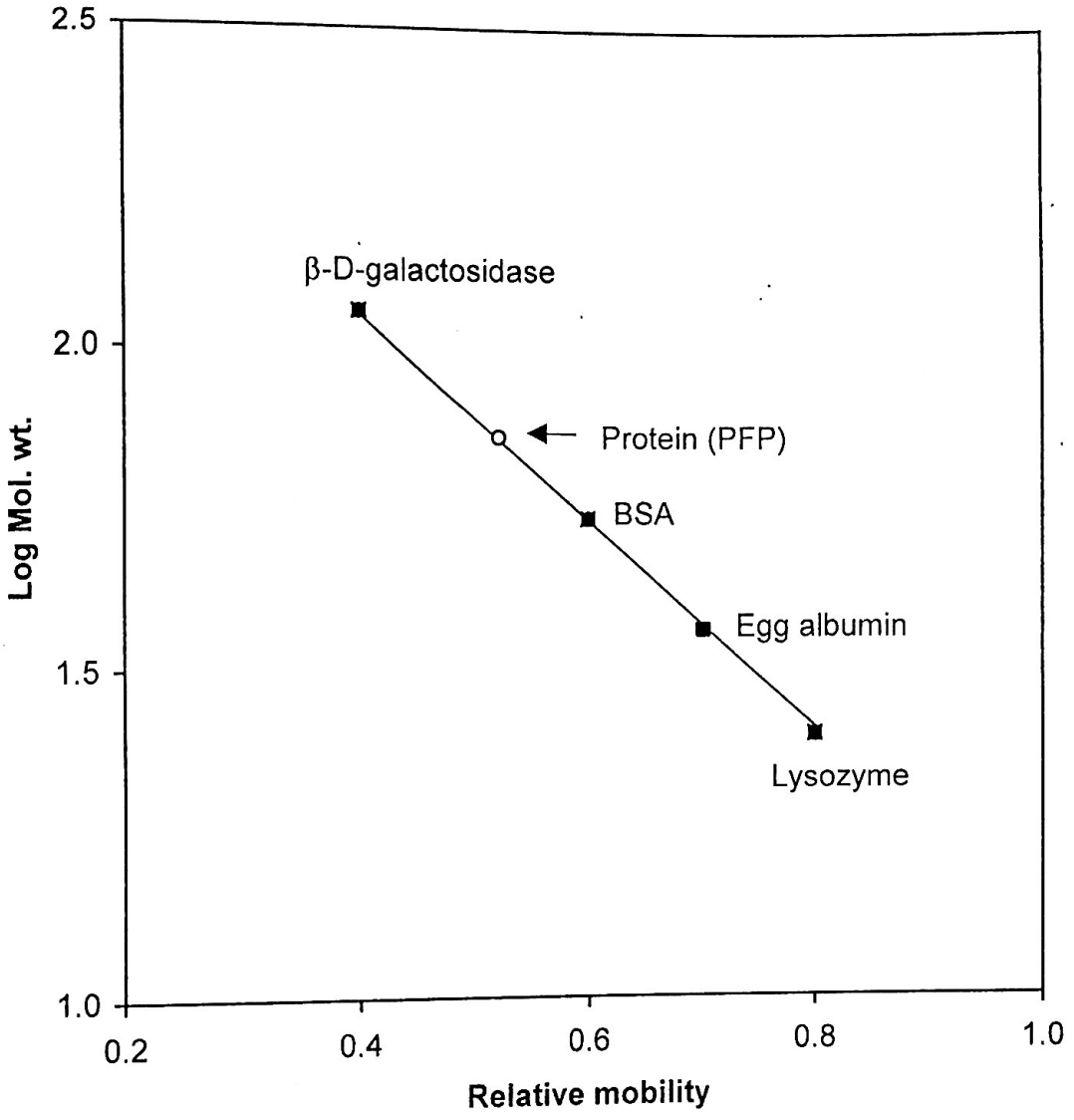


Fig. 10: Standard curve for the determination of molecular weight by SDS-polyacrylamide slab gel electrophoresis.

Fig. 11a: Native-Polyacrylamide slab gel electrophoretic pattern of the fractions F-1a[2] on the gel.

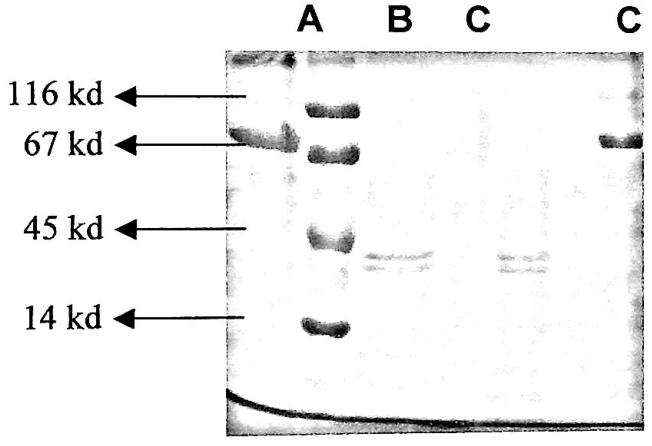


Fig.11 b: Determination of molecular weight and subunit structure of the protein (PFP) by SDS-polyacrylamide slab gel electrophoresis.

A = Purified protein

B = Marker protein

C = Subunit structure

13.3 Determination of the sub-unit structure of the protein by SDS polyacrylamide slab gel electrophoresis

The subunit structure of the protein was determined by SDS-polyacrylamide slab gel electrophoresis using β-marcaptoethanol. The result indicated that the protein gave a very close slightly distinct band, suggesting that the protein contained two subunits (Fig. 41). The molecular weight of the sub-units was estimated to be about 38 kd and 42 kd (Fig. 11 b).

3.13.4 Optical density vs. concentration relation of the purified protein

The absorbance of 1.0 at 280 nm for purified protein was found to be equal to 0.72 mg , as determined by the Lowry method (1951) using BSA as standard. This value was slightly lower as compared to that obtained by drying the protein solution under vacuum.

Table-2: Optical density (O.D.) and concentration relation of the protein:

Absorbance at 280 nm	mg of protein obtained by drying process	mg of protein obtained by Lowry method
1.0	0.75	0.72

3.13.4 Hemagglutinating Activity of the Protein

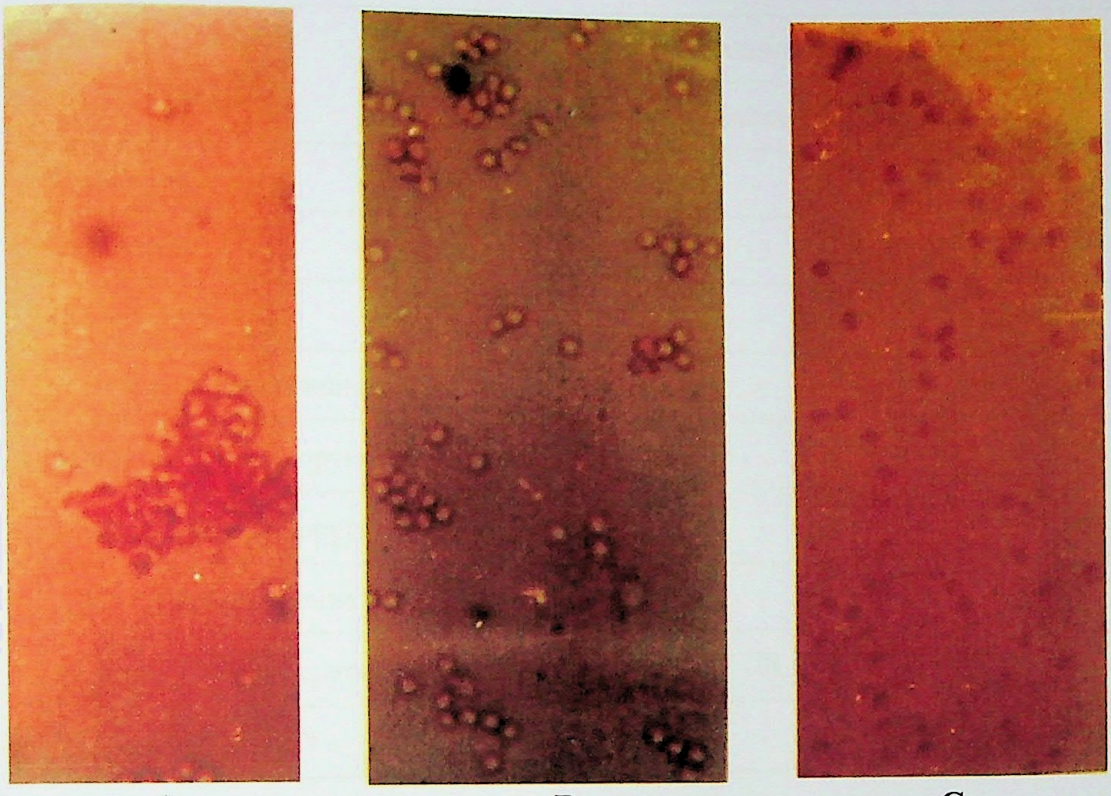
The purified protein agglutinated specifically albino rat red blood cells and the hemagglutination potency of rat red blood cells by the protein was shown photographically in Fig. 12. The minimum protein concentration at 280 nm needed for visible agglutination was taken as minimum hemagglutination does (MHD) and was found to be 0.015 μg/ml for Potca fish protein (Table-3) .

Table- 3: Hemagglutinating activities of the purified protein (PFP) with 2% red blood cells from albino rat.

Protein samples	Absorbance at 280 nm	Concentration (mg/ml)	Degree of Hemagglutination
PFP	0.050	0.070	3^+
	0.042	0.050	2^+
	0.025	0.035	1^+
	0.010	0.015	±
Control	0	0	—

3^+ Indicates complete aggregation of all most all the cells

± Indicates major cells were present in small aggregates.



A B C

Fig. 12: Agglutination Potency of albino rat red blood cells by PFP

A = 3^+ B = ± and C = Control

3.13.5 Glycoprotein test and estimation of sugar

The purified protein (PFP) gave yellow orange color in the presence of phenol-sulfuric acid, indicating that the protein contained sugar. The percentage of neutral sugar present in the glycoprotein was calculated from the standard graph of glucose and was found to be 0.35%.

3.13.6 Sugar specificity/ inhibition

Table-4 summarizes the results of hemagglutination inhibition test of PFP with haptenic sugars. It was found that PFP induced hemagglutination was inhibited in the presence of mannose and mannose containing saccharides.

Table- 4: Hemagglutination inhibition assay of potca fish Protein .

Sugar	Concentration (mM)		
	Maximum	Minimum	Inhibition
D-Glucose	110	-	No Inhibition
D-Mannose	-	13	Inhibition
D-Galactose	110	-	No Inhibition
N-Acetyl D-glucosamine	110	-	No Inhibition
Methyl-α-D-galactopyranoside	110	-	No Inhibition
Methyl-β-D-galactopyranoside	110	-	No Inhibition
N-acetyl-galactosamine	110	-	No Inhibition
Methyl-αD- Mannopyranoside	-	20	Inhibition
D-glucosamine-HCl	110	-	No Inhibition

3.13.7 Mitigenic assay

The lectin-mediated stimulation of the lymphocytes from mouse lymphnode was used to determine the mitogenic activity of the lectin. The stimulation was found to be more than 40-fold by the lectin PFL at the conc. of 0.1 μg/ml (Figure not shown).

3.13.8 Amino acid sequence

The sequences of subunit A upto 34 residues and for subunit B upto 48 residues were identified and shown in Fig 13 and 14. Among the sequences, the amino acid sequences of position 28 of subunit A and positions 21,39,42 and 44 of subunit B were not possible to be identified. Further the sequence homology of the N-terminal sequences of both the subunits was compared with those of the homologous proteins (Fig.13 and Fig. 14).

3.13.9 Homology alignment

Using N-terminal amino acid sequences of subunit A and B from PFL as a query, a ClustalX Blast Search of cDNA database of the homologous proteins.

Multi Sequence Allignment of PFL Subunit A

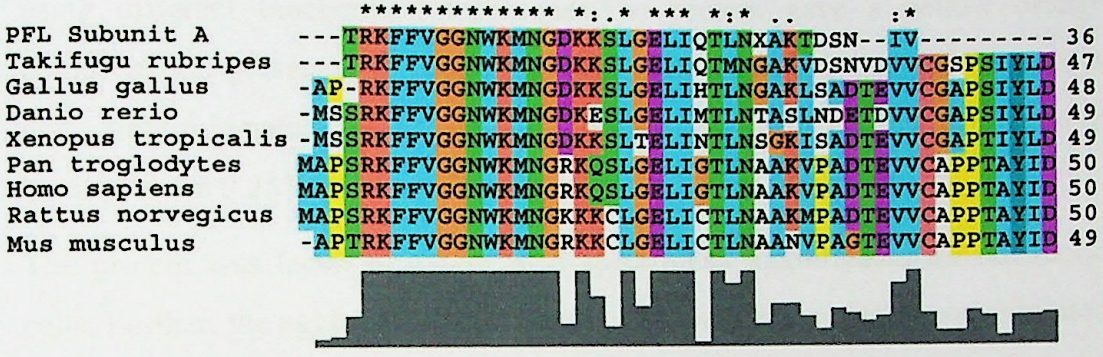


Fig. 13

Multi Sequence Allignment of PFL Subunit B

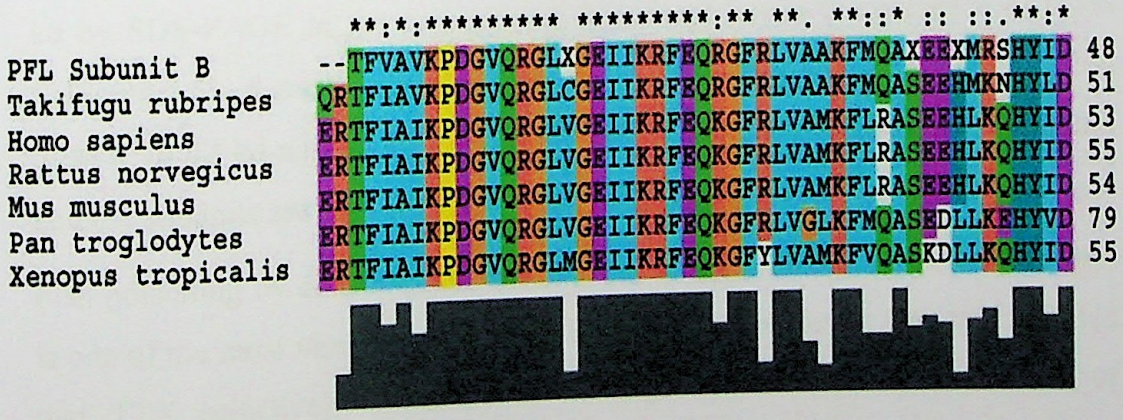


Fig. 14

Discussion

Potca fish stores high concentration of tetrodotoxin , a powerful neurotoxin that can cause death in approximately 60% of persons who ingest it. A TTX-binding protein has been purified from the plasma of the potca fish Kusafugu. *Taleifugu niphobles* . In the present study, a mannose specific lectin has been isolated and purified from the crude extract of ventral portion of potca fish by using different biochemical techniques.The protein gave a yellow orange colour in the presence of phenol sulfuric acid indicating that the protein is glycoprotein in nature and the neutral sugar content of the protein was found to be only about 0.35%.

The protein was lectin in nature as it specifically agglutinated rat red blood cells. Further, the agglutination of rat red blood cells by the lectin was inhibited specifically in the presence of mannose and its derivatives indicating that the purified lectin from potca fish is mannose specific. This finding was further substantiated from the data that the protein was purified finally by using affinity chromatography on ConA-Sepharose that is also specific for binding mannose-containing protein.

The lectin showed a single band and its molecular weight was estimated to 80 kd by SDS-PAGE in the presence of denaturant but in the presence of 0.1% SDS and 0.5% β-mercaptoethanol, the protein gave two subunits corresponding to MW of 42 kd and 38 kd indicating that the protein is heterodimer in nature and the subunits are held together by disulfide bond.

Fish poisoning by consumption of members of the order of Tetraodontoformes is one of the most violent intoxication from marine species. It was also reported that TTX selectively block-off the voltage sensitive sodium channels of excitable tissues and neural transmission in skeletal muscles (Noguchi and Mahmud, 2001). Our present data suggest that potca fish contained not only neurotoxin TTX but also a lectin of almost similar toxic properties. We confirm

from brine shrimp lethality bioassay and histopathological studies that TTX and the purified lectin showed similar toxic effect although the lectin is slightly less toxic than TTX.

An alignment of the N-terminal sequences of the subunit A and B of the purified mannose specific lectin, PFL from puffer fish with those of the homologous proteins such as *Takifugu rubripes* (Gene FRU P00000156180), *Danio rerio* (ENSDARG00000025012), *Xenopus tropicalis* (Gene XT10760), *Pan troglodytes* (GeneENSPTRG00000004595), *Mus musculus* (GeneENSMUSG00000020857) *Homo sapiens* (Hsap2, Gene TP11), *Homo sapiens* (Hsap1, Gene HIX0010385.1.1), *Homo sapiens* (Hsap0, Gene ENSG00000111669) and *Ratts norvegicus* (Gene ENSRNOG 00000026439) were compared and the results are shown in Fig. 6 and 7. The N-terminal sequences of both the subunits of PFL showed a higher degree of sequence homology with the previously reported homologous proteins.

The overall similarity is very high, using N-terminal amino acid sequences of subunit A and B from PFL as a query, a ClustalX Blast Search of cDNA database of the homologous proteins. The N-terminal sequences of the subunit A (residue. 1-35) and subunit B (residue. 1-48) are identical to the proteins FRU P00000156180, 88% and 85% , ENSDARG00000025012, 86% and 83%, Gene XT10760, 73% and 70%, ENSPTRG00000004595, 73%and 72%, Gene ENSMUSG00000020857, 75% and 11%, Gene TP11, 73% and 75% , Gene HIX0010385.1.1, 73% and 75% , Gene ENSG00000111669, 73% and 75% and Gene ENSRNOG 00000026439, 71% and 75% respectively.

Most recently, some lectins from fish have been purified in different laboratories. A lectin name Katsuwonus pelamis (KPL) was purified from hard roe of Skipjack Tuna (Jung W.k., Park P.J., Kim S.K, 2003). This lectin is a homolectin with molecular weight of 140,000 and it's activity i.e. agglutination inhibited by D-galactose, Lactose, N-acetyl-D-galactosamine etc. Antifreeze proteins (AFP) was purified from rainbow smelt and it was a dimer with

molecular weight of 22000 per subunit (John C. Achenbach and K. Vanya Ewart, 2002). Three L-rhamnose specific lectins were isolated from Chum salman eggs and all these lectins are monomer in nature with molecular weight of 22000–29000 (Nobuyuki Shiina and Hisao Kamiya, 2002). Two galectins isolated from the skin mucus of Conger eel named congerins II and I are dimer composed of two identical subunits of 136 and 135 amino acid residues respectively (Tomohisa and Muramoto, 2002). Galectins are a family of carbohydrate binding proteins defined by their affinity for β-galactosidase.

In conclusion, antifreeze protein purified from rainbow smelt and protein purified from potca fish is dimer in nature, which is specific for D-mannose. Further, like other lectins this lectin is very much toxic in nature. So besides being specific for rat red blood cells agglutination, PFL can be added as an addition to the list of the members of mannose specific lectins purified from other sources.

CHAPTER-4

COMPARISON BETWEEN THE PURIFIED POTCA FISH PROTEIN AND TETRODOTOXIN

Comparison between the purified Potca Fish Protein(PFP) and Tetrodotoxin (TTX)

4.1 Cytotoxicity study

4.1.1 Brine Shrimp Lethality Bioassay [76-79]

4.1.1.1 Principle and procedure

Methods

Brine shrimp lethality bioassay is a recent development in the bioassay for the bioactive compounds. Natural products (extracts, fractions and pure compounds) can be tested for their bioactivity by this method. Here the simple zoological organism (brine shrimp nauplii) is used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. This bioassay indicates cytotoxicity as well as a wide range of pharmacological activities of the compounds.

The brine shrimp assay has advantages of being rapid (24 hr), inexpensive and simple (e.g. no aseptic technique is required). It easily utilizes a large number of organisms for statistical validation and requires no special equipment and relatively small amount of sample is sufficient. Moreover, it does not require animal serum, as it is needed for the determination of cytotoxicity.

4.1.1.2 Materials

i. Artemia salinal Leach (brine shrimp eggs from store)
ii. Sea salt (NaCl)
iii. Small tank and lamp to hatch the shrimps
iv. Pipettes (5 ml and 1 ml)
v. Micro pipette (10 and 100 µl)
vi. Glass vial (10 ml)
vii. Magnifying glass.

4.1.1.3 Procedure

4.1.1.3.1 Preparation of seawater

38 g of NaCl was weighed, dissolved in one litre of distilled water and filtered off.

4.1.1.3.2 Hatching of shrimps

Seawater was kept in a small tank and shrimp eggs were added to the divided tank. Constant oxygen supply was carried out by the help of aqua pump and The light source maintained the constant temperature (around 37°C). Two days were allowed for the shrimps to hatch and mature as nauplii.

4.1.1.3.3 Preparation of sample

12.5 µg of TTX and PFL were dissolved separately in 5 ml of distilled water and used for experimental purposes.

4.1.1.3.4 Application of the sample on brine shrimp nauplii in the vials

Twelve clean vials were taken for the ventral, tail, head, dorsal and egg of the potca fish and for the control in order to test their comparative toxicity.

5 ml of seawater containing 30 brine shrimp nauplii was taken to each of the vials. Then with the help of a micropipette specific volume of samples were transferred from the stock solutions to the vials to get final concentration.

In the control vials same volume of seawater were taken.

4.1.1.3.5 Counting of nauplii

After 12 hours and 24 hours, the vials were observed for the number of survived nauplii in each vial and the results were noted.

4.1.1.3.6 Calculation

$$\chi^2 \text{ (Chi-squire)} = \Sigma \frac{(Op-Ep)^2}{Ep} \sim \chi^2 (r-1)(p-1)$$

Y = Regression line
OP = observed probit
EP = Expected probit

4.2 Toxicological studies

4.2.1 Introduction

Toxicology is the aspect of Pharmacology that deals with the adverse effect of bioactive substances on living creature along with their diagnosis and clinical use.

In order to develop and establish the safety and efficacy level of a new compound, toxicity studies are very essential experiment. No compound is used clinically without its clinical trial as well as toxicity studies. Toxicological data help to make decision whether a new compound is adopted for clinical use or not.

4.2.2 Types of toxicity

I. Acute toxicity:

Acute toxic effects occur rapidly as a result of exposure to a relatively large quantity of the compound administered as a single dose. Acute toxicity of a compound is characterized by untoward reactions having serious symptoms and a short course of existence, which may follow the administration of a single dose (or an over dose) of the drug.

II. Sub-acute toxicity:[96]

Sub-acute toxicities are those adverse reactions, which occurs after repeated administration of a compound for a period of 14-21 days. The response of the sub-acute study is to predict the toxic effects, which may occur in the animals during chronic administration of the test agent.

II. Chronic toxicity:

Chronic toxicity refers to the toxic effect of a compound over an extended period of time during which animals may receive repeated doses of apparently safe level. It causes slow accumulation of toxic symptoms of compound in the body.

Study of chronic exposure is often used to determine the carcinogenicity and mutagenic potentials of drugs.

4.2.3 Sub-acute toxicity studies

The sub-acute toxicity studies of pure compounds were performed on normal rats by giving a daily dose of 2.25 µg/rat/day for 14 consecutive days. The rats were kept under keen observations throughout the treatment period. The following parameters were studied during this test-

1. Gross general observation
2. Haematological profiles
3. Biochemical parameters of blood
4. Histopathology of liver, kidney, heart and lungs.

4.2.4 Collection of experimental rats

For this purpose, 15 rats of same sex (male) and age (7 week) were collected from the International Center for Diarrhoeal Diseases and Research, Bangladesh.

4.2.5 Maintenance of the rats

The rats were kept in properly numbered iron cages individually. They were given ideal food comprising the following ingredients per 100 g of mixture.

Composition	Amount (g)
Ata (flour)	40
Matar dal powder	25
Skimmed milk powder	28
Soyabean oil	05
Salt mixture	01
Vitamins mixture	01

The diet supplied to each rat was about 20 gm per day, which was approximately isocaloric. They were kept in a clean animal house with an optimal room temperature. The animals were maintained in this way for 15 days before administration of the compounds and continued upto the end of the experiment.

4.2.6 Grouping of rats

Weight of the individual rats were determined and they were grouped into three groups such as A, B and C. Each group contains 5 rats. Group-A received Tetrodotoxin(TTX), group B received PFL and group-C received water only for control.

Table-5: Dosage regimen adjustments for each group of rats.

Group	No. of rats	Average body weight (gm)	Sex	Average age (week)	Dose (i.p.) µL/rat/day
A	5	115	Male	7	300 µL containing PFT-1
B	5	114	Male	7	300 µL containing PFT-2
C	5	114	Male	7	300 µL of distilled water

4.2.7 Preparation of sample solution

The pure TTX and PFL were dissolved separately in distilled water with stirring and the conc. of the sample is 7.5 µg/ml .

4.2.8 Gross general observation after drug administration

The rat in different groups were injected intraperitonealy, as per dosase regimen (Table -8) with the help of 1 ml syringe while the control received water only.

They were observed daily very keenly to notify the following features:

i. Behaviour

ii. CNS excitation

iii. CNS depression

iv. Food intake

v. Salivation

vi. Diarrhoea

vii. Muscular weakness

4.2.9 Monitoring of body weight

The body weights of each rat of groups A, B and C were measured before administration of the compounds and at the completion of the treatment prior to sacrificing the animals.

4.2.10 Monitoring of haematological profiles

The haematological profiles of the control and experimental rats were done to check the haematological abnormalities after administration of the purified compounds intraperitoneally. For this purpose, the following parameters were observed:

i. Total R.B.C. count ,

ii. Total W.B.C. count ,

iii. Differential count of W.B.C.,

iv. Platelet count ,

v. Hemoglobin percentage and

vi. E.S.R. (Erythrocytic Sedimentation Rate)

Procedure

(i) Blood was drawn from the tail veins of the rat before the commencement of the administration of compound.

(ii) Blood smears were made on glass slides and stained with leishmen reagent to perform TC, DC and platelet count. With the use of capillary tubes, blood was drawn from each rat to estimate the hemoglobin percentage by Van Kampen-Zijlstra's method, which is the pre-haematological study on normal rats.

(iii) The pure compounds were administered intraperitoneally regularly to the rats of group A and B respectively, while group-C received vehicle only.

(iv) Post haematological studies were done on 7^{th} and 14^{th} days after the commencement of drug administration following the same procedure as that done on normal rats.

4.2.11 Monitoring the biochemical parameters of blood

The biochemical parameters such as SGOT (Serum-glutamate-oxaloacetate-transaminase), SGPT (Serum-glutamate-pyruvate-transaminase), SALP (Serum alkaline phosphatase) and serum bilirubin are associated with the condition of liver, while serum level of creatinine and urea are associated with the functioning of kidney. Serum levels of these parameters change with the pathological changes of these organs. In case of hepatic necrosis, cirrhosis and obstructive jaundice the serum level of SGOT and SGPT may increase upto 200 IU/L. If a compound possesses any effect on kidney, several pathological changes may occur and ultimately serum level of these parameters altered.

I. Liver function tests:

i. SGOT
ii. SGPT
iii. SALP and
iv. Serum bilirubin

I. Kidney function tests:

i. Creatinine and

ii. Urea

The tests were done using the procedure and reagents described in Boehrienger Mannheim GmbH Diagnostic.

Procedure

A. Collection of serum:

On the 14th day of treatment with the compounds, the rats of experimental and control groups were sacrificed by a surgical blade No. 22 and the blood was collected in plastic centrifuge tubes. These were then allowed to clot at 40°C for 4 hours. After clotting, the blood samples were centrifuged at 4000 rpm for 15 minutes using a WIFUNG centrifuge LABOR-50M. The clear straw color serum was then collected in vials with Pasteur pipette and stored at 20°C.

B. Analysis of the serum for enzyme content:[97-99]

The enzymes, SGOT, SGPT, SALP, serum creatinine and urea were determined by following the procedures using reagents as described in Boehringer Mannheim GmbH Diagnostica.

4.9.12 Histopathology of liver, kidney, heart and lung

Histopathology of liver, kidney, heart and lung were performed to observe any changes in the cellular structures (degradation and regeneration) of the rats after receiving purified compounds at a dose of 2.25 µg/rat/day for 14 consecutive days with respect to control group.

Reagents

i. Formaline (10%)

ii. Absolute alcohol (ethanol)

iii. Paraffin

iv. Xylene

v. D.P.X. mounting fluid

vi. Harris hematoxylin and eosin stain

Procedures

A. Collection and processing of the tissues:

The liver, kidney, heart and lungs were collected from different groups of treated and control rats. After sacrificing the rat on 14th day of observation, the tissues were sliced into pieces each measuring a few mm of thickness. The sliced tissues were then immersed in 10% formalin for three days. The tissues were then dehydrated in ethanol and embedded in paraffin. The blocks were sectioned with the help of rotating microtome at 6-micron thickness.

B. Staining:

The sections were deparaffinized by two changes of xylene (5 min each) and hydrated in alcohol (2-3 min each) and then cleaned in xylene (5 min each).

C. Mounting:

Glass slides containing the tissues were wiped, dried and then a drop of Canada balsam was put on the section and cover slip was gently placed on it. On the sections, thin film between the cover slip and the slide with the mounting medium (Canada balsam) was formed to attach them.

D. Histopathological examination was done under high magnifying power Microscope and photographs were taken.

4.3 Biological Study

In vitro antibacterial activity [72-75]

The antibacterial activity of the purified compounds can be measured *in vitro* by a number of techniques but the disc diffusion method,[44] is widely acceptable for the preliminary evaluation of antimicrobial activity. Disc diffusion technique is essentially a qualitative or semi quantitative test indicating the sensitivity or resistance of microorganisms to the test material. However, no distinction between bacteriostatic and bactericidal activity can be made by this method.

Principle of Diffusion Method[100]

Diffusion assay is based on the ability of antibiotics to diffuse from a confined source through the nutrient agar gel and create a concentrated gradient. If agar is seeded or streaked with a sensitive organism, a zone of inhibition will result where the conc. exceeds the minimum inhibitory concentration (MIC) for the particular organism.

4.3.2 Material

1. Filter paper disc (diameter, 5mm.)
2. Petridishes
3. Test tube
4. Inoculating loop
5. Bunsen burner
6. Sterile forceps
7. Sterile cotton
8. Laminar Air-Flow Unit
9. Micropipette (10µ, 100 µl)
10. Autoclave
11. Incubator
12. Refrigerator
13. Solvent (Methanol, Ethyl acetate)
14. Nutrient agar

4.3.3 Test organisms

Both gram-positive and gram-negative strains of bacteria were used as the test organisms to observe the anti-bacterial activity of the compounds. The bacterial strains used for this investigation are listed in the Table-6. These organisms were collected from the Microbiology laboratory of Pharmacy Department, R.U.

Table-6: List of pathogenic bacteria tested

Type of bacteria	Name of specific pathogenic bacteria
Gram-positive	*Bacillus subtilis* *Staphylococcus aureus*
Gram-negative	*Shigella sonnei* *Shigella dysenteriae* *Shigella flexinerie* *Escherichia coli*

4.3.4 Procedure

(a) Composition and preparation of culture media:

Nutrient agar media is most frequently used to demonstrate the antibacterial activity and to make subculture of the test organisms. Composition of nutrient agar media is shown in Table - 7.

Table-7: Composition of nutrient agar media

Ingredient	Amounts
Bacto peptone	0.5 g
Sodium chloride	0.5 g
Bacto yeast extract	1.0 g
Bacto agar	2.0 g
Distilled water	100 ml
P^H	7.2 ± 0.1 at 25°C

The instant nutrient agar media was accurately weighed and then reconstituted with distilled water in a conical flask according to specification (2.3%). It was then heated in water bath to dissolved the agar until a clear solution of media was obtained. The media was then transferred in prepared plates and slants, respectively, in a number of required test tubes respectively. These slants were used for making fresh culture of microorganisms, which in turn used for sensitivity test. The test tubes were then plugged with cotton and sterilised in an autoclave at a temperature of 121°C and a pressure of 15 lbs/sq inch for 15 minutes.

(b) Preparation of sub-culture:

With the help of an inoculating loop, the test organisms from the pure culture were transferred to the agar slants in an aseptic condition. The inoculated slants were then incubated at 37°C for 18-24 hrs to assure the growth of test organisms. This culture was used for sensitvity test.

(c) Preparation of the test plates:

The test organism was transferred from the subculture to the test tube containing 20 ml sterile media with the help of an inoculating loop in an aseptic area. The test tube was shaken well by rotation to get a uniform suspension. The bacterial suspensions were immediately transferred to the sterile petridishes in an aseptic area in such a way as to give a uniform depth of media (approximately 4 mm). The petridishes were rotated several times, first clockwise and then anticlockwise, to assure homogenous distribution of the test organisms and were kept in a refrigerator.

(d) Preparation of discs:

(i) Sample discs:

The filter paper discs (5 mm diameter) were sterilised using autoclave at 121°C and 15 lbs/sq inch pressure for 15 minutes. 10µl of each sample solution was applied aseptically to each disc with a micropipette.

TTX and PFL (5µg) were dissolved in 0.1ml of methanol.

(ii) Control Discs:

Control discs were prepared in the same manner applying only solvent to the discs for determining the antibacterial effects of the solvents used.

(iii) Standard discs:

Kanamycin-K (30μg/disc) was used as a standard sample.

(e) Placement of the discs, diffusion and incubation:

The sample discs, standard antibiotic discs and control discs were placed gently on the solidified agar plates freshly seeded with the test organism with the help of a sterile forceps to assure complete contact with medium surface. The spatial arrangement of the discs were such that the discs were not closer than 15 mm to the edge of the plate which was done to prevent the overlapping of zone of inhibition. The plates were then inverted and kept in a refrigerator for about 24 hrs at 4°C to obtain maximum diffusion. Finally, the plates were incubated at 37°C for 12-18 hrs.

(f) Determination of antibacterial activity:

After incubation, antibacterial activity of the test samples were determined by measuring the diameter of inhibitory zone in mm.

RESULT AND DISCUSSION

4.4 Toxicological studies

4.4.1 Brine-shimp lethality bioassay

From the investigation of Brine Shrimp Lethality Bioassay ,it was found that TTX which has the LC_{-50} of 1.82 and λ^2 =1.99 is more toxic than PFL which has the LC_{-50} of 2.764 and λ^2 =1.093 .

Table-8: Effect of TTX and PFL on the mortality of brine shrimp nauplii after 24 hours.

Sample	Conc. of sample µg/ml	Log conc.	No. of nauplii used	No. of nauplii death	% of mortality	Probit unit	$LC_{50\ (\mu g/ml)}$	λ^2
TTX	2.5	0.398	30	19	63.33	5.19	1.823	1.992
	5	0.699	30	24	80	5.94		
	7.5	0.999	30	28	93.33	6.19		
PFL	2.5	0.398	30	15	50.00	4.16	2.764	1.093
	5	0.699	30	21	68.66	5.72		
	7.5	0.999	30	23	76.66	5.86		

From the observation, it may be concluded that the severity of toxicity of the toxins TTX and PFL were increased with the increasing of concentration of toxins. At higher concentration (15µg/ml), all the nauplii was dead but at lower concentration , the toxicity was observed to be decrease sequentially.

Further, the toxicity of the purified toxins, TTX and PFL were found to be much higher than that of the crude fish extract .

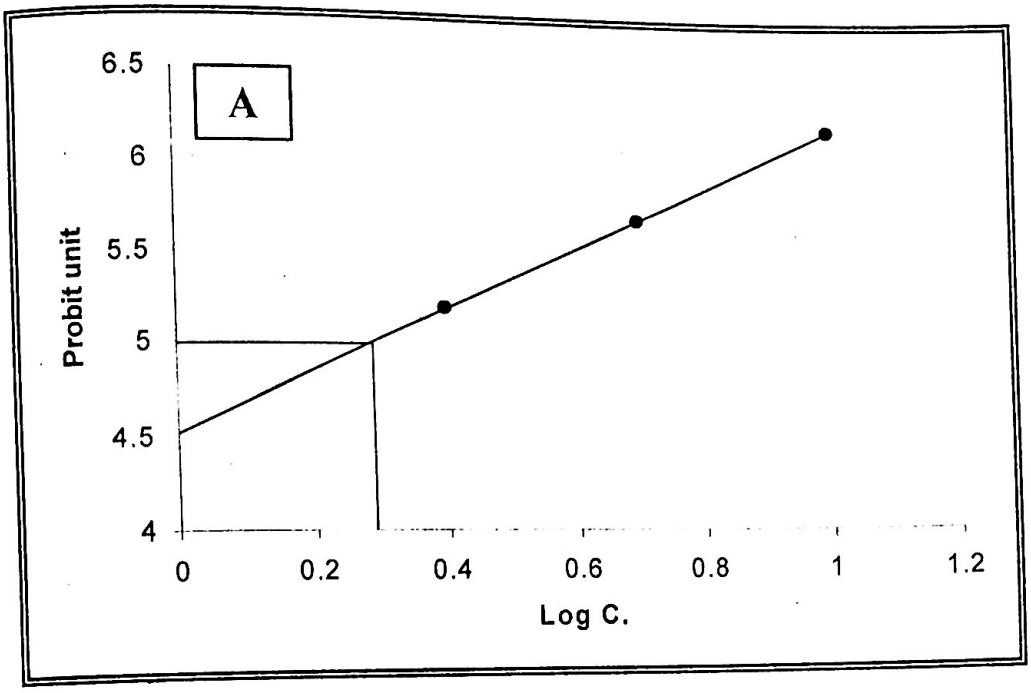


Fig. 15: Determination of LC_{50} of TTX against brine shrimp nauplii

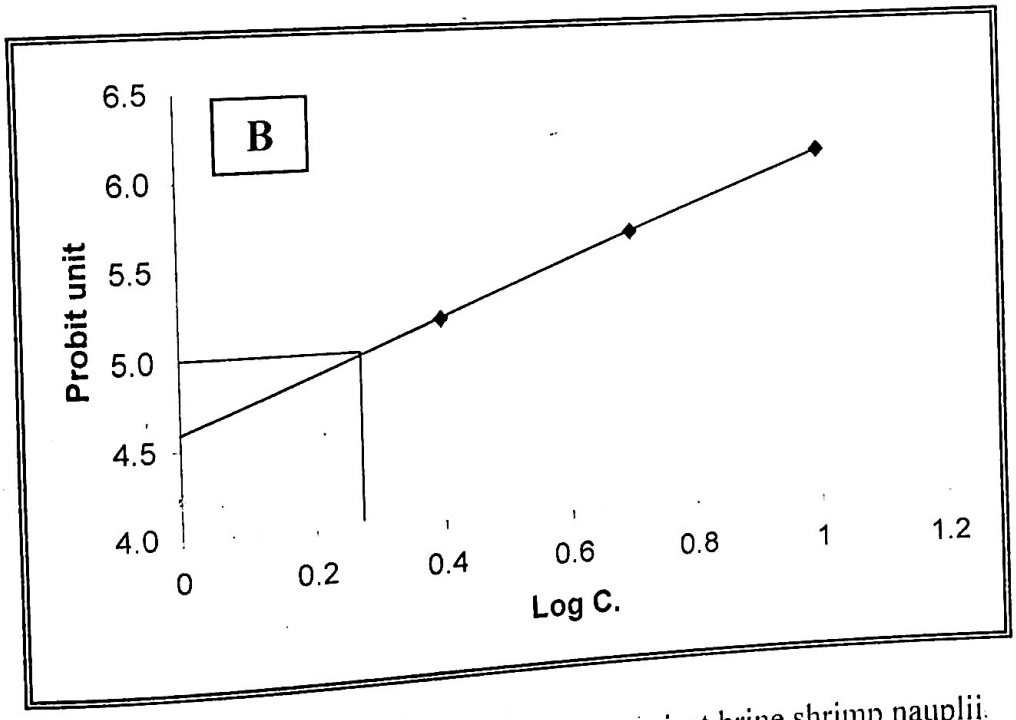


Fig. 16: Determination of LC_{50} of PFL against brine shrimp nauplii.

4.5 Subacute toxicity studies

4.5.1 Gross general observation

The experimental rats of group-A and group-B showed signs of tremor, convulsions and reflex abnormalities as well as muscular paralysis. Muscular numbness of the hind and four legs as well as salivation were also observed. Further, the food intake per day was also found to be much less than that of control group . On the otherhand, the rats of control group did not show any abnormalities and their food intake were also remained normal .

4.5.2 Monitoring the changes of body weight

As shown in the Table-9, the changes of body weight taken before and after adminstration of purified Toxins were found to be statistically significant .

Table- 9: Changes in body weight of control , TTX and PFL treated rats after intraperitoneal administration

Group	Dose µL/rat/day	Body weight (g) before administration n=4, M±SD	Body weight (g) after administration n=4, M±SD	% of change	t_c	t_s	Remark
A	300 µL (TTX)	123 ± 0.5	103 ± 0.5	16.26 (-)	10.68	12.26	S
B	300 µL (PFL)	121.5 ± 1.25	104.5 ± 1.20	13.99 (-)	9.25	12.26	S
C	300 µL (Water)	114 ± 1.58	115 ± 1.87	8.77 (+)	0.580	2.557	NS

t_c indicates calculated value; t_s indicates t value at 5% level of significance; M - sample mean value; SD- standard deviation; n- number of rats; NS- not significant and S-significant.

It was found that the body weight of TTX treated rats was decreased by about 16.26 % while that of the PFL treated rats was decreased by 10% as compared to that of their initial weight. On the otherhand, the body weight of the control rats increased about 9% .

4.5.3 Haematological profiles

Haematological profiles like total count of RBC and WBC, differential count of WBC, platelet counts and haemoglobin percentage were studied before treatment, after 7^{th} and 14^{th} days of experimental periods .The results were presented for control (Table 10), TTX and PFP treated rats respectively (Table-11) .

Table-10: Haematological profile of Group–C (Rat treated with vehicle)

Haematological parameters		Normal rat	Treated with vehicle	
		1^{st} day M ± SD	7^{th} day M ± SD	14^{th} day M ± SD
i. Total RBC count (million/cc)		5.02 ± 0.10	4.925 ±0.08	4.91 ± 0.1
ii. Total WBC count (no/cc)		6.52 ±0.149	6.12 ± 0.178	6.05 ± 0.229
iii. Differential count of WBC in %	a. Neutrophil	63.4 ± 1.87	64 ± 1.41	63.6 ± 0.70
	b. Lymphocyte	33.05 ± 2.04	32.95 ± 1.08	32.75 ± 0.82
	c. Monocyte	0.75 ± 0.43	0.76 ± 0.25	0.74 ± 0.90
	d. Eosinophil	2.75 ± 0.80	2.73 ± 0.5	2.74 ± 0.25
iv. Platelet count no/cc (thousand)		335 ±1. 24	337 ±1.12	336 ± 1.16
v. Hemoglobin (%)		65.25 ± 0.829	64 ± 0.707	63.25 ± 0.829
vi. ESR (mm/1^{st} hour)		11.25 ± 1.299	11.75 ± 1.299	11.75 ± 0.829

Table-11: Haematological profile of Group-A and Group-B (Rat treated with TTX and PFL)

Haematological parameters		Normal rat	Treated with TTX		Treated with PFL	
		1st day M ± SD	7th day M ± SD	14th day M ± SD	7th day M ± SD	14th day M ± SD
i. Total RBC count (million/cc)		5.05 ± 0.05	3.60 ± 0.05	2.40 ± 0.25	3.65 ± 0.25	3.075 ± 0.50
ii. Total WBC count (no/cc)		6.525 ± 0.51	5.80 ± 0.10	5.07 ± .005	6.02 ±1.20	5.275 ± .025
iii. Differential count of WBC in %	a. Neutrophil	63.5 ± 2.29	60.2 ± 0.05	57.5 ± 0.50	61.5 ± 1.25	59.75 ± 0.25
	b. Lymphocyte	33 ±2.121	31.20 ± 0.05	25.05 ±0.20	30.75 ± 1.25	26 ± 0.50
	c. Monocyte	0.75 ± 0.433	0.55 ± 0.25	0.52 ± 0.025	0.50 ± .025	0.51 ± .025
	d. Eosinophil	2.75 ± 0.829	2.12 ± 0.005	2.05 ± 0.025	2.325 ±0.125	2.08 ± .075
iv. Platelet count no/cc		335 ± 1.25	352 ± .52	367 ± 05	350 ± .52	363.25 ± 1.25
v. Hemoglobin %		65 ± 3.24	61.75 ± 3.344	60.2 ± 4.898	64.3 ± 0.25	64.0 ± 0.25
vi. ESR (mm/1st hour)		11.25 ± 1.29	13.5 ± 1.25	16.5 ± 1.20	12.75 ± 0.25	14.16 ± .50

From the experimental data ,it was found that the haematological profiles such as RBC, WBC, % hemoglobin and differential count of WBC of the rats treated with TTX and PFL were decreased after experimental periods while those parameters of the control group rats remained more or less very similar. On the other hand, ESR and the platelet were increased in toxic compounds treated rats but those of control rats remained almost unchanged .

4.5.4 Monitoring the biochemical parameters

Biochemical paramerers of blood e.g SGOT, SGPT, SALP, serum bilirubin, serum creatinine and urea levels of rat blood serum were determined after administration of TTX and PFL at a dose of 2.25 μg/rat/day for 14 consecutive days. It was found that after administration of the compounds, all the parameters examined were significantly increased as compared to those of control group (Table -12 and 13).

Table-12: Effect of TTX on some biochemical parameters of rat blood after i.p. administration of 2.25 μg/rat/day for 14 consecutive days.

Biochemical parameters	Group-C, n = 4 $M_1 \pm SD_1$	Group-A, n = 4 $M_1 \pm SD_1$	% of change	t_c	t_s	Remark
SGPT (IU/L)	8.75 ± 0.82	10 ± 0.50	14.28	2.31	12.26	S
SGOT (IU/L)	10 ± 0.70	11.70 ± 0.50	17	3.14	12.26	S
SALP (IU/L)	0.48 ± 0.027	0.57 ± 0.075	16.58	2.69	12.26	S
Serum bilirubin (m mol/L)	0.317 ± .048	0.38 ± 0.025	19.87	2.03	12.26	S
Creatinine (mg %)	0.571 ± .018	0.62 ± .075	8.77	2.65	12.26	S
Urea (mmol/L)	17.75 ± 0.84	21.25 ± .05	21.25	6.15	12.26	S

Table- 13: Effect of PFL on some biochemical parameters of rats blood after i.p. administration of 2.25 μg/rat/day for 14 consecutive days.

Biochemical parameters	Group-C, n = 4 $M_1 \pm SD_1$	Group-A, n = 4 $M_1 \pm SD_1$	% of change	t_c	t_s	Remark
SGPT (IU/L)	8.75 ± 0.82	9.70 ± 0.25	10.85	1.76	9.31	S
SGOT (IU/L)	10 ± 0.70	11.5 ± 0.50	15	3.03	9.31	S
SALP (IU/L)	0.48 ± 0.027	0.55 ± 0.04	12.75	3.66	9.31	S
Serum bilirubin (m mol/L)	0.317 ± .048	0.33 ± .075	6.45	0.624	9.31	S
Creatinine (mg %)	0.571 ± .018	0.61 ± 0.75	7.01	0.825	9.31	S
Urea (mmol/L)	17.75 ± 0.84	18.5 ± 0.50	4.51	5.71	9.30	S

'S' indicates significance

From the experimental data it was found that the amount of SGPT, SGOT, SALP, bilirubin, creatinine and urea in serum of TTX treated rats were increased by 14.28%, 17%, 16.58%, 19.87, 8.77% and 21.25%. While those of PFP treated rates were increased by 10.85%, 15%, 12.75%, 6.54%, 7.01% and 4.51% as compared to the amount of those present in the serum of control rats respectively .Further , the effects were found to be more pronounced by TTX than PFL.

4.5.5 Effect of puffer TTX and PFL on rat tissue (histopathological examinations)

The histopathological examinations of the liver, kidney heart and lung of control and experimental rats were performed after intraperitoneal administration of water to the control group and toxic compounds ,TTX and PFL (300µl contain 2.25µg toxins/rat/day) to the experimental groups for 14 consecutive days . A marked detectable histopathological differences among the organs of control and toxic compounds treated rats were observed and their histopathological photographs were presented in the Fig. 10-21. It can be observed from the photographs that the tissues such as kidney, liver, lung and heart of control rats were unchanged and there was no congestion of blood vessels, cellular inflammation and necrosis. On the otherhand all the tissues of TTX and PFL treated rats were seriously affected. The severity of all these symptoms were further aggravated when the experimental rats were injected by higher doses. Remarkably , it was found that all the experimental rats were died within 24 hours at a dose of 25 µg/ml by both TTX and PFL .

The severity of changes observed in the different tissues of control TTX and PFL treated rats were summarized in the Table -14.

Table-14: Effect of TTX and PFL on rat tissues.

Concentration of toxin (μg/ml)	Tissue	Types of effectiveness	
		TTX (Treated)	PFL (Treated)
7.5	Liver	Severe congestion of blood vessels, evids, deposition of fat in the hepatocyte. No inflammation and necrosis.	Mild congestion of blood vessels, accumulation of fat within the hepatocyte. No inflammation and necrosis.
	Heart	Necrosis, mild congestion of blood vessels, inflammation and accumulation of fat within the cardio vascular cells.	Necrosis, congestion of blood vessels and complexities in the cardiac system and the cells are collapsed.
	Kidney	Inflammation, stromal oedema, vascular congestion and mild fatty change and cells are blocked off .	Inflammation stromal oedema and mild congestion of blood vessels.
	Lung	Mild congestion of blood vessels, inflammation and deposition of fat.	Mild congestion of blood vessels, inflammation and deposition of fat.
Control (300 μL of distilled water injected intraperitoneally)	All the tissues	There was no inflammation, necrosis, stromal oedema and congestion of blood vessels of the liver, lung, heart and kidney.	

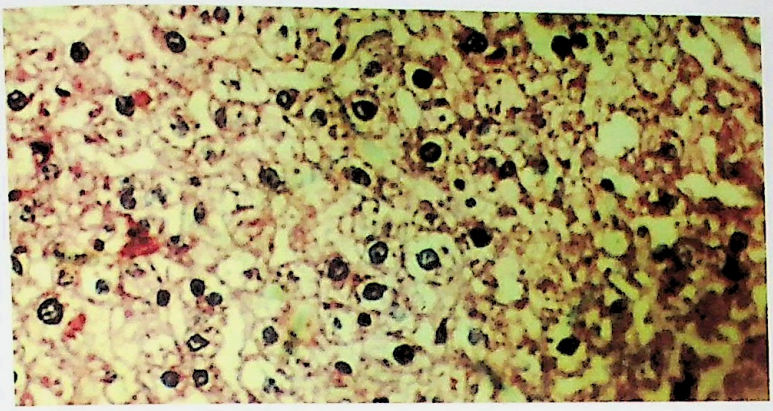


Fig. 17
Microphotograph of Liver (control) at **HE × 100**

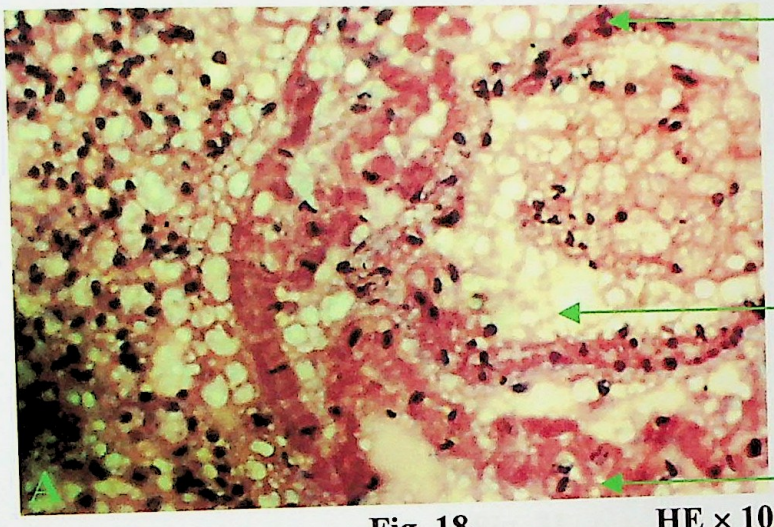


Fig. 18 **HE × 100**

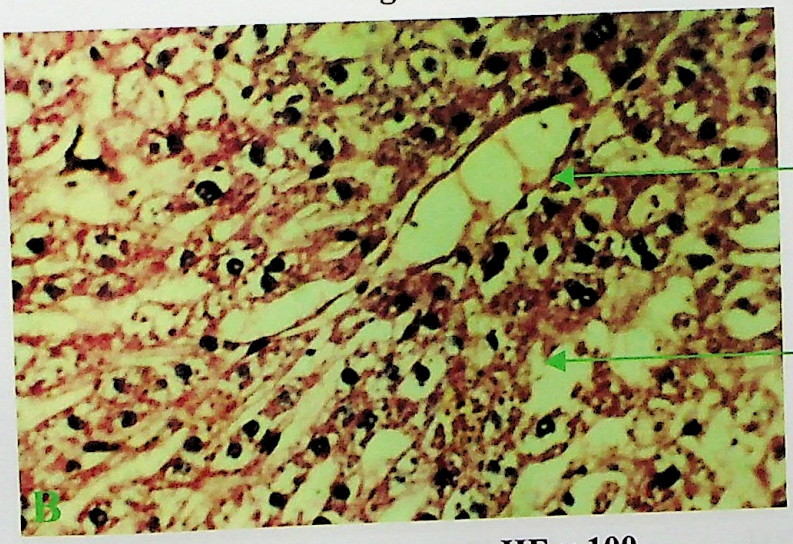


Fig. 19 **HE × 100**

Fig. 18 and 19: Microphotograph showing histopathological changes of liver of rat intraperitoneneally injected with 7.5 µg/mL of TTX and PFL. **A**: TTX treated and **B**: PFL treated

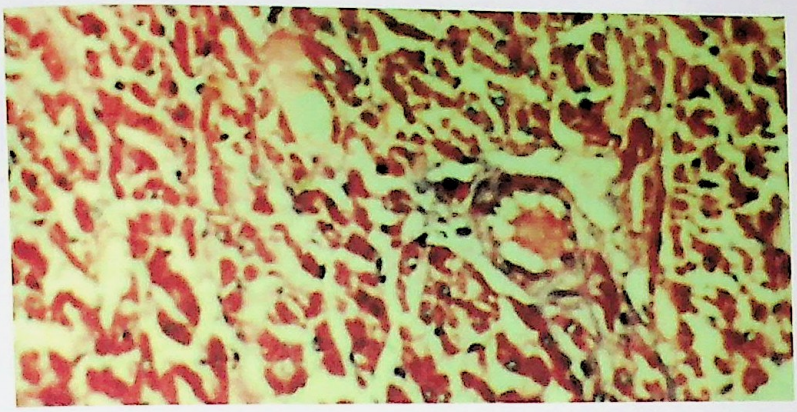


Fig. 20
Microphotograph of Heart (control) at **HE × 100**

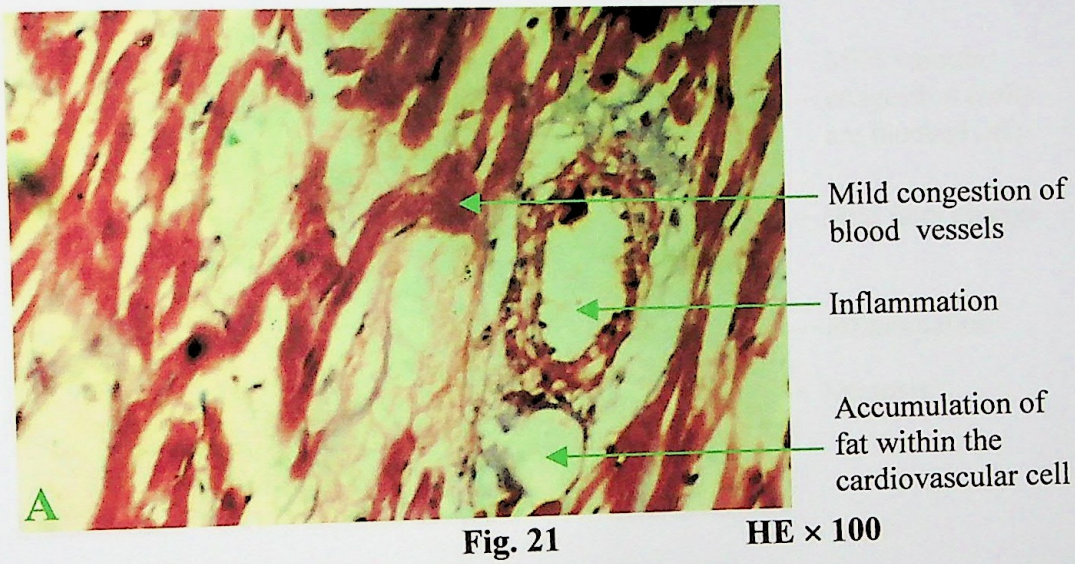


Fig. 21 **HE × 100**

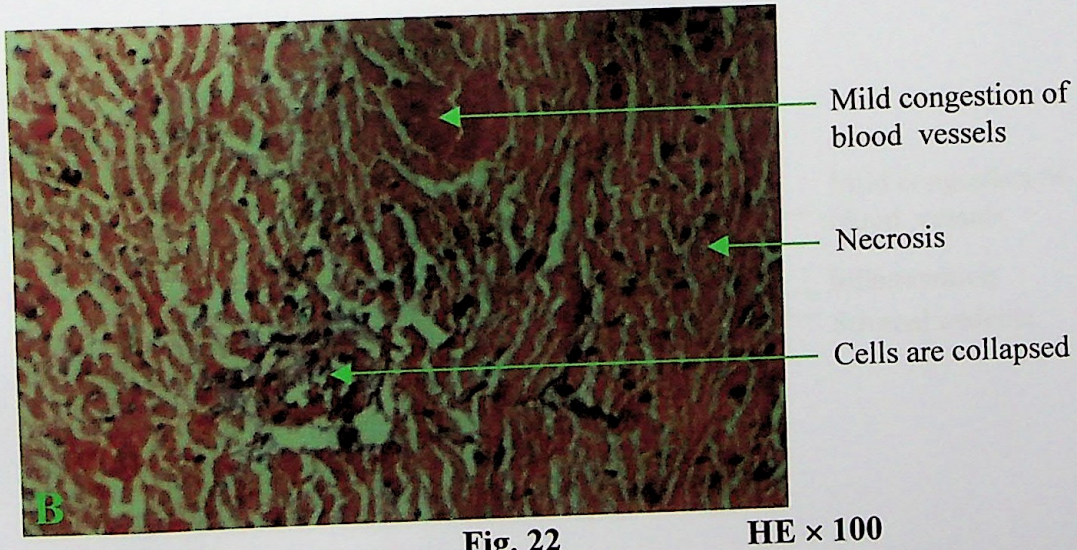


Fig. 22 **HE × 100**

Fig. 21 and 22: Microphotograph showing histopathological changes of heart of rat intraperitoneally injected with 7.5 µg/mL of TTX and PFL. **A**: TTX treated and **B**: PFL treated

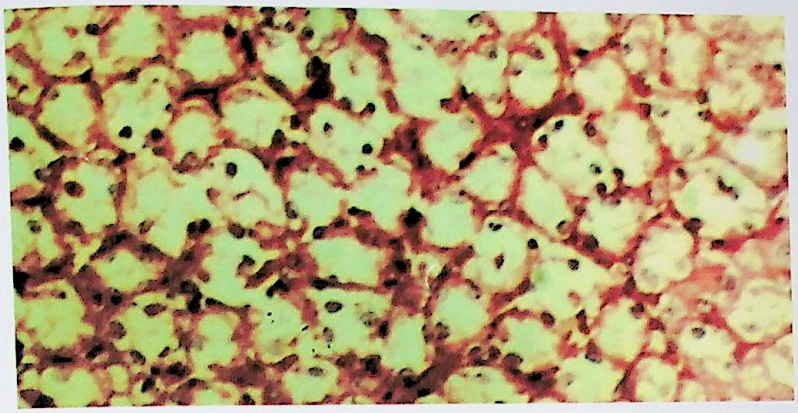


Fig. 23
Microphotograph of Kidney (control) at **HE × 100**

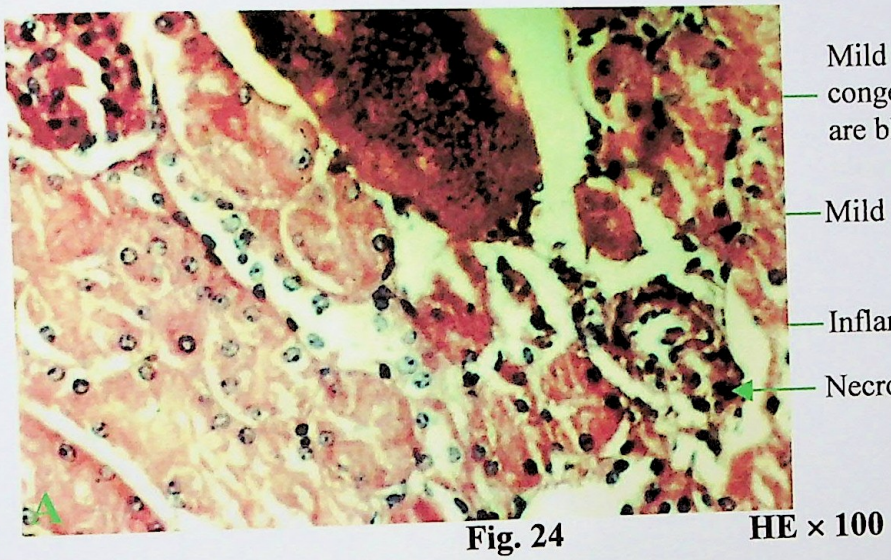


Fig. 24 **HE × 100**

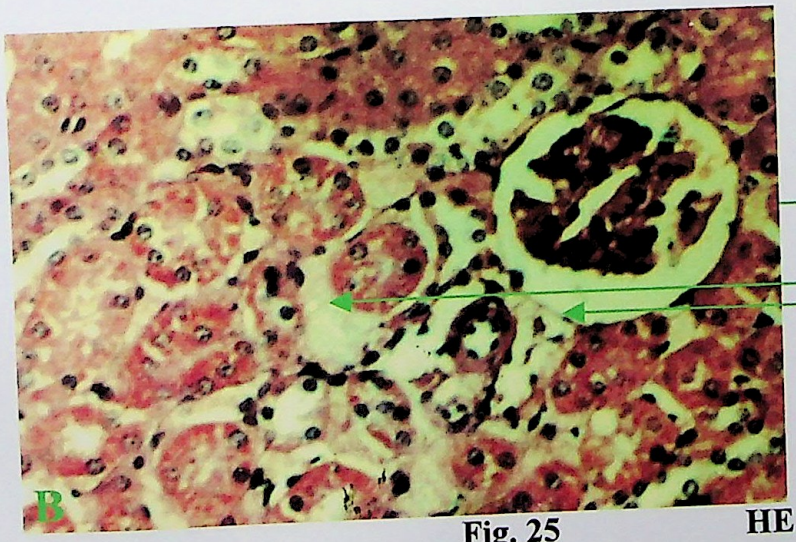


Fig. 25 **HE × 100**

Fig. 24 and 25: Microphotograph showing histopathological changes of kidney of rat intraperitoneally injected with 7.5 µg/mL of TTX and PFL. **A**: TTX treated and **B**: PFL treated

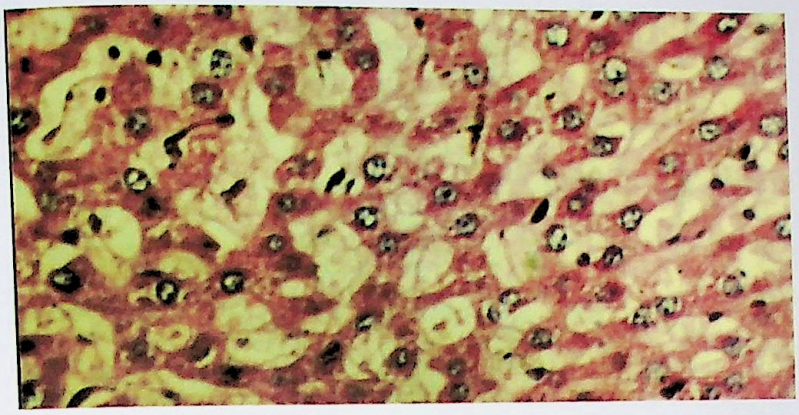


Fig. 26
Microphotograph of Lung (control) at **HE × 100**

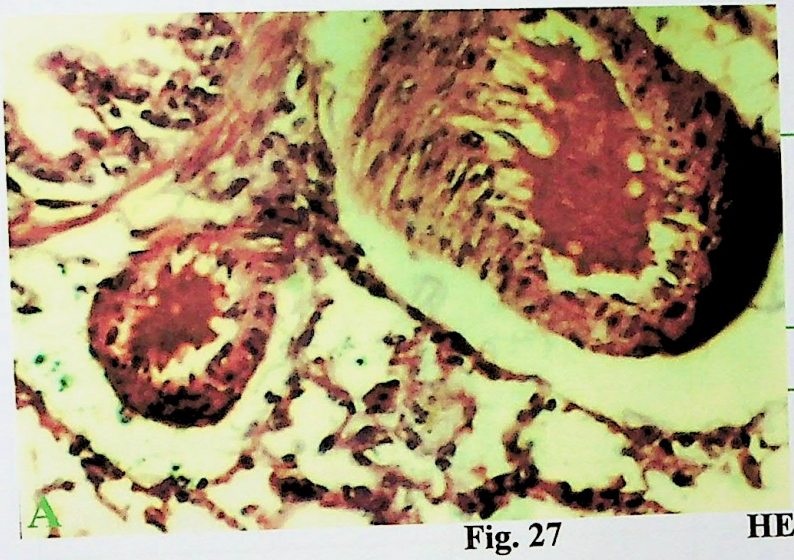


Fig. 27 **HE × 100**

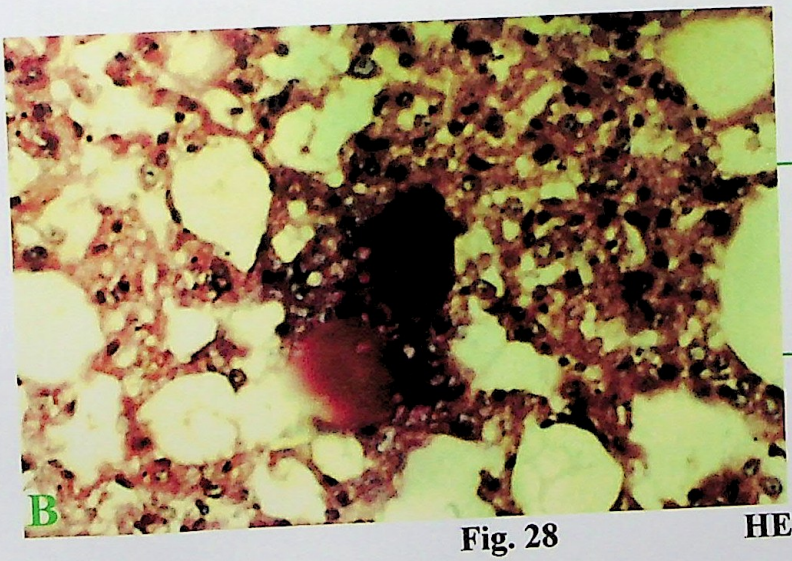


Fig. 28 **HE × 100**

Fig. 27 and 28: Microphotograph showing histopathological changes of lung of rat intraperitoneally injected with 7.5 µg/mL of TTX and PFL. **A**: TTX treated and **B**: PFL treated

4.6 Biological work

4.6.1 Invitro antibacterial activity

The toxins, TTX and PFP were tested for their antibacterial activity against both gram positive and gram negative bacteria in this study and the antibiotic, kanamycin-k was used as standard for comparison. As presented in the Table 20, both showed no detectable antibacterial activity.

Table-19: In vitro antibacterial activity of compounds, TXX and PFL.

Name of Bacteria	Diameter of the zone of inhibition (mm)		
	TTX (300 µg/disc)	PFL (300 µg/disc)	Kanamycin (30 µg/disc)
Gram-positive			
Bacillus subtilis	NS	NS	21
Staphylococcus aureus	NS	NS	17
Gram-negative			
Shigella sonnei	NS	NS	19
Shingle dysenteriae	NS	NS	17
Shigella flexinerie	NS	NS	17
Escherichia coli	NS	NS	19

'NS' Indicates no sensitivity

Fig. 29: Antibacterial activity against gram positive bacteria.

K = Kanamycin (antibiotic) zone

A = TTX and B = PFL

Fig. 30: Antibacterial activity against gram negative bacteria.

K = Kanamycin (antibiotic) zone

A = TTX and B = PFL

CHAPTER-5

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REFERENCE

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