STUDY OF TOXICITY AND HAEMAGGLUTINATING ACTIVITIY OF A PURIFIED INTRACELLULAR TOXIN AND EXTRACELLULAR CRUDE TOXIN ISOLATED FROM *PSEUDOMONAS AERUGINOSA*

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SUMMARY: The action of purified intracellular toxin (PIT) from Pseudomonas aeruginosa was examined in brine shrimp lethality bioassay. The LC_{50} of the PIT was calculated to be 25 µg/ml. The PIT agglutinated both albino rat and rabbit erythrocytes more potently than did extracellular crude toxin (ECT). Galactose and D-mannose, however, inhibited the agglutination property of PIT and ECT respectively. Intradermal injection of PIT caused changes on the tissues of rabbit skin at a lower dose than that of ECT.

Key Words: Pseudomonas aeruginosa toxin, Brine shrimp nauplli, Hemagglutination, Effect on rabbit tissue.

INTRODUCTION

Pseudomonas aeruginosa is one of the most common pathogens involved in hospital infection causing opportunistic infections in humans, particularly among immuno-compromised patients (1,2). This strain adheres to host cell surface, secretes noxious toxins and hydrolytic enzymes at the contact site. These factors seriously damage the host tissues and enable advanced infection (3). A remarkable number of bacterial factors have been postulated as playing a role in *Pseudomonas aeruginosa* infections, including exoenzyme S (ExoS), exoenzyme T (ExoT), exotoxin A, phospholipase C, alkaline protease, elastase, pili and non-pilus adhesins (4-8). Two of them such as phospholypase C and rhamnolipase produced by *Pseudomonas aeruginosa* may act synergistically to break down lipids and lecithin. Both may contribute to tissue invasion by their cytotoxic effects (9). Further, these strains are rich in adhesins which enable the bacterial adhesion to the target host cells. Most of the adhesins exhibit hemagglutinating activity (10). In addition to the fimbrial adhesins most *Pseudomonas aeruginosa* isolates grown to stationary phase at 28ºC exhibit prominent lectin activities (11). The hemagglutinating activity of the lectin was shown to be a result of their binding to erythrocyte receptors mainly associated with glycolipid (12).

In Bangladesh so far no work appears to have been conducted on the action of *Pseudomonas* toxin in rabbit tissue. In the present study, we have observed the effect of purified intracellular toxin (PIT) and extracellular crude toxin (ECT) causing histopathological changes after intradermally injecting into the rabbit tissue. The hemagglutinating activity of the toxins were investigated on both albino rat and rabbit erythrocyte. The action of PIT was examined on brine shrimp nauplii.

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Table 1: Brine shrimp lethality bioassay of PIT.

Test sample	Concentration of sample (μg/ml)	Log of concentration (log C)	Number of shrimp (each vial)	Number of survivor (Average)	% of mortality	LC_{50} μg/ml
PIT	10	1	10	8	20	25
	20	1.3	10	6	40	
	40	1.6	10	3	70	
	80	1.9	10	1	90	
	120	2	10	0	100	
Control	0	0	10	10	0	

Table 2: Hemagglutinating activities of PIT with 4% erythrocyte from albino rat and rabbit.

Protein sample	Absorbance at 280 nm	Concentration (mg/ml)	Degree of Hemagglutination	
			Rat	Rabbit
PIT	0.1	0.094	3^+	2^+
	0.08	0.069	2^+	1^+
	0.033	0.029	1^+	±
	0.02	0.015	±	-

Table 3: Hemagglutinating activities of ECT with 4% erythrocyte from albino rat and rabbit.

Protein sample	Absorbance at 280 nm	Concentration (mg/ml)	Degree of Hemagglutination	
			Rat	Rabbit
PIT	0.1	0.094	1^+	±
	0.08	0.069	±	-
	0.033	0.029	-	-
	0.02	0.015	-	-

3^+ Indicates complete aggregation of almost all the cells
2^+ Indicates lesser degree of agglutination where smaller number of cells remained free
1^+ Indicates all the cells were present in small aggregation of varying sizes.
± Indicates majority of cells were present in small aggregates.
- Indicates no agglutination.

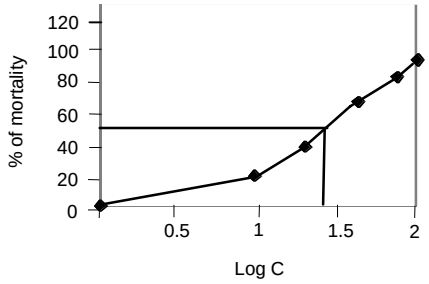
MATERIALS AND METHODS

The PIT and ECT used in the present study were isolated from no. 14 strain following the procedure previously reported by Shahanara and co-workers (Unpublished Ph.D thesis). The PIT was purified to homogeneity from the cells of *Pseudomonas aeruginosa* as confirmed by polyacrylamide disc gel electrophoresis (13). The molecular weight of the PIT was calculated to be about 62.000 by gel filtration on G-150 Sephadex (14). The PIT appeared to be a dimer on SDS-PAGE under denaturing and reducing conditions (15) and the molecular weights of the subunits were estimated to be 31.000 and 30.500.

Brine shrimp lethality bioassay of PIT

Brine shrimp lethality bioassay a rapid general bioassay for cytotoxic effect of bioactive compound, (16,17) has been used in the present study. *Artemia salina* leach (brine shrimp eggs) were hatched in small tank containing sea water as mature as nauplii (larvae) and these nauplii were used in the bioassay. The PIT sample of 10, 20, 40, 80 or 120 μg/ml were added in different vials containing 10 brine shrimp nauplii each. Three vials were used for each concentration and the control vials contained 10 nauplii in 5 ml sea water only. After 24 hours the number of survivors in each vial were counted.

Figure 1: Determination of LC_{50} value of the PIT.

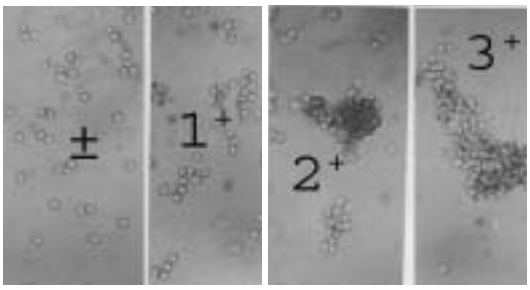


Hemagglutination study of PIT and ECT

Hemagglutinating activities of PIT and ECT were determined by using albino rat and rabbit erythrocytes following the method as described by Lin *et al.* (18). Each sample (0.2 ml) at 0.094, 0.069, 0.029 or 0.015 mg/ml was added to 0.2 ml of 4% erythrocyte suspension after dialysis against 5 mM phosphate buffer saline (PBS), pH 7.2. A control containing 0.2 ml PBS, pH 7.2, instead of sample solution and 0.2 ml of 4% erythrocyte suspension was used as reference. After incubation for 1 hour at 34^{o}C, the degree of hemagglutination was examined under microscope.

Hemagglutination inhibition study

The hemagglutination-inhibition test was performed in the presence of six different sugars with 20 to 110 mM each. Protein solution containing minimum concentration of protein needed for visible agglutination was used. Reactions were compared with a positive control (0.1 ml protein + 0.1 ml sugar solution + 0.2 ml 4% erythrocyte in PBS pH 7.2) and a negative control (0.2 ml PBS pH 7.2 and 0.2 ml of 4% erythrocyte) as reported by Atkinson and Trust (19).



± = Minimum Agglutination with 0.015 mg/ml,
1^+ = Agglutination with 0.029 mg/ml,
2^+ = Agglutination with 0.069 mg/ml,
3^+ = Agglutination with 0.094 mg/ml.

Figure 2: Photomicrograph showing agglutination of albino rat erythrocyte by PIT. The degree of agglutination increased with the increase of toxin concentration (mg/ml).

Histopathological study of PIT and ECT on rabbit tissue

The histopathological changes caused by PIT and ECT on rabbit tissue was observed using rabbits weighing between 400-550g (Animal Resources Division of ICDDRB, Mohakhali, Dhaka, Bangladesh). Two groups of 10 rabbits each was challenged with intradermal injection of 1 ml PIT and ECT at 0.01, 0.03, 0.06 or 0.1 mg/ml after dialysis against 5 mM phosphate buffer, pH 7.2. The control rabbits were injected with 1 ml of 5 mM phosphate buffer, pH 7.2 only. After 72 hours the rabbits were sacrificed under ether anesthesia. A wedge piece of skin was taken from the site of injection and preserved in 10% formalin. Tissue processing was done following the method described by Carleton (20) and Preece N (21). The Haematoxylin and Eosin stained slides were prepared according to Burkitt (22) and Humason (23) and were critically examined under microscope to evaluate histological changes.

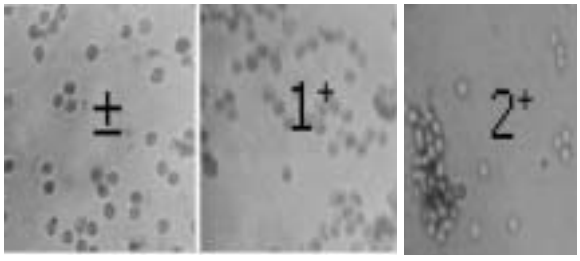
RESULTS

Cytotoxicity assay of PIT (Brine shrimp lethality bioassay)

In the brine shrimp lethality bioassay the protein showed positive result, indicating that the PIT was cytotoxic in nature. As shown in Table 1, the toxicity increased with the increase of sample concentration and a plot of log of concentration versus percentage of mortality gave an almost linear correlation (Figure 1). From the graph, the LC_{50} was determined by extrapolation and the LC_{50} value of the PIT was calculated to be 25 μg/ml.

Hemagglutinating activities of PIT and ECT

The PIT and ECT agglutinated specifically albino rat and rabbit erythrocytes. The hemagglutination potency of



± = Minimum Agglutination with 0.029 mg/ml,
1^+ = Agglutination with 0.069 mg/ml,
2^+ = Agglutination with 0.094 mg/ml.

Figure 3: Photomicrograph showing agglutination of rabbit erythrocyte by PIT.

Table 4: Hemagglutination inhibition assay of PIT and ECT using albino rat erythrocyte by different sugars.

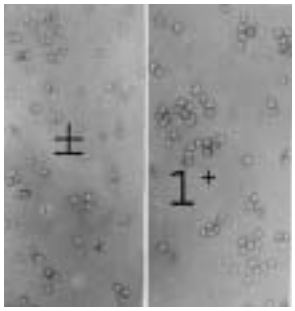
Protein sample	Sugar	Concentration (mM)							Inhibition
		20	30	35	40	50	80	110	
PIT	D-glucose	-	-	-	-	-	-	-	NI
	D-mannose	-	-	-	-	-	-	-	NI
	D-galactose	-	+						I
	N-acetyl-D-glucosamine	-	-	-	-	-	-	-	NI
	Methyl-α-D-galactopyranaside	-	-	-	-	+			I
	Methyl-β-D-galactopyranaside	-	-	-	-	+			I
ECT	D-glucose	-	-	-	-	-	-	-	NI
	D-mannose	-	+						I
	D-galactose	-	-	-	-	-	-	-	NI
	N-acetyl-D-glucosamine	-	-	-	-	-	-	-	NI
	Methyl-α-D-galactopyranaside	-	-	-	-	-	-	-	NI
	Methyl-β-D-galactopyranaside	-	-	-	-	-	-	-	NI

Table 5: Hemagglutination inhibition assay of PIT and ECT using rabbit erythrocyte by different sugars.

Protein sample	Sugar	Concentration (mM)							Inhibition
		20	30	35	40	50	80	110	
PIT	D-glucose	-	-	-	-	-	-	-	NI
	D-mannose	-	-	-	-	-	-	-	NI
	D-galactose	-	+						I
	N-acetyl-D-glucosamine	-	-	-	-	-	-	-	NI
	Methyl-α-D-galactopyranaside	-	-	-	-	+			I
	Methyl-β-D-galactopyranaside	-	-	-	-	+			I
ECT	D-glucose	-	-	-	-	-	-	-	NI
	D-mannose	-	+						I
	D-galactose	-	-	-	-	-	-	-	NI
	N-acetyl-D-glucosamine	-	-	-	-	-	-	-	NI
	Methyl-α-D-galactopyranaside	-	-	-	-	-	-	-	NI
	Methyl-β-D-galactopyranaside	-	-	-	-	-	-	-	NI

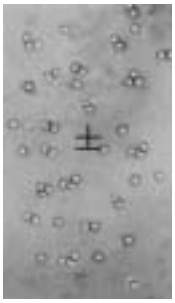
NI : No inhibition I : Inhibition

Figure 4: Photomicrograph showing agglutination of albino rat erythrocyte by ECT.



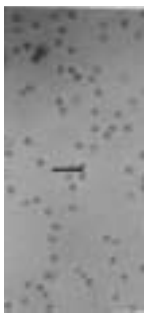
± = Minimum Agglutination with 0.069 mg/ml,
1^+ = Agglutination with 0.094 mg/ml.

Figure 5: Photomicrograph showing agglutination of rabbit erythrocyte by ECT.



± = Minimum Agglutination with 0.094 mg/ml.

Figure 6: Photomicrograph showing no agglutination of the erythrocyte by 5 mM PBS pH 7.2 (Control).



PIT was more pronounced than ECT and are shown by photographic representation in Figures 2-6 and the results are indicated in Tables 2 and 3. The minimum hemagglutination dose was calculated to be 0.015 and 0.029 mg/ml for PIT and 0.069 and 0.094 mg/ml for ECT using albino rat and rabbit erythrocytes respectively.

Hemagglutination inhibition Studies

Hemagglutination inhibition of albino rat and rabbit erythrocytes by PIT and ECT are given in the Tables 4 and 5. The results showed that the hemagglutination activity of PIT was inhibited specifically with galactose and galactose containing saccharides, while that of the ECT was inhibited specifically by D-mannose.

Effects of intradermal injection of PIT and ECT on rabbit tissue

Histopathological examination of the tissue of the rabbit showed clear distinction between control and experimental rabbits and the results are presented photographically in the Figures 7 - 12. Different concentration of toxins exhibited distinctly different effects on the tissues of rabbit's skin suggesting that the toxicity is dose dependent. The results, summarized in the Table 6, indicated that the PIT caused changes at any concentration applied, while the ECT exerted comprehensive changes at higher concentrations only. In control rabbits all the normal structure of epidermis and dermis were well preserved.

DISCUSSION

The PIT showed strong cytotoxic activity in brine shrimp lethality bioassay indicating that PIT was cytotoxic in nature. The increase in the toxin concentration increased the mortality rate indicating that toxicity is dose dependent. Iglewisky and Kabat (24) reported that *Pseudomonas aeruginosa* toxin (PA toxin) inhibited protein synthesis in a reticulocyte cell-free system by blocking an elongation step of polypeptide assembly. Another report showed that the mature, water soluble, acidic 29-KDa *Pseudomonas aeruginosa* cytotoxin attacked plasma membrane of a great variety of eukaryotic cells resulting in a channel of 1-2 nm in diameter (25) and forms ion channel in planar lipid bilayers (26). Their study suggested that our PIT might have exhibited similar effects to PA toxin or pore-forming cytotoxin of *Pseudomonas aeruginosa* as it exhibited lethal effect on brine shrimp nauplii.

Though both the PIT and ECT agglutinated albino rat and rabbit erythrocyte, but the agglutinating property of PIT was more pronounced than that of ECT. The hemagglutinating action of ECT may be due to the presence of adhesins in *Pseudomonas aeruginosa* (10). Further, the erythrocyte of albino rat was agglutinated more readily than that of rabbit. Pseudomonas aeruginosa produced lactin (11) of which hemagglutinating activity was shown

Table 6: Dose dependent effects of PIT and ECT on rabbit tissues.

Concentration of toxin (mg/ml)	Types of effectiveness	
	PIT	ECT
0.01	Slight changes i.e., vascular congestion in upper dermis, mild stromal oedema involving upper dermis, perivascular round cell infiltrate upper dermis.	No significant change occured. Epidermis: Intact, no evidence of ulceration. Dermis: No congestion, cellular inflammatory infiltrate, oedema or necrosis.
0.03	Mild changes i.e., vascular congestion in upper dermis, mild stromal oedema involving whole dermis, perivascular and patchy focal round cell infiltrate all through dermis.	No significant change occurred. Epidermis: Intact, no evidence of ulceration. Dermis: No congestion, cellular inflammatory infiltrate, oedema or necrosis.
0.06	Moderate changes i.e., vascular congestion, whole extravasation of red blood cells, oedema involving dermis, subcutaneous area; perivascular and diffuse inflammatory cellular infiltrate involving dermis.	Slight changes i.e., vascular congestion in upper dermis, mild stromal oedema involving upper dermis, perivascular round cell infiltrate upper dermis.
0.1	Severe changes i.e., marked vascular congestion and extravasation of red cells involving upper and lower dermis; vasculitis, perivascular and diffuse round cell infiltrate involving whole dermis with extention into underline muscle coat; oedema involving whole dermis, subcutaneous region and muscle coat; areas of necrosis.	Mild changes i.e., vascular congestion in upper dermis, mild stromal oedema involving whole dermis, perivascular and patchy focal round cell infiltrate all through dermis.
Control (1ml 5mM sodium phosphate buffer pH 7.2)	Epidermis: Intact, no evidence of ulceration. Dermis: No congestion, cellular inflammatory infiltrate, oedema or necrosis.	Epidermis: Intact, no evidence of ulceration. Dermis: No congestion, cellular inflammatory infiltrate, oedema or necrosis.

to be a result of their binding to erythrocyte receptors mainly associated with glycolipid (12). Their study suggested that the saccharides present in albino rat erythrocyte is more preferable for binding the lectins than that of rabbit erythrocyte.

The PIT and the ECT are quite different from each other in protein structure since the agglutinating property of ECT was inhibited by mannose while that of the PIT was inhibited by galactose. The results suggested that the PIT might be a type of galactophilic lectin. It was reported that *Pseudomonas aeruginosa* produced galactophilic lectin (PA-IL) that preferentially bind I-antigen on human erythrocytes (27). Similarly, though the ECT was not purified, it could be regarded as a mannose binding lectin, since the agglutination of erythrocyte was inhibited in the presence of mannose. Gilboa-Garber (11) reported the presence of a lectin (PA-IIL) in *Pseudomonas aeruginosa*, which was fucose- and mannose-binding.

Again the PIT and ECT from *Pseudomonas aeruginosa* showed intense toxic activity on the tissues of rabbit skin. Severity of toxicity was more pronounced with the increase of dose concentration. Though the PIT acted well at low concentration as compared to ECT, they showed similarity in their action. This action of PIT and ECT could be related to that of phospholipase C and rhamnolipase produced by *Pseudomonas aeruginosa* which break down lipids and lechithin and contribute to tissue invasion by their cytotoxic effects (9). Another report also described by Nicas *et al.* (3) that *Pseudomonas aeruginosa* possesses a variety of exoproducts (enzyme, toxins) which are responsible for direct tissue destruction in lung.

Figure 7: Photomicrograph showing no significant change of the rabbit skin intradermally injected with 0.01 mg/ml (a) and 0.03mg/ml (b) of ECT.

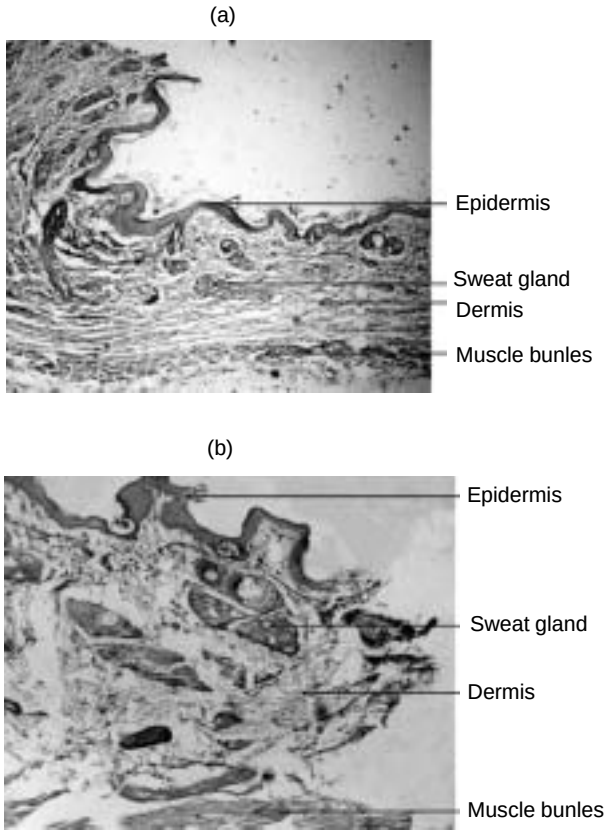


Figure 8: Photomicrograph showing slight histopathological changes of the rabbit skin intradermally injected with 0.01 mg/ml of PIT (a) and 0.06 mg/ml of ECT (b).

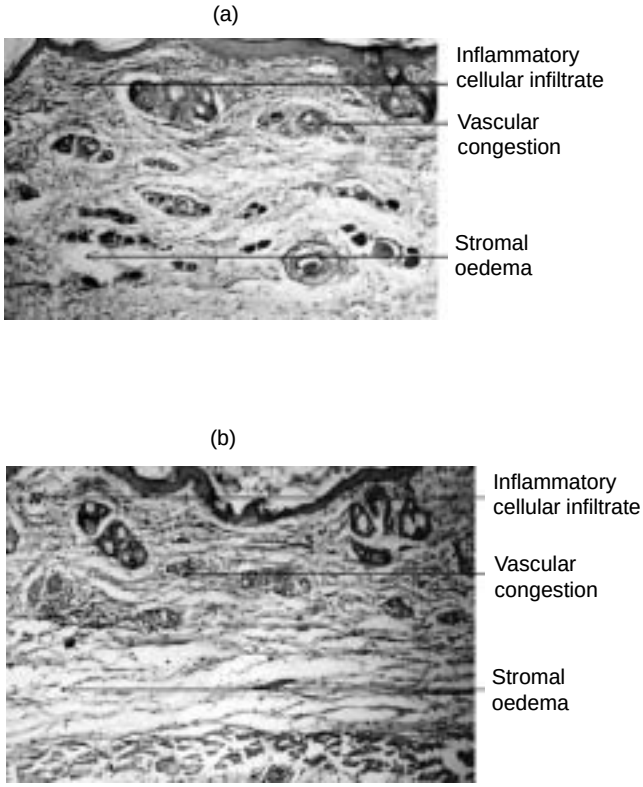
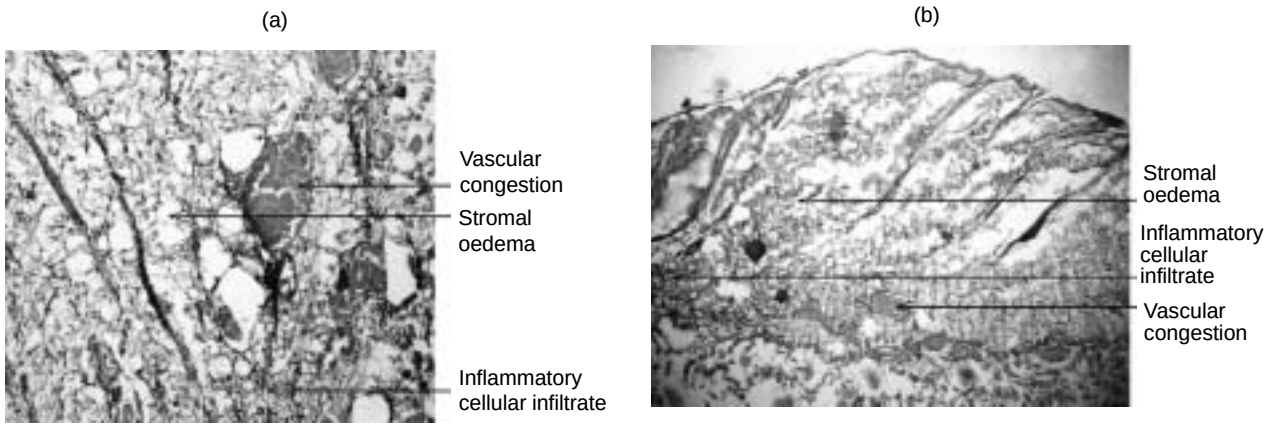


Figure 9: Photomicrograph showing mild histopathological changes of the rabbit skin intradermally injected with 0.03 mg/ml of PIT (a) and 0.1 mg/ml of ECT (b).



In the present study, at higher dose level (0.1 mg/ml) of PIT, the histopathological changes observed in rabbit tissue were marked vascular congestion and extravasations of red blood cells involving upper and lower dermis. There were vasculitis, perivascular and diffuse round cell infiltrate involving whole dermis with extension into underline muscle coat. Oedema involving whole dermis, subcutaneous region and muscle coat was observed including

Figure 10: Photomicrograph showing moderate histopathological changes of the rabbit skin intradermally injected with 0.06 mg/ml of PIT.

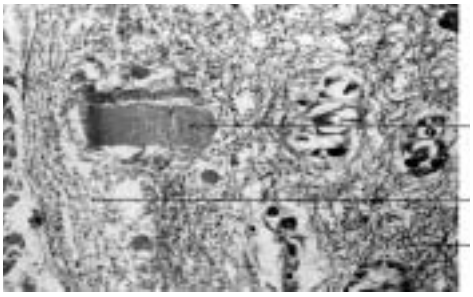


Figure 11: Photomicrograph showing severe histopathological changes of the rabbit skin after intradermally injected with 0.1 mg/ml PIT.

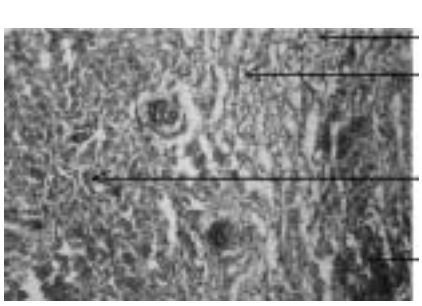
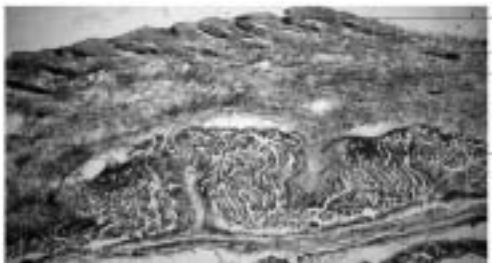


Figure 12: Figure 12 Photomicrograph showing histopathological features of rabbit skin intradermally injected with 1 ml of 5 mM sodium phosphate buffer pH 7.2 (Control).



areas of necrosis. Kuo *et al.* (28) reported that the *S. pyogenes* A-20 produced streptococcal pyrogenic exotoxin B appeared to cause necrosis of epidermis, dermis and subcutaneous fat on skin tissues of a mouse model 48 hours after inoculation in air pouches with the strains.

Finally, we can conclude that the PIT could have toxic as well as lectin properties though the function of its subunits was not clarified separately.

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