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Effects of Purified Indian Cattle Tick *Rhipicephalus microplus* Saliva Toxins on Various Enzymes in Blood Serum, Liver and Muscles of Albino Mice

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Abstract

In the present investigation, *in vivo* effects of purified ticks' saliva toxin were evaluated on the level of certain important cellular metabolic enzymes *i.e.* acid phosphatase (ACP), alkaline phosphatase (ALP), glutamate pyruvate transaminase, glutamate oxaloacetate transaminase and lactic dehydrogenase. For this purpose, sub-lethal doses, 40% and 80% of 24 h LD_{50} purified saliva toxins of *Rhipicephalus microplus* (Canestrini, 1888) were injected subcutaneously in the albino mice. In treated mice saliva toxins targeted membrane-bound enzymes *i.e.* serum acid phosphatase and alkaline phosphatase, its level was increased from 118.30% to 163.63% at the 6th hr in comparison to the control. Besides this, the levels of serum glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT) and lactic dehydrogenase (LDH) also increased up to 161.11% (at 6th hr), 148.27 (at 8th hr) and 125.45% (at 6th hr) respectively in comparison to control. An increase in the level of LDH showed insufficient oxygen supply, massive disintegration of cells and leakage of the enzyme into the circulation. It clearly indicated the toxic effects of saliva toxins on the membrane of blood cells, hepatocytes and myocardial muscle cell functions in albino mice. On the other hand activity of acetyl cholinesterase was reduced by 65.51% at the 6th hr of the saliva toxin injection in comparison to the control. This inhibition of acetyl cholinesterase activity caused the accumulation of acetylcholine molecules at the synaptic junctions and led to prolonged activation of acetylcholine receptors. It caused permanent stimulation of nerves and muscle cells that may result in muscular paralysis and finally death of the animal.

Keywords

Rhipicephalus microplus, Serum, Liver and Rectus Abdominis, Gastrocnemius, Muscle, Atria and Ventricle Acid Phosphatase (ACP),

Alkaline Phosphatase (ALP), Glutamate Pyruvate Transaminase (GPT) and Glutamate Oxaloacetate Transaminase (GOT), Lactic Dehydrogenase (LDH) and Acetylcholinesterase (AchE)

1. Introduction

Ticks are obligate hematophagous ectoparasites which rely on host blood to derive nutrition. Ticks are major vectors of a number of pathogens *i.e.* viruses, rickettsiae, spirochetes, bacteria, fungi, protozoa and filarial nematodes in humans, livestock and wild animals [1]. These affect the health and survival of both domestic and wild animals worldwide [1]. Ticks obtain nutrients from blood meals to fulfill their metabolic requirements [1]. During feeding ticks discharge salivary secretion or cocktail of diverse molecules in the blood of the host. Tick saliva especially secretes anti-coagulatory molecules mainly salivary proteins which inhibit blood clotting and assist in uninterrupting blood feeding by rupturing host skin [2]. Through blood feeding these transfer or inject pathogens into the bloodstream and easily transmit various disease pathogens [3] [4]. Transmission of pathogens results in various diseases and morbidities causing more serious economic losses to humans and livestock [5]. The main factors of tick parasitism are blood-sucking and saliva secretion, host immune status, age, breed and local ecology [6].

The tick's fat body plays an essential role in energy storage and utilization. Salivary protein insists ticks have blood meal and the whole process depends upon the injection of tick saliva and its mixing in the blood [7]. Ticks saliva contains so many proteins which display strong pharmacological and immunological activities. These proteins are mainly used in host invasion for blood feeding and are known as evasions. Salivary proteins assist ticks in feeding for more than 8 - 10 days without being noticed by the host animal. These block secretion of the host's chemokines and prevent painful inflammation. Ticks also generate iron-bound proteins *i.e.* ferritins during blood feeding [8] [9]. Lipocalins are abundant proteins in the saliva of both soft and hard ticks. Lipocalin isolated from *Ixodes ricinus* (LIR) was associated with the modulation of inflammation [10]. Tick saliva proteins are involved in several physiological roles, including egg development, transportation of proteins, immunity and anti-microorganism, anti-coagulant, and adhesion. Thus, tick saliva not only controls host hemostasis and wound healing but also subverts the host immune response to avoid tick rejection that creates a favorable niche for the survival and propagation of diverse tick-borne pathogens [1].

Tick saliva toxins cause significant alterations in various biomolecules after *in vivo* injection in albino mice. These significantly target various metabolic enzymes and severely affect the host's physiological and metabolic functions. Similar to other toxic substances these severely affect the activity of various metabolic

enzymes mainly acetylcholinesterase [11], Lactate dehydrogenase (LDH) level [12] and impose cytotoxicity in muscles, serum and liver cells (LDH) level [12]. These cause cell necrosis and death, display hepatoxicity and damage to cardiac muscles and subsequently increase the LDH activity in the serum [13]. Similarly, plasma and blood cholinesterases level also get increased that also displays toxicity in the liver [14]. Two important enzymes glutamic oxaloacetic transaminase (GOT) and serum glutamic pyruvic transaminase (GPT) were found in the liver, heart cells, muscle tissue, pancreas and kidney. Alteration in levels of these enzymes displays damage to these tissues [15]. Herbicides such as atrazine severely affect lipid peroxidation in fish *Channa punctatus* (Bloch.) that also targets liver hepatocytes [16]. Ammonia poisoning also causes liver damage and alters the levels of these enzymes [17]. Liver is also damaged due to the effect of pollutants like bisphenol A that induces hepatotoxicity and causes severe oxidative stress [18]. In the present study level of various metabolic enzymes was determined after injecting a sub-lethal dose of purified tick saliva toxins in mice models. Most of these enzymes are involved in toxicity reduction and their level displays toxic effects in blood serum, liver, tissue, kidney damage and neuronal effects.

2. Experimental

2.1. Isolation and Purification of Ticks' Saliva Toxins

The living *Rhipicephalus microplus* was collected from rural areas of the Gorakhpur district. Living ticks were collected in sterilized plastic vessels and immobilized by quick freezing at −20˚C. Whole body homogenate was prepared in phosphate buffer saline (50 mm, pH 6.9) with the help of a power homogenizer. The homogenate was centrifuged at 24,000 XG at 4˚C for 30 minutes and the supernatant was used as crude saliva toxins.

2.2. Preparation of Homogenate

Rhipicephalus microplus were homogenized properly in a glass-glass homogenizer in 5 ml of different solubilizing buffers such as Triton X-100, PBS buffer (pH 6.9), 10% TCA, Tris-EDTA and absolute ethanol separately. Homogenate was centrifuged at 12,000 rpm in cold for 30 minutes and the supernatant was separated out. Total protein contents were estimated in the different supernatants according to Lowry's (1951) method [19].

Besides this, proteins (tissue) were solubilized in other solubilizing agents (Triton X-100, PBS, 10% TCA, and EDTA + Tris) in different combinations. Homogenate was centrifuged at 10,000 XG for 30 min and proteins were estimated in supernatant according to Lowry's (1951) method [19].

2.3. Purification of Saliva Toxins/Proteins from *Rhipicephalus microplus*

Proteins were eluted on a Sepharose CL-6B-200 a double cavity gel filtration column with sintered disc filtered in the bottom having a height of 1 meter in 25

mm diameter. A known volume *i.e.*, 5 ml of toxin proteins solubilized in PBS was loaded in the column. The flow rate was maintained between 1 ml/minute by a continuous supply of PBS buffer (pH 6.7) in a cold room. Eluted fractions were collected at a fixed time interval using a Pharmacia fraction collector and the values of protein concentration in different eluted fractions will be plotted on the graph; absorbance in each fraction was determined at 280 nm using Shimadzu spectrophotometer (UV 2001 PC). Further, the absorbance of the same fractions was taken at 640 nm after protein estimation by Lowry's (1951) method [19].

2.4. Elution of the Saliva Toxin Protein through Gel Filtration Column

The column was tightly held erect with a stand. Elution of the saliva toxin proteins through the gel filtration column was done at the flow rate of 5 ml/minute.

2.5. Fraction Collection

Eluted fractions of saliva protein/toxins were collected manually at a fixed time interval at a constant flow rate. Total of 140 fractions were collected. The eluted fractions were observed for the detection of the presence of saliva toxin protein at a wavelength of 280 nm. Absorbance was taken on a Shimadzu spectrophotometer (UV 2001 PC) at 640 nm after protein estimation of the eluted fraction by Lowry's (1951) method [19]. A graph was plotted between absorption at 280 nm and fraction numbers to show the elution pattern of *Rhipicephalus microplus* saliva toxins/proteins.

2.6. Molecular Weight Determination of Purified Saliva Toxin Proteins

The range of molecular weight of different proteins/toxins in the purified tick saliva toxins/proteins was determined by running the proteins of known molecular weight through the Sepharose CL-6B gel column as done previously at the same flow rate. A calibration curve was drawn between Ve/Vo log M between elution volume in fractions and molecular weight of different known proteins and compared with elution of proteins from *Rhipicephalus* (*Boophilus*) *microplus* saliva proteins/toxins at the same flow rate and same fraction.

2.7. Lyophilization of Eluted Saliva Toxins/Proteins

The eluted fractions of saliva toxin were pooled and lyophilized to get the desired concentration of saliva toxin.

3. Biological Activity of the Purified Saliva Proteins/Toxins

Biological activity testing of *Rhipicephalus microplus* saliva protein/toxins was determined in albino mice serially known volumes of the purified saliva toxins were injected intra-peritoneal.

3.1. Determination of Lethality of *Rhipicephalus microplus* Toxins/Proteins

The albino mice were injected subcutaneously with the purified saliva toxin of different serial concentrations and LD_{50} was determined at the intervals of 24 hours. Deformities such as paralysis and neurotoxic effects were also recorded. Six albino mice were injected with serial concentrations of the saliva toxin to determine LD_{50}. Mortality was determined by using Abbot's formula. The LD_{50} values were calculated at which half of the test animals died. The lethal concentration for 40% and 80% of the LD_{50} was determined with the doses-mortality regression line plotted on the log by using the Probit method (Spier, R.E. 1982) [20]. The confidence limits were calculated at 95% probability levels.

3.2. Dialysis of Lyophilized Saliva Toxin

The dialysis bag of cellulose membrane was boiled for 10 min in a large volume of 2% (w/v) sodium bicarbonate and 1 mM EDTA (pH 8.0) and then the membrane was rinsed thoroughly in distilled water. The membrane was then cooled and stored at 4˚C. The membrane was washed again with distilled water inside and outside before use. The lyophilized saliva toxin protein was filled in the dialyzing bag and dialyzed against three changes of phosphate buffer (50 mM, pH 6.9) to remove the excess salt from the lyophilized saliva toxin protein solution.

3.3. Effect of Purified *Rhipicephalus microplus* Saliva Protein/Toxins on Certain Serum Enzymes (*in Vivo*)

The albino mice were injected with sub-lethal doses (40% and 80% of 24-h LD_{50}) of purified *Rhipicephalus microplus* saliva protein/toxins. The injected mice were sacrificed at 2 hours, 4 hours, 6 hours, 8 hours and 10 hours after the treatment and blood was collected to get the serum. For comparison mice treated with only PBS buffer were sacrificed and considered as control. The same method was followed for the collection of blood and isolation of serum as mentioned before.

3.4. Determination of Acid Phosphatase

Changes in acid phosphatase level were determined according to the method of Andrech and Szeypiaske, (1947) [21] and modified by Bergmeyer (1967) [22]. For this purpose, 0.2 ml of serum was taken in a test tube and 1.0 ml of acid buffer substrate solution (prepared by dissolving 0.41 gm citric acid, 1.125 gm sodium citrate and 165 mg p-nitrophenyl phosphate sodium salt to 100 ml of double distilled water) was added. Contents were mixed thoroughly and incubated for 30 minutes at 37˚C. Then 4.0 ml of 0.10-N NaOH solution was added to the incubated mixture. A yellow color developed which was measured at 420 nm. Standard curves were drawn with p-nitrophenol. Enzyme activity was expressed as the amount of p-nitrophenol formed/30 min/mg protein.

3.5. Determination of Alkaline Phosphatase

Changes in alkaline phosphatase levels were determined according to the method of Andrech and Szeypiaske (1947) [21], and modified by Bergmeyer (1967) [22]. For this purpose, 0.10 ml of serum was taken in a test tube and added 1.0 ml of alkaline buffer substrate to it. Alkaline buffer substrate was prepared by addition of 375 mg glycine, 10 mg $MgCl_2 \cdot 6H_2O$ and 165 mg p-nitrophenyl phosphate sodium salt in 42 ml of 0.1 N NaOH. The mixture was made up to 100 ml with double distilled water. The incubation mixture was mixed thoroughly and incubated for 30 minutes at 37˚C. 10.0 ml of 0.02 N NaOH was then added to the incubation mixture. The reaction was stopped due to excess NaOH. In reaction p-nitrophenol was formed as a result of hydrolysis of p-nitrophenyl phosphate, it provided a yellow color with NaOH. Optical density was measured at 420 nm. The standard curve was drawn with the help of different concentrations of p-nitrophenol. Enzyme activity was expressed as μ moles of p-nitro phenol formed/30 min/mg protein.

3.6. Determination of Serum Glutamate Pyruvate Transaminase

Changes in serum glutamate pyruvate transaminase (GPT) levels were measured according to the method of Reitman and Frankel (1957) [23]. For this purpose 0.10 ml of non-hemolysed serum was taken and 0.50 ml of GPT substrate (0.292 gm of *α*-ketoglutaric acid and 17.8 gm of DL alanine was taken and added 1 N NaOH slowly with mixing until all solids were dissolved and sufficient buffer was added to adjust the pH 7.4) The buffer solution was prepared by dissolving 13.97 gm K_2HPO_4 and 2.69 gm KH_2PO_4 in 1000 ml distilled water. The contents were mixed well and incubated at 37˚C for one hour. Now 0.50 ml of 2 - 4 dinitrophenyl hydrazine solution (dissolved 0.198 gm of 2,4-dinitrophenyl hydrazine in 1 N HCl to make 1000 ml.) was added and contents were left to stand for 15 minutes at room temperature. Then 5.0 ml of 0.4 N NaOH (1.6 gm NaOH dissolved in 100 ml distilled water) was added and mixed well and allowed to stand at room temperature for 20 minutes. The optical density was read at 505 nm setting the instrument to zero with water. The standard curve was prepared by using oxaloacetic acid as a working standard. The enzyme activity was expressed in units of glutamic-pyruvate transaminase activity/mg of serum.

3.7. Determination of Serum Glutamate Oxaloacetate Transaminase Activity

Changes in glutamic-oxaloacetate transaminase (GOT) levels were measured according to the method of Reitman and Frankel (1957) [23]. For this purpose, 0.10 ml of serum was taken and 0.50 ml of GOT substrate was added to it. GOT substrate (0.292 gm of *α*-ketoglutaric acid and 26.6 gm of DL-aspartic acid) was taken and 1 N NaOH was added slowly with mixing until all solids were dissolved and sufficient buffer was added to adjust the pH 7.4). The buffer solution was prepared by adding 13.97 gm K_2HPO_4 and 2.69 gm KH_2PO_4 in 1000 ml dis-

tilled water. The content was mixed well and incubated at 37˚C for one hr. To this tube, 0.50 ml of 2 - 4 dinitrophenyl hydrazine solution (Dissolved 0.198 gm of 2, 4-dinitrophenyl hydrazine in 1 N HCl to make 1000 ml.) was added and kept standing for 15 minutes at room temperature. Then 5.0 ml of 0.4 N NaOH (1.6 gm NaOH dissolved in 100 ml distilled water) was added and mixed well. Contents were mixed and left for 20 minutes at room temperature. Optical density was recorded at 505 nm by setting the blank with distilled water. The standard curve was prepared by using oxaloacetic acid as the standard. Enzyme activity was expressed in units of glutamate oxaloacetate transaminase/30 min/mg protein.

3.8. Determination of Lactic Dehydrogenase

Changes in serum lactic dehydrogenase activity were measured by the method of Annon, T. M. (1984) [24]. For this purpose 0.05 ml of blood serum was added to 0.50 ml of pyruvate substrate. The pyruvate substrate was prepared by mixing 10 ml of pH 7.5 pyruvate buffer (dissolved 10 gm of K_2HPO_4 and 0.2 gm pyruvic acid in enough water to make 1000 ml and added one drop of chloroform as preservative.) with 0.010 gm $NADH_2$. Now the contents were incubated at 37˚C for 45 minutes. 0.50 ml of 2,4-dinitrophenyl hydrazine solution (0.2 gm of 2,4-dinitrophenyl hydrazine and 85 ml concentrated HCl make up the volume of 1litre with distilled water) was added and the contents of the mixture were kept at the room temperature. After 20 minutes, 5.0 ml of 0.4 N NaOH (dissolved 1.6 gm sodium hydroxide in 100 ml of distilled water) was mixed and left for 30 minutes at room temperature. The optical density was measured at 540 nm and converted to LDH unit by means of a specially prepared standard curve. Enzyme activity has been expressed as μ moles of pyruvate reduced/min/mg protein.

3.9. Determination of Acetylcholinesterase

Changes in serum acetylcholinesterase (AchE) level were measured according to the method of Ellman *et al.*, 1961 [25]. For estimation of AchE, 0.05 ml of enzyme source was pipetted to a 10 mm path length cuvette. To this 0.10 ml (5×10^{-4} M) of freshly prepared acetylcholine thioiodide solution, 0.05 ml of DTNB reagent (chromogenic agent) and 1.45 ml of PBS (pH 6.9) were added. The changes in optical density at 412 nm were monitored regularly for three minutes at 25˚C. Enzyme activity has been expressed as μ moles "SH" hydrolysed per minute per mg protein.

4. Results

4.1. Solubilization of Tick Saliva Toxins

Before isolation of tick salivary gland secreted toxins, whole body extract of ticks was prepared, was homogenized and solubilized in different solublizing buffers *i.e.* Triton was isolated by making homogenization of Triton X 100 (0.01%), Tris + EDTA (0.1 Mm), PBS buffer, TCA 5% and Absolute alcohol, among Triton

X-100 (0.1%) was proved to be a good solubilizing agent for the *Rhipicephalus microplus* salivary proteins than any other solublizing buffer used. A higher protein solubilization was obtained in the supernatant than in the residue except TCA (Figure 1).

4.2. Purification

For isolation and purification of saliva toxins adults of Ticks; *Rhipicephalus microplus* was homogenized in 5 mL of PBS (pH 7.2) by using a glass-glass homogenizer 5 ml solubilizing buffer. Homogenate was centrifuged in cold at 4˚C for 30 min at 15,000 rpm and the supernatant was gently isolated. It was subjected to load on a Sepharose CL-6B 200 column for separation of salivary toxins. The elution pattern of tick saliva toxins homogenate exhibited five major peaks at 280 nm. One soon after the void volume from fraction numbers three peaks 31 - 37, 38 - 47 and 50 - 60 while the fourth and fifth peaks were present 67 - 73 (Figure 2). Further, the concentration of tick saliva toxin was determined in each and every tube by using Lowry's method (1951). Again, two similar protein peaks were resolved at 640 nm (Figure 3). The first peak was a minor one present between 50 - 60 while the second peak was a major peak and located between fraction numbers 67 - 77 (Figure 3). Both peaks were eluted with PBS buffer (pH 7.2). The total yield of tick saliva toxins in eluted fractions was calculated at 69.21%.

4.3. Molecular Weight Determination of Saliva Toxins

The molecular weight of *Rhipicephalus microplus* saliva toxins/proteins was determined by Sepharose CL6B 200 gel column chromatography using standard marker proteins of known molecular weight. The calibration curve indicates that the molecular weight of purified saliva toxins proteins ranges from 14.3 - 63 kDa (Figure 4). From the calibration curve, the molecular weight of purified saliva

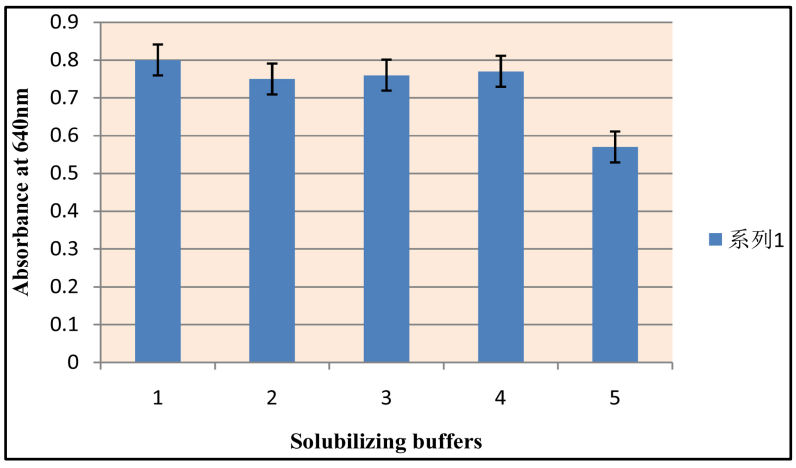

Figure 1. Solubilization of saliva proteins from tick *Rhipicephalus microplus* in different buffer solutions. Absorbance of solubilized protein was taken at 640 nm. Solubilizing buffers on X-axis are (1) Triton X 100 (0.01%), (2) Tris + EDTA (0.1 Mm) (3) PBS buffer (4) TCA 5% and (5) Absolute alcohol.

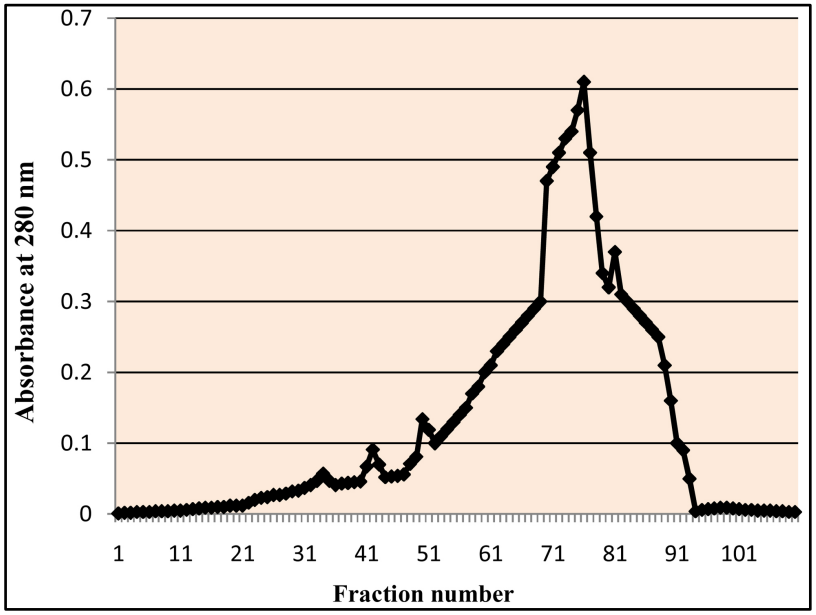

Figure 2. Elution pattern of PBS extractable saliva proteins of tick *Rhipicephalus microplus* chromatographed on a Sepharose CL-6B 200 column. Absorbance taken at 280 nm.

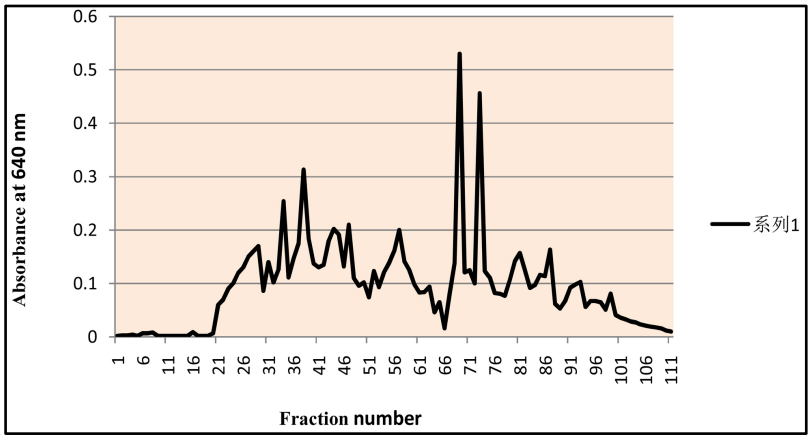

Figure 3. Elution pattern of PBS extractable proteins of *Rhipicephalus microplus* tick chromatographed on a Sepharose CL-6B 200 column. Absorbance taken at 640 nm.

toxins proteins was obtained in a range of 14 - 63.0 kDa (**Figure 4**).

4.4. Effect of Purified Tick Saliva Toxins on Certain Blood Serum Enzymes

Changes in the level of various serum enzymes *i.e.* acid phosphatase (ACP), alkaline phosphatase (ALP), glutamate, pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT), lactic dehydrogenase (LDH) and acetylcholinesterase (AchE) were observed in albino mice after providing injections of 40% and 80% of 24-h LD_{50} at different time periods (0 hr, 2 hr, 4 hr, 6 hr, and 8 hr,) in control groups mice were injected only with PBS buffer (pH 6.9).

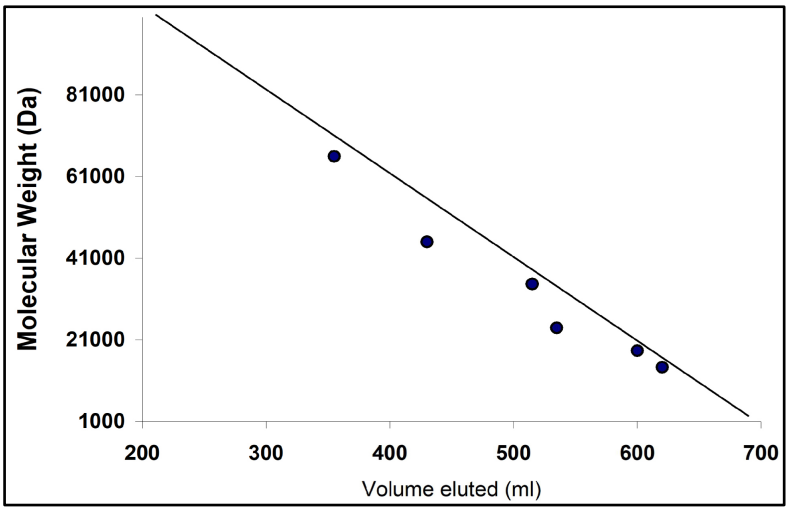

Figure 4. Standard proteins chromatographed on Sepharose CL-6B 200 column for determining the molecular weights of tick saliva toxins/proteins/peptides isolated from *Rhipicephalus microplus.* Proteins used were bovine albumin mol. wt 66,000, egg albumin mol. wt. 45,000, pepsin mol. wt. 34,700, trypsinogen mol. wt. 24,000, beta lactoglobulin mol. wt 18,400 and lysozyme mol. wt. 14,300. Elution volumes of unknown proteins were compared with log values on the X-axis for estimation of molecular weights.

Acid phosphatase level was found to be significantly ($p < 0.05$) increased up to 136.36% and 142.30% at 4th hr of 40% and 80% of 24-h LD_{50} of ticks saliva toxins injection in comparison to control respectively. Further, it recovered 109.09% and 113.63% at the 8th hr in comparison to control respectively (Table 1 & Table 2; Figure 5).

Similarly, a significant ($p < 0.05$) elevation in alkaline phosphatase level obtained was 125.40% and 126.61% at the 6th hr of the 40% and 80% of 24-h LD_{50} of tick saliva toxins treatment in comparison to control respectively. Later on, it was found 117.85% and 28.57% at the 8th hr (Table 1 & Table 2; Figure 6).

Maximum elevation *i.e.* 123.35% in the level of glutamate pyruvate transaminase was found to be 123.35% at the 6th hr of 40% of 24-h LD_{50} treatment while it was found 152.63% at the 4th hr of 24-h 80% of LD_{50} of saliva toxin injection. Later on it was recorded 104.65% and 142.105% at the 8th hr of 40% and 80% of 24-h LD_{50} treatment in comparison to control respectively (Table 1 & Table 2; Figure 7).

Similarly, glutamate oxaloacetate transaminase level significantly ($p < 0.05$) increased up to 129.03% and 140.625% at the 6th hr of 40% and 80% of 24-h LD_{50} treatment respectively. Further, it was recorded at 122.58% and 131.25% at the 8th hr in comparison to control respectively (Table 1 & Table 2; Figure 8).

A significant ($p < 0.05$) increase in lactic dehydrogenase obtained in blood serum was 126.126% and 128.46% at the 6th hr of 40% and 80% of 24-h LD_{50} of ticks saliva toxins treatment (Table 1 & Table 2; Figure 9).

Contrary to this, acetylcholinesterase level was found to be decreased significantly ($p < 0.05$) up to 70.96% and 57.14% at the 4th hr of treatment with 40% and

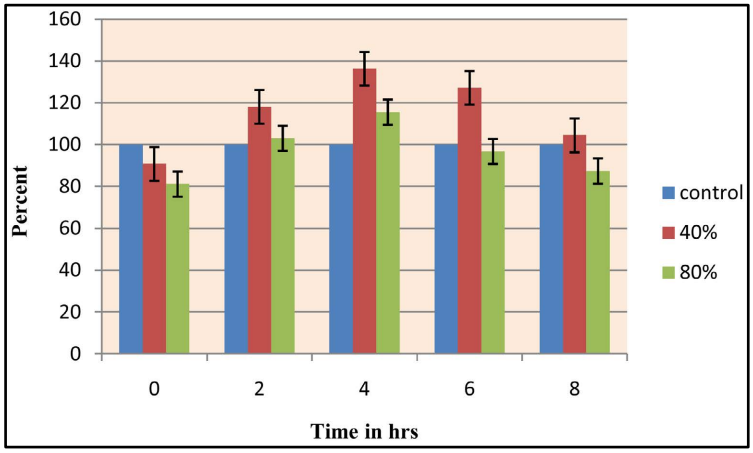

Figure 5. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of serum acid phosphatase in albino mice.

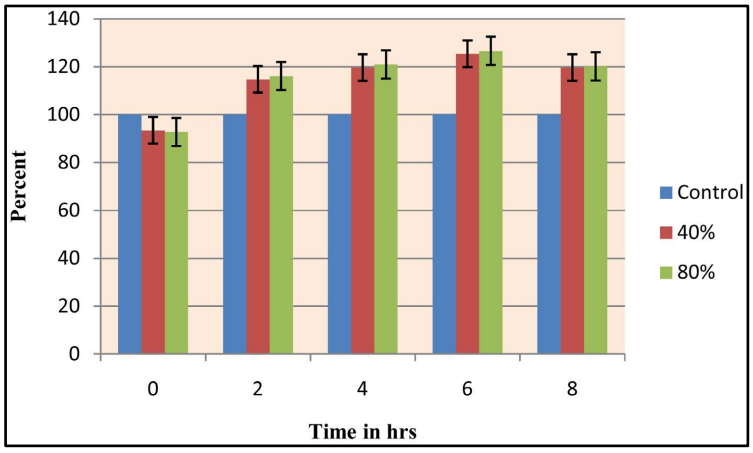

Figure 6. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of serum alkaline phosphatase in albino mice.

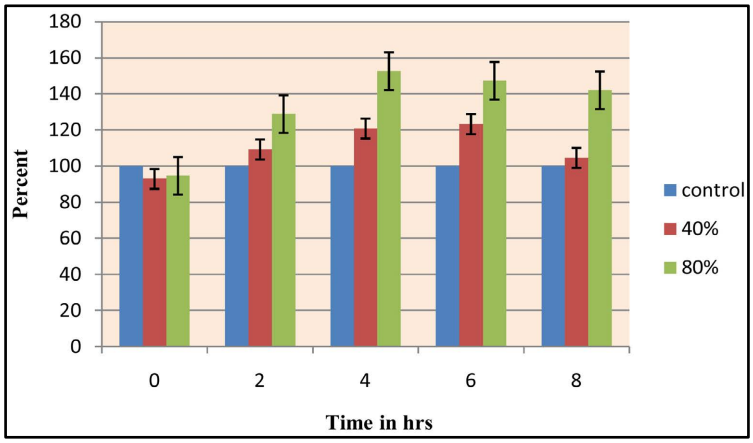

Figure 7. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of serum glutamate pyruvate transaminase in albino mice.

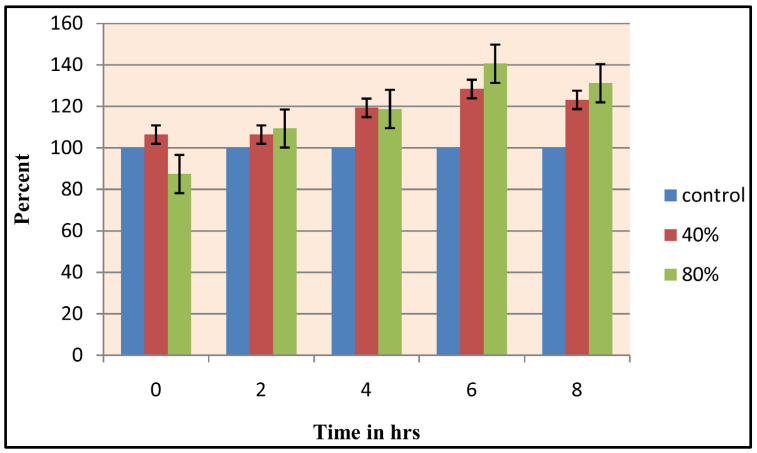

Figure 8. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of serum glutamate oxaloacetate transaminase in albino mice.

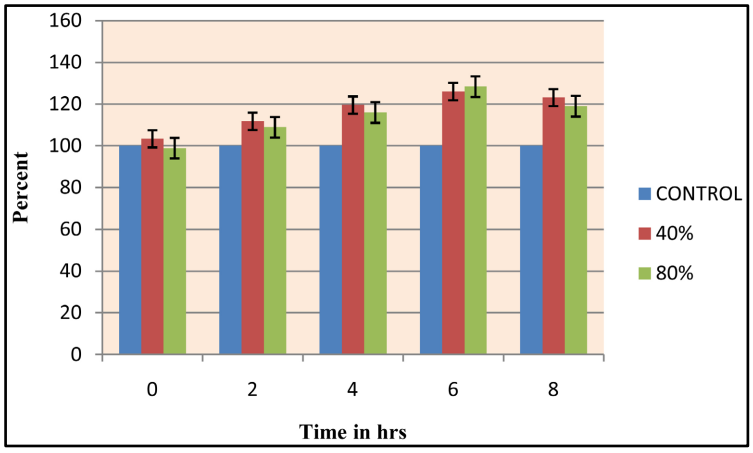

Figure 9. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of serum lactic dehydrogenase in albino mice.

80% of 24-h LD_{50} of purified ticks saliva toxins respectively. Further, it was found 80.64% and 68.57% at the 8th hr after the treatment with same dose of the saliva toxin in comparison to control (Table 1 & Table 2; Figure 10).

4.4.1. Effect of Purified Ticks Saliva Toxins on Certain Enzymes in Gastrocnemius Muscle of Albino Mice

This section deals with the effect of 40% and 80% of 24-h LD_{50} purified ticks' saliva toxins on different enzymes in mice gastrocnemius muscle of albino mice. After the above treatment of albino mice levels of various enzymes such as acid phosphatase, alkaline phosphatase, glutamate, pyruvate transaminase, glutamate oxaloacetate transaminase, lactic dehydrogenase and acetylcholinesterase were measured in gastrocnemius muscles at 2, 4, 6, and 8, hrs of treatment. Mice, injected only with buffer were considered as control. Following changes in enzyme level were found in gastrocnemius muscles of albino mice.

Table 1. *In vivo* effect of 40% of LD_{50} purified tick saliva toxins on different enzymes *i.e.* acid phosphatase, alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, lactic dehydrogenase and acetylcholinesterase in mice serum.

Enzyme	Control	0 hrs	2 hrs	4 hrs	6 hrs	8 hrs
ACP	0.22 ± 0.043 (100)	0.20 ± 0.0454 (90.90)	0.26 ± 0.0216 (118.18)	0.30 ± 0.0216 (136.36)	0.28 ± 0.0141 (127.27)	0.23 ± 0.0294 (104.54)
ALP	1.23 ± 0.1202 (100)	1.14 ± 0.0216 (93.44)	1.40 ± 0.0216 (114.75)	1.46 ± 0.029 (119.67)	1.53 ± 0.014 (125.40)	1.46 ± 0.050 (119.67)
GPT	0.043 ± 0.0216 (100)	0.040 ± 0.160 (93.023)	0.047 ± 0.00 (109.30)	0.052 ± 0.00 (120.93)	0.053 ± 0.02 (123.25)	0.045 ± 0.03 (104.65)
GOT	0.31 ± 0.0294 (100)	0.33 ± 0.0141 (106.45)	0.33 ± 0.021 (106.45)	0.37 ± 0.068 (119.35)	0.40 ± 0.021 (129.03)	0.38 ± 0.016 (122.58)
LDH	6.43 ± 0.02616 (100)	6.65 ± 0.0565 (103.42)	7.19 ± 0.0163 (111.18)	7.69 ± 0.0216 (119.59)	8.40 ± 0.0216 (126.126)	7.92 ± 0.0141 1(123.17)
AchE	0.031 ± 0.0043 (100)	0.029 ± 0.0035 (93.54)	0.025 ± 0.0014 (80.64)	0.022 ± 0.0021 (70.96)	0.024 ± 0.0064 (77.41)	0.025 ± 0.0014 (80.64)

Values are mean ± SE of three replicates; Values in parentheses indicate per cent enzyme activity with respect to control taken as 100%; *Significant ($p < 0.05$, Student t-test); Acid phosphatase (ACP) and Alkaline phosphatase (ALP): μ moles of p-nitrophenol formed/30minute/mg protein; Glutamate pyruvate transaminases (GPT): Units of Glutamate pyruvate transaminase activity/hour/mg protein; Glutamate oxaloacetate transaminase (GOT): Units of Glutamate oxaloacetate transaminase activity/hour/mg protein; Lactic dehydrogense (LDH): μ moles of pyruvate reduced/45 minute/mg protein; Acetylcholinesterase (AchE): μ moles "SH" hydrolysed/minute/mg protein.

Table 2. *In vivo* effect of 80% of LD_{50} purified tick saliva toxins on different enzymes *i.e.* acid phosphatase, alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxalo acetate transaminase, lactic dehydrogenase and acetylcholinesterase in mice serum.

Enzyme	Control	0 hrs	2 hrs	4 hrs	6 hrs	8 hrs
ACP	0.26 ± 0.0141 (100)	0.32 ± 0.0141 (123.07)	0.33 ± 0.0081 (126.92)	0.37 ± 0.0216 (142.30)	0.31 ± 0.0081 (119.23)	0.28 ± 0.0141 (107.69)
ALP	1.24 ± 0.0216 (100)	1.15 ± 0.0282 (92.74)	1.44 ± 0.0216 (116.12)	1.50 ± 0.021 (120.96)	1.57 ± 0.014 (126.61)	1.49 ± 0.021 (120.16)
GPT	0.38 ± 0.0163 (100)	0.36 ± 0.0216 (94.73)	0.49 ± 0.028 (128.94)	0.58 ± 0.008 (152.63)	0.56 ± 0.021 (147.36)	0.54 ± 0.014 (142.105)
GOT	0.32 ± 0.0141 (100)	0.28 ± 0.0374 (87.5)	0.35 ± 0.008 (09.375)	0.38 ± 0.016 (118.75)	0.45 ± 0.014 (140.625)	0.42 ± 0.014 (131.25)
LDH	6.66 ± 0.0535 (100)	6.59 ± 0.0282 (98.94)	7.26 ± 0.0282 (109.009)	7.73 ± 0.0282 (116.06)	8.26 ± 0.0355 (128.46)	7.93 ± 0.0535 (119.06)
AchE	0.035 ± 0.0081 (100)	0.031 ± 0.0069 (88.571)	0.025 ± 0.0141 (71.42)	0.020 ± 0.0021 (57.14)	0.026 ± 0.004 (74.285)	0.024 ± 0.0037 (68.57)

Values are mean ± SE of three replicates; Values in parentheses indicate per cent enzyme activity with respect to control taken as 100%; *Significant ($p < 0.05$, Student t-test); Acid phosphatase (ACP) and Alkaline phosphatase (ALP): μ moles of p-nitrophenol formed/30 minute/mg protein; Glutamate pyruvate transaminases (GPT): Units of Glutamate pyruvate transaminase activity/hour/mg protein; Glutamate oxaloacetate transaminase (GOT): Units of Glutamate oxaloacetate transaminase activity/hour/mg protein; Lactic dehydrogense (LDH): μ moles of pyruvate reduced/45 minute/mg protein; Acetylcholinesterase (AchE): μ moles "SH" hydrolysed/minute/mg protein.

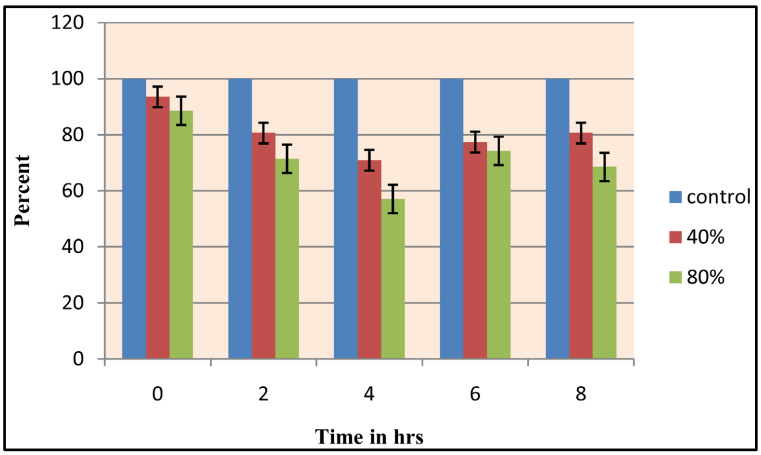

Figure 10. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of serum acetylcholinesterase in albino.

Acid phosphatase level was found to be increased significantly ($p < 0.05$) up to 134% and 138.23% at the 6th hr of 40% and 80% of 24-h LD_{50} of ticks saliva toxins injection in comparison to control respectively. Later on, it recovered up to 126% and 123.52% at the 8th hr (Table 3 & Table 4; Figure 11).

While alkaline phosphatase level was found to be reduced up to 76.92% at the 6th hr of 40% of 24-h LD_{50} and 52.94% at the 4th hour of 80% of 24-h LD_{50} of ticks saliva toxins injection in comparison to control respectively. Later on, it recovered up to 76.92% and 62.74% at the 8th hr (Table 3 & Table 4; Figure 12).

In a similar treatment glutamate pyruvate transaminase level was found to be increased significantly ($p < 0.05$) up to 108.28% at the 4th hr of treatment with 40% of 24-h LD_{50} and 117.5% at the 6th hr of treatment 80% of 24-h LD_{50} of purified ticks saliva toxins treatment in comparison to control respectively. Later on, it recovered up to 103.18% and 110.625% at the 8th hr (Table 3 & Table 4; Figure 13).

Similarly, glutamate oxaloacetate transaminases level was found to be increased significantly ($p < 0.05$) up to 115% at the 4th hr of treatment with 40% and 138.88% at the 6th hr of 80% of 24-h LD_{50} of purified ticks' saliva toxins compared to the control. Then, at the 8th hour it was found to be 92.5% and 122.22% at the same dose (Table 3 & Table 4; Figure 14).

More specifically, lactic dehydrogenase level was slightly elevated up to 101.128% and 102.19%, at the 6th hr of treatment with 40% and 80% of 24-h LD_{50} of purified *Rhipicephalus microplus* saliva protein/toxins in comparison to control. Further, no significant elevation was observed in LDH level at the 8th hr as it was found to be 99.75% and 101.10% (Table 3 & Table 4; Figure 15).

Contrary to this, acetylcholiesterase level was found to be decreased significantly ($p < 0.05$) up to 48.14% at the 4th hr of treatment with 40% of 24-h LD_{50} and 48% at the 6th hr of 80% of 24-h LD_{50} purified ticks saliva toxins in comparison to control respectively. At the 8th hr, it was found to be recovered up to 51.85% and 48% at the same dose in comparison to control (Table 3 &

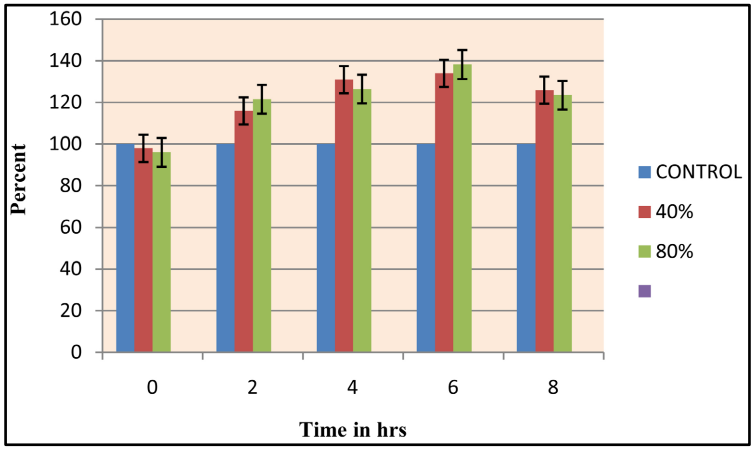

Figure 11. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of gastrocnemius acid phosphatase in albino mice.

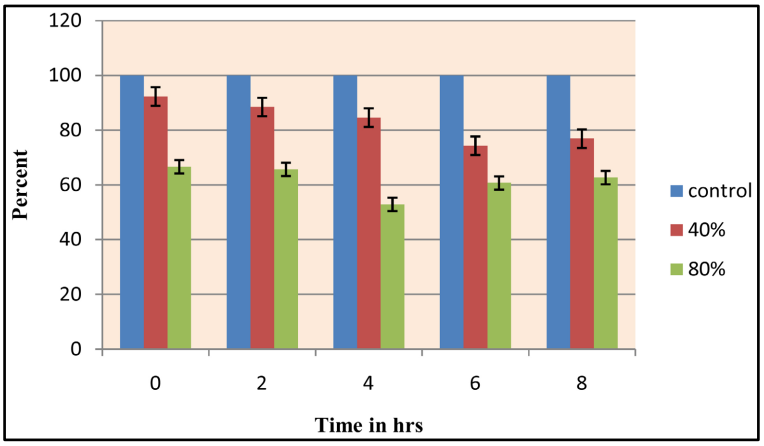

Figure 12. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of gastrocnemius muscle alkaline phosphatase in albino mice.

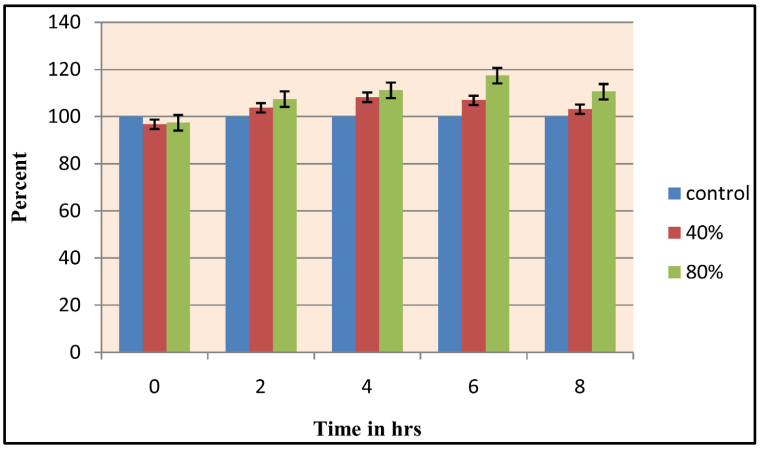

Figure 13. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of gastrocnemius muscle glutamate pyruvate transaminase albino mice.

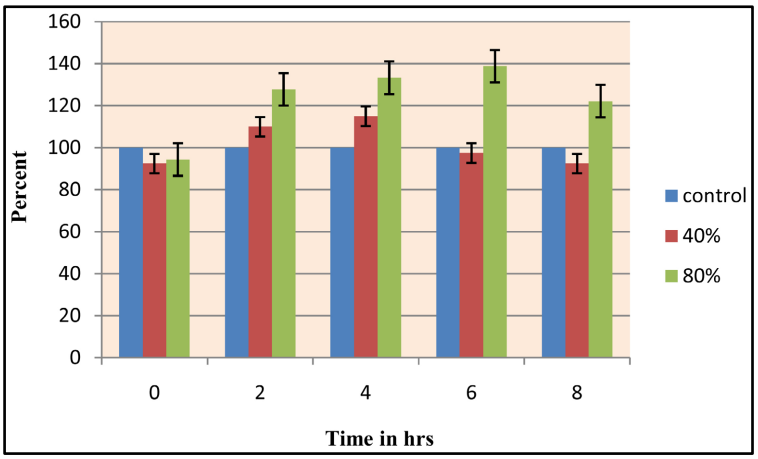

Figure 14. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of gastrocnemius muscle glutamate oxaloacetate transaminase in albino mice.

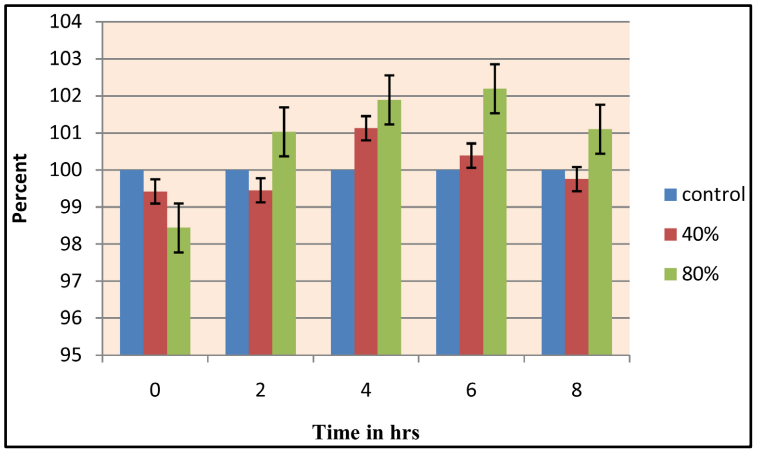

Figure 15. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of gastrocnemius muscle lactic dehydrogenase in albino mice.

Table 4; Figure 16).

4.4.2. Effect of Purified *Ticks Saliva* Toxins on Certain Enzyme in Liver of Albino Mice

This section deals with the effect of purified *Rhipicephalus microplus* saliva proteins/toxins on different enzymes in the liver of albino mice. When mice were treated with 40% and 80% of 24-h LD_{50} of purified ticks saliva toxins caused a significant elevation in the level of acid phosphatase, glutamate, pyruvate transaminase, glutamate oxaloacetate transaminase and lactic dehydrogenase while the reduction in alkaline phosphatase and acetyl cholinesterase.

The acid phosphatase level was found to be elevated up to 106.57% and 112.41% at the 6th hr of the 40% and 80% of 24-h LD_{50} of purified ticks' saliva toxins injection. Later on, at the 8th hr, it was recovered to 103.28% and 108.49% respectively in comparison to control (Table 5 & Table 6; Figure 17).

Table 3. *In vivo* effect of 40% of LD_{50} purified tick saliva toxins on different enzymes *i.e.* acid phosphatase, alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxalo acetate transaminase, lactic dehydrogenase and acetylcholinesterase in gastrocnemius.

Enzyme	Control	0 hrs	2 hrs	4 hrs	6 hrs	8 hrs
ACP	1.0 ± 0.0216 (100)	0.98 ± 0.0081 (98)	1.16 ± 0.041 (116)	1.31 ± 0.1208 (131)	1.34 ± 0.0571 (134)	1.26 ± 0.0216 (126)
ALP	0.78 ± 0,041 (100)	0.72 ± 0.0216 (92.30)	0.69 ± 0.0216 (88.46)	0.66 ± 0.0141 (84.61)	0.58 ± 0.02081 (74.35)	0.60 ± 0.0216 (76.92)
GPT	1.57 ± 0.0216 (100)	1.52 ± 0.0141 (96.81)	1.63 ± 0.0216 (103.82)	1.70 ± 0.0216 (108.28)	1.68 ± 0.0081 (107.00)	1..62 ± 0.0081 (103.18)
GOT	0.40 ± 0.0216 (100)	0.37 ± 0.0141 (92.5)	0.44 ± 0.0211 (110)	0.46 ± 0.0509 (115)	0.39 ± 0.0282 (97.5)	0.37 ± 0.0216 (92.5)
LDH	92.19 ± 0.0015 (100)	91.66 ± 0.9637 (99.42)	91.69 ± 0.3137 (99.45)	93.23 ± 0.2336 (100.39)	92.55 ± 0.0454 (101.128)	91.75 ± 0.1080 (99.75)
AchE	0.27 ± 0.0141 (100)	0.19 ± 0.0216 (70.37)	0.015 ± 0.0216 (55.55)	0.13 ± 0.0374 (48.14)	0.14 ± 0.0141 (51.85)	0.14 ± 0.0282 (51.85)

Values are mean ± SE of three replicates; Values in parentheses indicate per cent enzyme activity with respect to control taken as 100%; *Significant ($p < 0.05$, Student t-test); Acid phosphatase (ACP) and Alkaline phosphatase (ALP): μ moles of p-nitrophenol formed/30 minute/mg protein; Glutamate pyruvate transaminases (GPT): Units of Glutamate pyruvate transaminase activity/hour/mg protein; Glutamate oxaloacetate transaminase (GOT): Units of Glutamate oxaloacetate transaminase activity/hour/mg protein; Lactic dehydrogense (LDH): μ moles of pyruvate reduced/45 minute/mg protein; Acetylcholinesterase (AchE): μ moles "SH" hydrolysed/minute/mg protein.

Table 4. *In vivo* effect of 80% of LD_{50} purified tick saliva toxins on different enzymes *i.e.* acid phosphatase, alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxalo acetate transaminase, lactic dehydrogenase and acetylcholinesterase in gastrocnemius.

Enzyme	Control	0 hrs	2 hrs	4 hrs	6 hrs	8 hrs
ACP	1.02 ± 0.0141 (100)	0.98 ± 0.0081 (96.07)	1.24 ± 0.0216 (121.56)	1.29 ± 0.0216 (126.47)	1.41 ± 0.104 (138.23)	1.26 ± 0.0216 (123.52)
ALP	0.76 ± 0.041 (100)	0.68 ± 0.0081 (66.66)	0.67 ± 0.0141 (65.68)	0.54 ± 0.0216 (52.94)	0.62 ± 0.0141 (60.78)	0.64 ± 0.0141 (62.74)
GPT	1.60 ± 0.0216 (100)	1.56 ± 0.01419 (97.5)	1.72 ± 0.0141 (107.5)	1.78 ± 0.0081 (111.25)	1.88 ± 0.0141 (117.5)	1.77 ± 0.0244 (110.625)
GOT	0.36 ± 0.0216 (100)	0.34 ± 0.0216 (94.44)	0.46 ± 0.0141 (127.77)	0.48 ± 0.0141 (133.33)	0.50 ± 0.0216 (138.88)	0.44 ± 0.041 (122.22)
LDH	91.45 ± 0.8166 (100)	90.03 ± 0.3066 (98.44)	92.40 ± 0.4598 (101.03)	93.18 ± 0.0711 (101.89)	93.46 ± 0.2974 (102.19)	92.46 ± 0.0294 (101.10)
AchE	0.025 ± 0.0014 (100)	0.021 ± 0.0081 (84)	0.015 ± 0.0014 (60)	0.014 ± 0.0282 (56)	0.012 ± 0.0014 (48)	0.012 ± 0.0014 (48)

Values are mean ± SE of three replicates; Values in parentheses indicate per cent enzyme activity with respect to control taken as 100%; *Significant ($p < 0.05$, Student t-test); Acid phosphatase (ACP) and Alkaline phosphatase (ALP): μ moles of p-nitrophenol formed/30 minute/mg protein; Glutamate pyruvate transaminases (GPT): Units of Glutamate pyruvate transaminase activity/hour/mg protein; Glutamate oxaloacetate transaminase (GOT): Units of Glutamate oxaloacetate transaminase activity/hour/mg protein; Lactic dehydrogense (LDH): μ moles of pyruvate reduced/45 minute/mg protein; Acetylcholinesterase (AchE): μ moles "SH" hydrolysed/minute/mg protein.

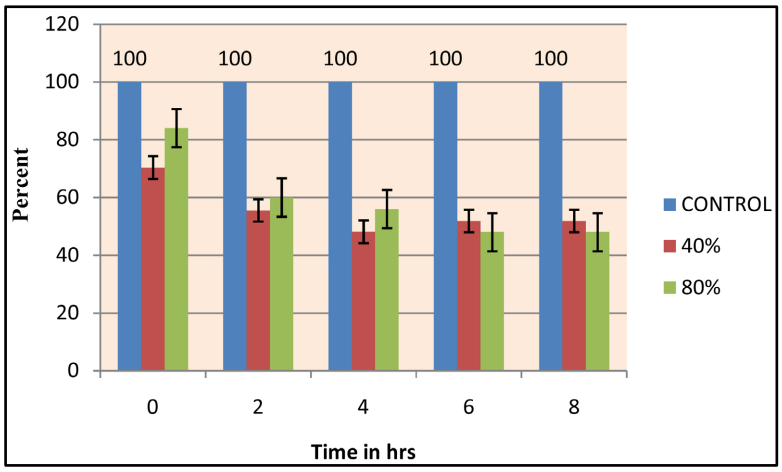

Figure 16. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of gastrocnemius muscle acetylcholinesterase in albino mice.

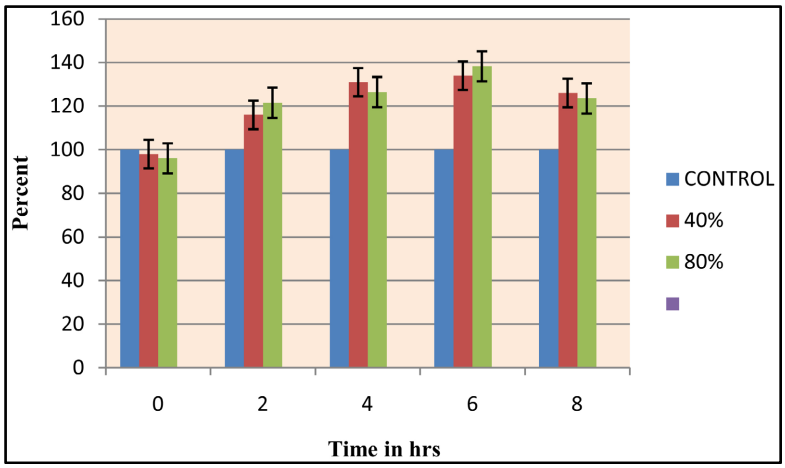

Figure 17. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of liver acid phosphatase in albino mice.

Alkaline phosphates level was found to be decreased significantly ($p< 0.05$) up to 75% and 61.96% at the 6th hr of treatment with 40% and 80% of 24-h LD_{50} of purified *Rhipicephalus microplus* saliva toxins. Further, it was recovered up to 83.92% and 62.74% at the 8th hr in comparison to control respectively (**Table 5** & **Table 6**; **Figure 18**).

A significant ($p < 0.05$) elevation in glutamate pyruvate transaminase observed was 115.38% and 142.028% at the 4th hr of 40% and 80% of 24-h LD_{50} of the ticks saliva toxin injection, in comparison to control respectively. It was recovered 88.46% and 126.08% at the 8th hr (**Table 5** & **Table 6**; **Figure 19**).

Similarly, glutamate oxaloacetate transaminase level was found to be elevated significantly ($p < 0.05$) up to 112.63% at 6th hr and 126.31% of 40% of 24-h LD_{50} and 80% of 24-h LD_{50} of purified ticks saliva toxins treatment. Later on, it recovered up to 109.20% and 123.68% at the 8th hr of the treatment in comparison to

Table 5. *In vivo* effect of 40% of LD_{50} purified tick saliva toxins on different enzymes *i.e.* acid phosphatase, alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxalo acetate transaminase, lactic dehydrogenase and acetyl cholinesterase in liver.

Enzyme	Control	0 hrs	2 hrs	4 hrs	6 hrs	8 hrs
ACP	1.52 ± 0.0141 (100)	1.43 ± 0.0081 (94.07)	1.48 ± 0.0141 (97.36)	1.54 ± 0.0454 (101.3)	1.62 ± 0.0141 (106.57)	1.57 ± 0.0216 (103.28)
ALP	2.24 ± 0.0081 (100)	2.15 ± 0.0141 (95.98)	2.11 ± 0.0281 (94.19)	1.88 ± 0.0244 (83.92)	1.68 ± 0.0081 (75)	1.88 ± 0.0141 (83.92)
GPT	0.78 ± 0.0081 (100)	0.73 ± 0.0081 (93.58)	0.82 ± 0.0294 (105.12)	0.90 ± 0.0216 (115.38)	0.79 ± 0.0216 (101.28)	0.69 ± 0.029 (88.46)
GOT	1.163 ± 0.0216 (100)	1.10 ± 0.0216 (94.58)	1.15 ± 0.0374 (98.88)	1.30 ± 0.0216 (111.77)	1.31 ± 0.0355 (112.63)	1.27 ± 0.0216 (109.20)
LDH	1.80 ± 0.0216 (100)	1.68 ± 0.0081 (93.33)	1.86 ± 0.0509 (103.33)	1.97 ± 0.0216 (109.44)	2.21 ± 0.282 (122.77)	1.90 ± 0.0216 (105.55)
AchE	0.31 ± 0.0081 (100)	0.028 ± 0.0035 (83.87)	0.026 ± 0.0081 (83.87)	0.019 ± 0.0026 (61.29)	0.024 ± 0.0028 (77.41)	0.029 ± 0.0021 (93.54)

Values are mean ± SE of three replicates; Values in parentheses indicate per cent enzyme activity with respect to control taken as 100%; *Significant ($p < 0.05$, Student t-test); Acid phosphatase (ACP) and Alkaline phosphatase (ALP): μ moles of p-nitrophenol formed/30 minute/mg protein; Glutamate pyruvate transaminases (GPT): Units of Glutamate pyruvate transaminase activity/hour/mg protein; Glutamate oxaloacetate transaminase (GOT): Units of Glutamate oxaloacetate transaminase activity/hour/mg protein; Lactic dehydrogense (LDH): μ moles of pyruvate reduced/45 minute/mg protein; Acetylcholinesterase (AchE): μ moles "SH" hydrolysed/minute/mg protein.

Table 6. *In vivo* effect of 80% of LD_{50} purified tick saliva toxins on different enzymes *i.e.* acid phosphatase, alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxalo acetate transaminase, lactic dehydrogenase and acetylcholinesterase in liver.

Enzyme	Control	0 hrs	2 hrs	4 hrs	6 hrs	8 hrs
ACP	1.53 ± 0.0216 (100)	1.44 ± 0.0571 (94.11)	1.60 ± 0.0216 (104.57)	1.69 ± 0.0216 (110.45)	1.72 ± 0.0141 (112.41)	1.66 ± 0.0141 (108.49)
ALP	2.55 ± 0.355 (100)	2.17 ± 0.0282 (85.09)	1.92 ± 0.0141 (75.29)	1.84 ± 0.0282 (72.15)	1.58 ± 0.0081 (61.96)	1.60 ± 0.0216 (62.74)
GPT	0.76 ± 0.216 (100)	0.69 ± 0.024 (110.144)	0.84 ± 0.0282 (121.73)	0.96 ± 0.0216 (139.13)	0.98 ± 0.0081 (142.028)	0.87 ± 0.122 (126.08)
GOT	1.14 ± 0.0294 (100)	1.09 ± 0.282 (95.61)	1.26 ± 0.355 (110.52)	1.39 ± 0.053 (121.92)	1.44 ± 0.0294 (126.31)	1.41 ± 0.0282 (123.68)
LDH	1.85 ± 0.0282 (100)	1.74 ± 0.0509 (94.05)	1.93 ± 0.0216 (104.32)	2.17 ± 0.0282 (117.29)	2.61 ± 0.0282 (141.081)	1.97 ± 0.0266 (106.48)
AchE	0.032 ± 0.0216 (100)	0.029 ± 0.0028 (90.625)	0.22 ± 0.0021 (68.75)	0.019 ± 0.0028 (59.375)	0.015 ± 0.0049 (48.75)	0.025 ± 0.0028 (78.12)

Values are mean ± SE of three replicates; Values in parentheses indicate per cent enzyme activity with respect to control taken as 100%; *Significant ($p < 0.05$, Student t-test); Acid phosphatase (ACP) and Alkaline phosphatase (ALP): μ moles of p-nitrophenol formed/30 minute/mg protein; Glutamate pyruvate transaminases (GPT): Units of Glutamate pyruvate transaminase activity/hour/mg protein; Glutamate oxaloacetate transaminase (GOT): Units of Glutamate oxaloacetate transaminase activity/hour/mg protein; Lactic dehydrogense (LDH): μ moles of pyruvate reduced/45 minute/mg protein; Acetylcholinesterase (AchE): μ moles "SH" hydrolysed/minute/mg protein.

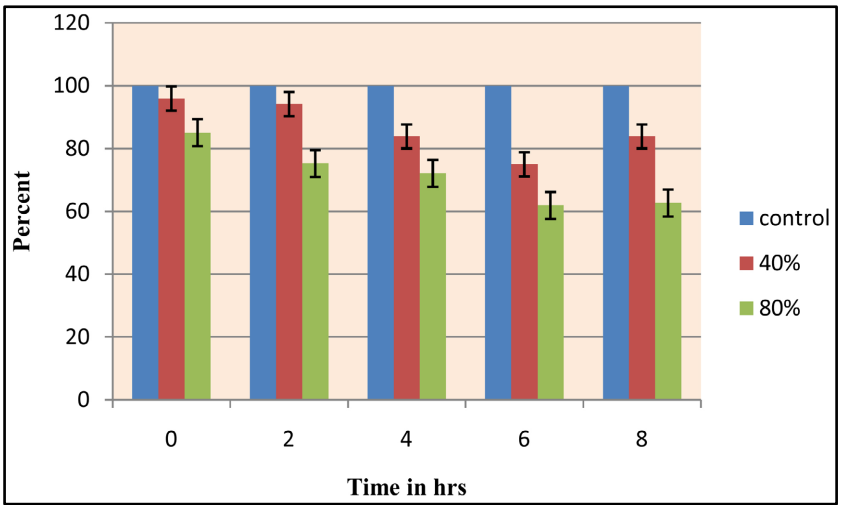

Figure 18. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxin Indian tick, *Rhipicephalus microplus* on activity of liver alkaline phosphatase in albino mice.

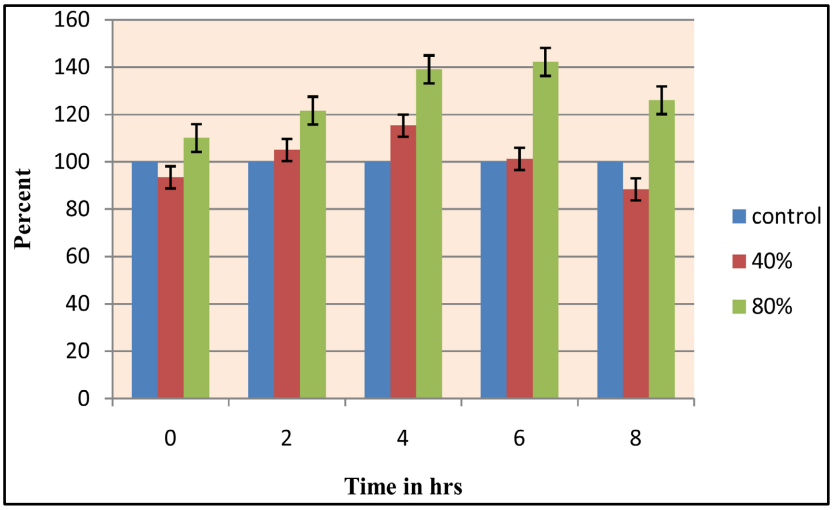

Figure 19. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of liver glutamate pyruvate transaminase in albino mice.

control respectively (Table 5 & Table 6; Figure 20).

At a similar dose of the saliva toxins lactic dehydrogenase level was found to be elevated significantly ($p < 0.05$) up to 122.77% and 141.081% at the 6th hr of 40% and 80% of 24-h LD_{50} of purified tick saliva toxins injection in comparison to control respectively. Later on, it recovered up to 105.55% and 106.48% at the 8th hr in comparison to the control (Table 5 & Table 6; Figure 21).

More specifically, acetylcholinesterase level was found to be decreased significantly ($p < 0.05$) up to 61.29% at 4th hr and 48.75% at the 6th hr of treatment with 40% and 80% of 24-h LD_{50} of purified ticks saliva toxins in comparison to control respectively. Later on, it improved up to 93.54% and 78.12% at the 8th hr in comparison to the control (Table 5 & Table 6; Figure 22).

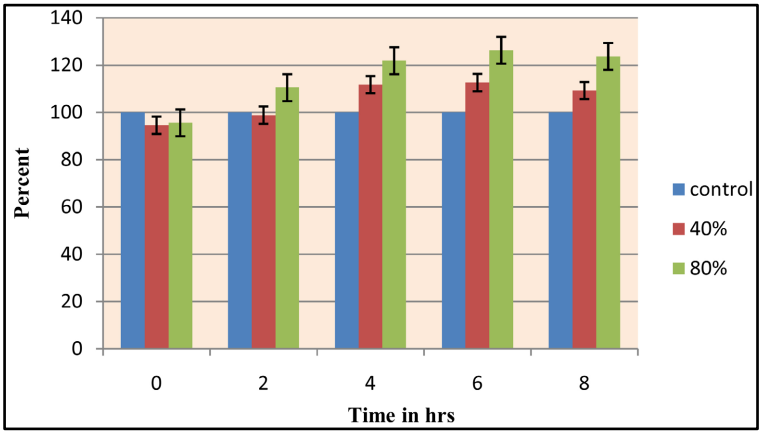

Figure 20. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of liver glutamate oxaloacetate transaminase in albino mice.

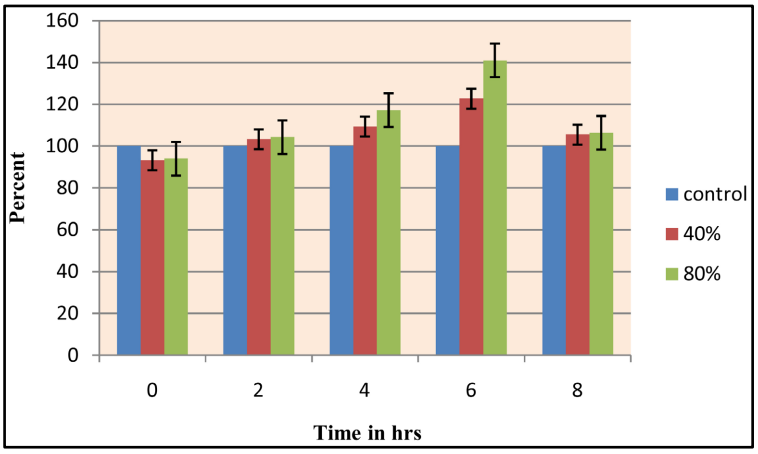

Figure 21. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of liver lactic dehydrogenase in albino mice.

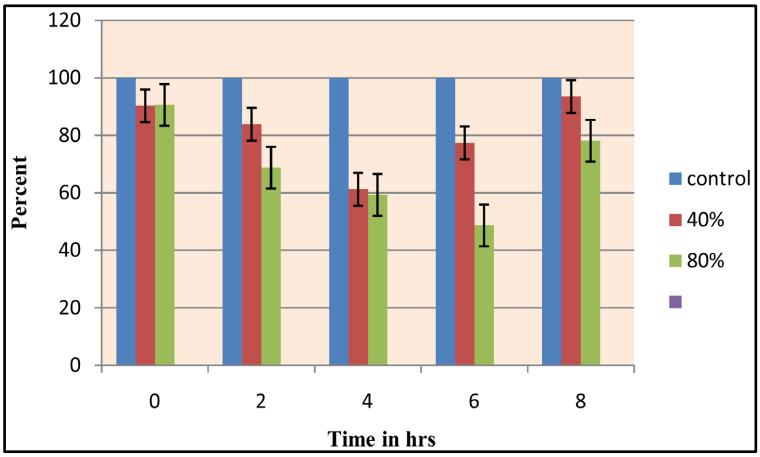

Figure 22. *In vivo* effect of 40% and 80% of 24 h-LD_{50} of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of liver acetylcholinesterase in albino mice.

5. Discussion

In the present study level of various metabolic enzymes was determined after injecting a sub-lethal dose of purified tick toxins *Rhipicephalus microplus* saliva toxins into the albino mice. Most of these metabolic enzymes are involved in toxicity reduction and their level displays toxic effects in blood serum, liver, tissue, kidney damage and neuronal effects. In the present investigation activity of serum acid phosphatase was increased up to 136.36% at 6th hr in comparison to the control (**Table 1** & **Table 2**, **Figure 5**). Similarly, the level of acid phosphatase was found to be increased up to 134% and 106.57% in gastrocnemius muscle and liver, respectively (**Table 3** & **Table 5**; **Figure 11** & **Figure 17**). Acid phosphatase is a lysosomal enzyme that plays an important role in catabolism, pathological necrosis, autolysis and phagocytosis [26]. There are so many reasons for an increase in the level of acid phosphatase level. This might be due to the intoxication of liver cells and lysosomal disintegration. After cell destruction, most of the enzyme leaks out from liver and muscle cells into the circulation which results in elevation of enzyme level [27]. In the present study ticks saliva toxins have significantly increased the level of serum acid phosphatase which may be due to the induction of liver ischemia and hypoxia [28]. Acid phosphatase level is also increased due to bone demineralization and the generation of defective osteoclasts. It acts as a marker of bone disorders, such as Paget's disease of bone. Metastasis of internal organs mainly glandular tissues and cells also cause a rise in acid phosphatase level.

Serum alkaline phosphatase level was found to be increased up to 125.40% at the 6th hr of the saliva toxin injection in comparison to control (**Table 1** & **Table 2**, **Figure 6**). This elevation may be due to cytolysis. In the present study, ACP and ALP levels were also found to be increased significantly ($p < 0.05$) up to 136.36% and 125.40% *in vitro* saliva toxin treatment (**Table 1**; **Figure 5** & **Figure 6**). This elevation in ACP and ALP is due to the hemolytic activity of the saliva toxin. Contrary to this the level of ALP was found to be reduced up to 52.94% and 75% in gastrocnemius and liver muscle at the 4th and 6th hr respectively in comparison to control (**Table 3** & **Table 5**; **Figure 12** & **Figure 18**). This inhibition may retard the protein synthesis in tissues and release excess free amino acids into the circulation, thereby, increasing amino acid level in the serum.

Alkaline phosphatase is an important membrane-bound enzyme found in all body tissues. It mediates the transport of metabolites across the membrane. It also plays an important role in protein synthesis [29]. It also causes a breakdown of proteins and is found in different forms, depending on where it originates. It is also found in the liver, gall bladder and bones. The main causes of its increase are malnutrition, metastasis of the kidney, intestine and pancreas, or a serious microbial infection. Pesticides elsan induced an increase in acid phosphatase activity in the liver, kidney and intestine [30]. Similarly, benzene hexachloride increased the levels of acid phosphatase, alkaline phosphatase and glutamate oxa-

loacetate transaminase (42%, 2% and 18% respectively) in blood serum. These toxicants cause hepatocyte necrosis and increase liver enzyme synthesis [31].

A significant elevation was observed in serum acid phosphatase and alkaline phosphatase levels. Both these enzymes are detoxifying enzymes and their level increased after tissue poisoning [32]. These enzymes are mainly found in the blood, liver, plasma and intestine of human beings [33] [34]. Toxins isolated from *Gymnapistes marmoratus* (Soldier fish) have displayed a higher level of serum acid phosphates, alkaline phosphatase and phosphodiesterase in human victims [35]. Similar, frequent elevation was noted in alkaline and acid phosphatase in Turkey hens after a dose of 1,2,4-triasole derivative (3-(2-pyridil)-4phenyl-1, 2,4-triasole-5-carboxilic acid) [36].

Further, GPT and GOT were also found to increase up to 108.28% and 115% in the gastrocnemius muscle at the 4th hr (**Table 3**; **Figure 13** & **Figure 14**). Its level in the liver of albino mice was recorded at 115.38% (at 4th hr) and 112.63% (at 6th hr) after providing a sub-lethal injection of purified Ticks saliva toxins respectively of the control (**Table 5**; **Figure 19** & **Figure 20**). Increased GPT and GOT level are due to damage to the liver, heart cells, muscle tissue, pancreas and kidney tissues [15]. This enzyme catalyzes the reversible transfer of amino groups between an amino acid, and α-keto acids are called aminotransferase or transaminases. [37]. Glutamate pyruvate transaminase (GPT) plays a key function in carbohydrate metabolism. It catalyzes the amination of α-ketoglutarate from alanine to produce pyruvate and glutamate. This enzyme participates in cellular nitrogen metabolism and also in liver gluconeogenesis starting with precursors transported from skeletal muscles. Ingestion of Pb^{2+} induced significant stimulation in glutamic-pyruvic transaminase (ALT) and glutamic-oxalacetic transaminase (AST) activity [38].

Hemolysis also increases AST and ALT because of this enzyme activity in the RBCs compared to serum. GPT and GGT are expressed in hepatocytes. As well as in the liver, GOT is expressed in the myocardium, skeletal muscle, kidneys, brain, pancreas, lung, leukocytes, and erythrocytes [39]. These enzymes also occur in small concentrations in plasma, which may be delivered from the regular physiological shedding of cells [40]. Therefore, any detectable increase in their activity in plasma can be used as a reliable indicator of changes in metabolic functions and structural damage in tissues [40]. Therefore, it functions as a link between carbohydrate and protein metabolism by catalyzing the conversion of alanine to pyruvate. Glutamate pyruvate transaminase, glutamate oxaloacetate transaminase and lactic dehydrogenase are cellular metabolic enzymes with no evident function in vertebrate plasma. Thus pyruvate is utilized in the process of gluconeogenesis for glucose production [41]. Tick saliva toxin caused massive cellular toxicity in liver cells and caused significant alterations in cell permeability of myocardial, liver and smooth muscle cells, which may facilitate the release of certain metabolic enzymes out of the cells into circulation [27].

Besides this, the levels of serum glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT) and lactic dehydrogenase (LDH)

was also found to be increased up to 123.35% (at 6th hr), 129.03% (at 6th hr) and 126.126% (at 6th hr) respectively in comparison to control (**Table 1**; **Figures 7-9**). Elimination of glutamate through enzymatic degradation is an alternative to glutamate receptor blockade in preventing excitotoxic neuronal injury. Level of SGOT/SGPT severely altered in alcoholic hepatitis and cirrhosis patients. The elevation in GPT level may be due to the stress that was created after *Rhipicephalus microplus* saliva toxin. Therefore, saliva toxin induced stress may be the causative factor for the elevation in GPT concentration [27]. It is evident that during stress conditions the energy requirement becomes high, which results in very high utilization of glucose and massive breakdown of stored glycogen that leads to decrease in glycogen level [42]. Similarly, tick saliva exhibit pharmacological effects and causes a significant decrease in Na^+-K^+ ATPase level and obstruct the glycogen synthesis in mice [43]. Similarly, Egyptian scorpion venom after administration of 100 - 400 μg/kg dose of lyopholysed venom caused a significant increase in serum glucose, creatinine, glutamate pyruvate transanimase, glutamate oxaloacetate transanimase and lactic dehydrogenase level in mice [44].

More specifically, lactic dehydrogenase level was slightly elevated up to 101.128% and 102.19% in gastrocnemius muscles at the 6th hr of treatment with 40% and 80% of 24-h LD_{50} of purified *Rhipicephalus microplus* saliva protein/toxins in comparison to control. Further, no significant elevation was observed in LDH level at the 8th hr as it was found to be 99.75% and 101.10% (**Table 3** & **Table 4**; **Figure 15**). At a similar dose of the saliva toxin lactic dehydrogenase level was found elevated significantly ($p < 0.05$) up to 122.77% and 141.081% in liver at the 6th hr of 40% and 80% of 24-h LD_{50} of purified tick saliva toxins injection in comparison to control respectively. Later on, it recovered up to 105.55% and 106.48% at the 8th hr in comparison to control (**Table 5** & **Table 6**; **Figure 21**). Lactate dehydrogenase is an enzyme that is present in almost all body tissues.LDH is a cytoplasmic enzyme that is present in almost all tissues but at high concentrations in muscle, liver, and kidney. Lactic dehydrogenase is an intracellular enzyme found at high level in tissues, particularly, in liver, heart, skeletal muscle, kidney, brain and lungs. LDH exhibits five isomeric forms assembled in tetramers of either of the two types of subunits, namely muscle (M) and heart (H). This is one of the H transfer (oxidoreductase) enzymes, which catalyzes the reversible conversion of pyruvate to lactate using NADH. Basically, LDH get involved in the anaerobic metabolism of glucose when oxygen is absent or in short supply. Red blood cells also contain moderate concentrations of LDH enzyme.

It is well known that pyruvic acid is the main end product of glycolysis in those tissues, which are supplied oxygen in abundance, but in those tissues where oxygen supply is insufficient or in anaerobic state e. g. skeletal muscles lactic acid form the usual end product of glycolysis [45]. In such cases pyruvic acid is reduced to lactic acid under the influence of lactic dehydrogenase. The main function of lactate dehydrogenase is that either it converts excess pyruvate to lactate or regenerates pyruvate from lactate for use as a fuel or biosynthetic

substrate. Normal level and utilization indicates metabolic and energetic harmony [46]. Therefore, increased level of lactic dehydrogenase in muscles and liver shows very low oxygen tension after tick saliva envenomation Thus pyruvate utilization decides level of LDH in state of no oxygen supply.

This elevated or high LDH level shows stress and hypertension and increase the rate of oxidation in animals. This is also a marker of intravascular hemolysis, reduced haptoglobin, and unconjugated hyperbilirubinemia [47]. Increase in the level of LDH also shows massive disintegration of cells and leakage of the enzyme in to the circulation [40]. Besides this, increased level of LDH in muscle and liver cells shows insufficient oxygen supply. Therefore, elevation in LDH level increases the glucose catabolism for energy production especially in anaerobic condition. LDH level also increase in a state of disease, anemia, heart attack, bone fractures, muscle trauma, cancers, infection and intoxication or poisoning of body tissues.

Herbicides such as atrazine [16], ammonia poisoning [15], pollutants like bisphenol A that induce hepatotixicity and cause severe oxidative stress [18] Similarly *para*-aminophenol (PAP) cause cytotoxicity in muscles, serum and liver and increases the level of Lactate dehydrogenase (LDH) level [12]. An elevation in the LDH enzyme activity in the serum correlated with a decrease in the activity of cardiac muscle LDH in all the age groups of isoproterenol-treated rats [13]. Snake venom causes significant alterations in AST, LDH, glucose and urea levels, and total protein content, as well as important tissue alterations in the liver, kidneys and lungs [48].

On the other hand activity of serum acetylcholiesterase was found to be reduced up to 70.96% and 57.14% respectively (**Table 1** & **Table 2**; **Figure 9**). It clearly indicates binding of the saliva toxin AchE and inhibit competitively. Similarly, ticks saliva toxins inhibit the action of lactic dehydrogenase, acetylcholinesterase thrombokinase and transaminases in envenomated patients [49]. Thymoquinone 10 mg/kg increased the activity of plasma and blood cholinesterases and reduced DZN-induced alternations of the liver [14].

On the other hand activity of acetylcholiasterase was reduced 70.96% at 4th hr of the saliva toxin injection in comparison to control (**Table 1**; **Figure 9**). It's level in gastrocnemius and liver was also found to be decreased significantly up to 48.14% (at 4th hr) and 61.29% at (at 4th hr), respectively in comparison to control (**Table 3** & **Table 5**; **Figure 16** & **Figure 22**). It clearly indicates binding of certain saliva toxin components with this enzyme. This inhibition of acetylcholinesterase activity causes accumulation of acetylcholine molecules at the synaptic junctions. This accumulation of acetylcholine may lead to prolonged activation of acetylcholine receptors and a permanent stimulation of nerves and muscle cells resulting in muscular paralysis and finally death of animal [50]. Tissue poisoning and intoxication causes also affect acetyl cholinesterase activity in mice [11]. The cholinesterase activity was inhibited, but the activities of alkaline and acid phosphates and lactate dehydrogenase were stimulated [40].

Organophosphate (OP) pesticides are known as nerve agents, these are major neuroinhibitors of acetylcholinesterase (AChE) activity. Both carbamate and organophosphate poisons decreased AChE level. Similarly, diazinon poisoning also influence acetylcholinesterase and butyrylcholinesterase level in affected animals and man [51]. Inhibition of acetylcholinesterase results in accumulation of acetylcholine in the central and peripheral nervous system. This excess acetylcholine generates a predictable cholinergic syndrome consisting of copious respiratory and oral secretions, diarrhea and vomiting, sweating, altered mental status, autonomic instability, and generalized weakness that can progress to paralysis and respiratory arrest. Similarly, acetylcholinesterase enzyme (ACHE) level was found to be increased in workers' blood of a poison-producing industry. AChE level in workers' blood was lowered with increasing at the exposure years due to irreversible effects. It is also observed that growth at the PPE application acted as an important parameter to decline exposure to poisons and subsequently various health complications [52].

Glutamate pyruvate transaminase (GPT) plays a key function in carbohydrate metabolism. It makes a way for the delivery of skeletal muscle alanine to the liver. In skeletal muscle pyruvate is transaminated to alanine and transported to the liver. Inside liver glutamate pyruvate transaminase transfers the ammonia to the α-ketoglutarate and regenerates pyruvate. This pyruvate is then utilized in the process of gluconeogenesis for glucose production [41]. Therefore, it functions as a link between carbohydrate and protein metabolism by catalyzing the conversion of alanine to pyruvate. The elevation in GPT level may be due to stress that was created after *Rhipicephalus microplus* toxin injection. Therefore, saliva toxin induced stress may be the causative factor for the elevation in GPT concentration [27]. It is evident that during stress condition the energy requirement becomes high, which results in very high utilization of glucose and a massive breakdown of stored glycogen that leads to a decrease in glycogen level [52]. Similarly, tick saliva exhibits pharmacological effects and causes a significant decrease in Na^+-K^+ ATPase level and obstructs glycogen synthesis in mice [53]. Similarly, administration of lyophilized venom of Egyptian scorpion caused a significant increase in serum glucose, creatinine, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase and lactic dehydrogenase level in mice [54].

In the present study, it is clear that tick salivary toxin molecules target metabolic enzymes in blood serum, liver and muscle tissues of experimental mice. Among them, feeding inhibitors, enzyme inhibitors or inducers and strong pathogen-secreted noble antigens may be there. These molecules have an important role in catabolism, pathological necrosis, lysosomal disintegration or autolysis and phagocytosis. Tick Saliva toxins also cause liver ischemia and hypoxia and impose oxygen deficient state. Massive disintegration of glycogen inside the liver and an increase in glucose level increase the chance to catabolize glucose molecules very rapidly which leads to an increase in the production of pyruvate that renders an increase in LDH level. There are two main reasons to study tick

salivary toxins, first to search for suitable inhibitors of blood feeding and second to find unique and novel protein antigens that can be used for the generation of antibodies to prohibit digestion of blood so that ticks can be deterred from feeding.

6. Conclusion

From results, it is clear that tick saliva toxins impose toxic effects on various tissues of experimental mice. These severely cause hemolysis of RBCs, liver ischemia and hypoxia, which increased in the level of serum acid phosphatase and alkaline phosphatase. Both these enzymes are detoxifying enzymes and their level increased after tissue poisoning. These enzymes are mainly found in the blood, liver, plasma and intestine of human beings. Saliva toxins target membrane-bound enzymes which mediate the transport of metabolites across the membrane and play an important role in protein synthesis. Tick saliva toxins also have targeted cellular metabolic enzymes serum glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT) and lactic dehydrogenase (LDH) found in the liver, heart cells, muscle tissue, pancreas and kidney. Alteration in levels of these enzymes displays damage to these tissues. An increased level of LDH in muscle and liver cells also displays insufficient oxygen supply and pyruvic acid is reduced to lactic acid under the influence of lactic dehydrogenase. Saliva toxin inhibited acetylcholinesterase activity that results in the accumulation of acetylcholine molecules at the synaptic junctions. This accumulation of acetylcholine may lead to prolonged activation of acetylcholine receptors and a permanent stimulation of nerves and muscle cells resulting in muscular paralysis and finally death of the animal.

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Authors' Contributions

Ravi Kant Upadhyay and Nidhi Yadav were responsible for conception, experiments, writing and revising the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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