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Effects of Purified Tick Saliva Toxins on Various Bio-Molecules in Blood Serum, Liver and Muscles of Albino Mice

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Abstract: In the present study, salivary toxins from *Rhipicephalus microplus* were isolated and purified on a Sepharose CL 6B 200 gel column at a constant flow rate with regular fractions of 5 mL/min. Pooled fractions were lyophilized and LD_{50} was then determined in albino mice. The LD_{50} value of the protein toxin of *Rhipicephalus microplus* was found 36.11 mg/kg body weight of albino mice. To determine physiological effects, a sub-lethal dose of *Rhipicephalus microplus* toxin and 40-80% 24 h LD_{50} of salivary toxin were injected into albino mice. In different biological assays, total serum protein, free amino acids, uric acid, cholesterol, pyruvic acid, total serum lipids and glucose levels were determined at different stages. In addition, the effects of toxins purified from tick saliva were observed on the rectus abdomens, gastrocnemius, muscles, atria and ventricles of albino rats and muscle glycogen. A significant increase in bimolecular activity was observed in the serum, liver and muscle of albino mice. The group injected with the saliva toxin showed an increase in serum protein content of 79.70% while the content of free amino acids, uric acid, cholesterol, pyruvic acid, total lipids and glucose all increased correspondingly. Toxins from tick saliva broke down glycogen stores in the liver, abdominal muscles, stomach muscles, atria and ventricles of albino mice. Toxins in tick saliva significantly reduced glycogen concentrations (67-82%) in the serum, liver, muscle and ventricles of albino mice. These effects were due to the presence of low molecular weight proteins that act on the membrane and interfere with muscle cell function in albino mice. These proteins could be used for drug development and target molecules for the generation of transmission-blocking vaccines or anti-tick vaccines.

Keywords: *Rhipicephalus microplus*, Serum Protein, Free Amino Acid, Uric Acid, Cholesterol, Pyruvic Acid, Serum Total Lipid, Glucose, Liver, Rectus Abdominis, Gastrocenimus, Muscle, Atria and Ventricle

Introduction

Ticks act as reservoirs for pathogens that are transmitted to the host during blood feeding. They are obligate blood-sucking parasites, dependent on their host's blood for food. To feed on blood, ticks cling to the host's skin for days, even weeks and secrete saliva to counteract the host's defenses. Ticks secrete anticoagulant molecules mixed with saliva that prevent blood clotting and allow the tick to continuously suck blood. This is one of the most important host defense mechanisms. Ticks use their hypostome to penetrate the skin and use their attachment organs to make feeding marks on the host's skin. They enter the nutrient duct inside the skin to suck blood (Fogaca *et al*., 2021). Ticks secrete saliva into the host's bloodstream while feeding and easily transmit pathogens (Vancová *et al*., 2020). During feeding, hard ticks use different parts of the mouth to suck blood. Ticks, mainly *Ixodes scapularis* nymphs, feed on host blood for longer periods of time. To achieve this purpose, they use adhesive biomaterials or cementum to approach the mouthparts to the host's skin and prevent the host from slipping out (Mulenga *et al*., 2022). The feeding tube may have barbs that help hold the tick in place. In all life stages, nymphs and adults feed exclusively on a variety of hosts, mainly domestic animals, wild animals, zoos and dairy farms. Ticks also act as pathogens

and disease vectors. The important causes that are responsible for tick parasitism are blood feeding and salivation, level of immune resistance, age, variety and regional ecology (Hrnková *et al*., 2021). To feed properly, ticks bypass or suppress the host's defense mechanisms, including the immune system. To do this, ticks secrete specialized immunomodulatory proteins in their saliva, like many other blood-sucking parasites (Denisov and Dijkgraaf, 2021). Tick saliva also contains Kunitz-like protease inhibitors that help ticks target host homeostasis and immunity (Jmel *et al*., 2023). These substances inhibit and/or block host homeostatic responses most relevant to protease inhibitors such as cystatin or serpin, also ensuring successful blood feeding by the tick. Ticks also contain Serine Protease Inhibitors (SPIs) which play an important role in the regulation of inflammation, blood clotting, wound healing, vasoconstriction and the modulation of host defense mechanisms (Blisnick *et al*., 2017).

Tick salivary glands produce toxins that contribute to the blood supply and play a significant role in the transport, release and pathogenesis of tick-borne pathogens in humans and other animals. ticks' saliva contains many pathogens such as viruses, rickettsiae, spirochetes and bacteria, fungi, protozoa and filariasis and transmits them through the blood to humans, livestock and wild animals. These pathogens, after entering the host's blood, stimulate the immune response and the host body begins to prepare innate and acquired immune defense mechanisms. These pathogens induce affording immune protection after interacting with cells, organs, proteins and tissues related to immunity. ticks' salivary secretions therefore transmit various pathogens to humans, pets and wildlife (Perveen *et al*., 2021). Ticks cause damage directly by affecting the site of entry or indirectly as vectors of a number of protozoa, bacteria and viruses. Tick-borne diseases ultimately lead to reduced productivity of livestock populations.

Two types of ticks, argasid or soft ticks and ixodids or hard ticks, have been reported. There is another large family of ticks, the “soft ticks” and the family argasidae which do not have a hard shield. Ixodidae is a family of hard ticks or scale ticks (Hrnková *et al*., 2021). Hard ticks have hard shields or scales. They feed very slowly but establish a long-term blood supply and widespread parasitism and exhibit host-specific responses (Blisnick *et al*., 2017). Tick menace is everywhere as tropical and temperate species are differently adapted but infest livestock and human populations in America, Europe, Africa, Australia and Asian countries. Currently, 904 valid tick species have been listed throughout the world. Ticks are the major cause of morbidity and mortality; they generate significant economic losses for dairy owners (Van Oosterwijk and Wikel, 2021). Major blood-sucking species of ticks are *R. microplus*, *Rhipicephalus (Boophilus) microplus*, *Amblyomma* and *Ixodes* sp which transmit so many tick-borne pathogens in humans. These carry a number of pathogenic, commensal and symbiotic microorganisms. These are the main vectors of disease for pets and people.

Although ticks are small, slow-moving creatures, their distribution depends on the movement of their host. These are more abundant in residential areas (Keesing *et al*., 2022). They are transported by wild and domestic animals from one place to another. Migrating birds carry them long distances as they cling to their bodies while feeding on blood (Zhang *et al*., 2022). Most commonly, the Western black-legged tick, *Ixodes pacificus*, transmit Lyme disease, a spirochete pathogen, *Borrelia burgdorferi* (Arthur and Snow, 1968). These pathogens circulate in the environment through a variety of hosts, including small mammals, birds, livestock and pets (McVicar *et al*., 2022). Ticks also harbor endosymbionts that influence their body physiology by influencing nutritional adaptations, fitness and immunity (Hussain *et al*., 2022). Recently, new tick-borne microorganisms and symbionts with unknown zoonotic potential have been reported in many parts of the world (Stanko *et al*., 2021). This can lead to more serious economic losses for people and their pets. This could be a potential vector of pathogens (Obaid *et al*., 2022).

Significance of the Study

Generally, for control of tick different categories of acaricides such as organochlorines, organophosphates, formamidines (e.g., amitraz), synthetic pyrethroids, macrocyclic lactones, fipronil and fluazuron have been used. But unfortunately ticks have developed resistance against most of them. This resistance in ticks was developed in different ways, including amino acid substitutions that resulted in morphological changes in the acaridae target, metabolic detoxification and reduced acaridae entry through the outer layer of the tick body (Sgroi *et al*., 2022). Ticks also have evolved adaptations to chalk out the immune functions of crossbreed livestock and challenge its genetic constitution (Lock, 2016). In response to control agent’s ticks have developed resistance to them and ticks also subvert the immune defense prepared by various hosts (Chmelař *et al*., 2019). This also lower downs the efficacy of recombinant vaccines and antibiotics. There is a need to focus on the search for new noble antigens, biologicals, signaling molecules, complement molecules, cytokines, death programmers, feeding inhibitors, antimicrobials, antibodies and vaccines so that a significant and long term control against tick bites and infestation could be achieved (Fogaca *et al*., 2021).

Tick saliva has a large number of proteins, including iron-binding proteins i.e., ferritins (Galay *et al*., 2014; Hajdusek *et al*., 2009) and lipocalins, anticoagulants and evasions (Chmela *et al*., 2019). These proteins are primarily used by ticks for blood feeding during host invasion and are known as evasions. These proteins assist ticks in feeding for more than 8-10 days without

being noticed by the host animal. These substances interact with biological molecules in the blood, mainly enzymes, creating an immune response and regulating inflammation (Chmela *et al*., 2019). Ticks secrete specialized immunomodulatory proteins in their saliva, as do many other blood-sucking parasites (Chmela *et al*., 2017). These substances block the secretion of host chemokines and prevent painful inflammation. These have important pharmacological and therapeutic values. There are two main reasons to study tick saliva: The first is to search for suitable vaccines that block pathogen transmission or "tick repellents" in the form of new and unique protein antigens. Second to have novel therapeutics derived from tick salivary components (Chmela *et al*., 2019).

Aim of the Study

This study was conducted to evaluate their percussions of toxins purified from tick saliva on various blood bio-molecules in serum, liver and muscle. This study was conducted to find out the main causes of disease, invasion routes and physiological effects that commonly occur in pets, humans and animals after being bitten by ticks and initiating infection. These toxins are responsible for creating toxic stress and thereby altering the levels of glycogen, proteins, lipids and amino acids and affecting the function of various metabolic enzymes. Most notably, low molecular weight proteins are unique molecules from tick saliva that can act as stable antigens and help develop antibodies. These new molecules induce a strong innate immune response in the host and induce their own immunity and anti-pathogen efficacy (Fogaca *et al*., 2021).

This study will help identify toxins in tick saliva as antigens for the synthesis of appropriate antibodies, anti-sera and vaccines to prevent strong tick feeding. In the present investigation, efforts have been made to isolate salivary proteins from ticks, mainly focusing on bio-molecular targets, structure-activity relationships and development prospects for their development into therapeutic methods. Ticks and other blood-sucking arthropods can be considered rich sources of proteins with unique biological activities against vertebrate homeostasis.

Experimental Procedure

Isolation and Purification of Tick Salivary Toxins

Live *Rhipicephalus microplus* was collected from rural areas of the Gorakhpur district. Live ticks were collected in sterile plastic containers and fixed by flash freezing at -20°C. Whole-body homogenates were prepared in phosphate-buffered saline (50 m Mol, pH 6.9) using a strong homogenizer. The homogenate was centrifuged at 24,000× G at 4°C for 30 min and the supernatant was used as crude toxin for saliva.

Making the Homogenate

Liver *Rhipicephalus microplus* were homogenized 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 min and supernatant was separated out. Total protein contents were estimated in the different supernatants according to the Lowry (1951) method.

Additionally, proteins (tissues) were solubilized with other solubilizes (Triton ×100, PBS,110% TCA, EDTA + Tris) in various combinations. The homogenate was centrifuged at 10,000× g rpm for 30 min and the proteins in the supernatant were analyzed according to Lowry (1951).

Purification of Salivary Toxin Protein from Rhipicephalus microplus

Proteins were eluted on a dual-chamber Sepharose CL-6B-2 hundred gel filtration column with afiltered sintered disc at the bottom of 1mheight and 25 mm diameter. 5 mL of toxic protein dissolved in PBS, was loaded onto the column. An uninterrupted flow rate wasmaintained at about 1 mL/min by continuously offering PBS buffer (pH 6.7) in a chilly room. Eluted fractions had been gathered at fixed periods the usage of a pharmacia fraction collector and protein content in different eluted fractions had been plotted; absorbance in every fraction was determined at 280 nm using a shimadzu spectrophotometer (UV 2001 laptop). Moreover, the absorbance of the identical sections was taken at 640 nm (Lowry, 1951).

Elute Salivary Toxin Proteins Through Gel Filtration Column

The column is held vertically by the stand. Elution of protein toxins through a gel filtration column was performed at a flow rate of 5 mL/min.

Elution of Saliva Toxins

Eluted salivary proteins/toxin fractions were collected manually at set time intervals and constant rates. A total of 140 fractions was collected. The eluted fraction was detected at 280 nm for salivary toxin proteins. After estimating the protein of the eluate using the Lowry method (Lowry, 1951) the absorbance was measured at 640 nm in a Shimadzu spectrophotometer (UV 2001 PC). A plot was drawn between absorbance obtained at 280 nm is generally intended to show the elution pattern of proteins/toxins in the saliva of *Rhipicephalus microplus.*

Molecular Weight Determination of Purified Salivary Proteins

Purified toxins/proteins from tick saliva were determined by running proteins of known molecular

weight through a sepharose CL-6B gel column as previously performed in the laboratory same flow rate. A calibration curve was plotted between Ve/Vo log M between elution volume fractions and molecular weights of different known proteins and compared with protein elution from salivary proteins/toxins *Rhipicephalus* (*Boophilus*) *microplus* at the same flow rate and in the same fraction (Chaubey and Upadhyay, 2008).

Lyophilization of Eluted Salivary Proteins

Eluted fractions of saliva toxins were pooled and lyophilized to obtain the desired concentration of salivary toxins.

Biological Activity of Purified Salivary Protein/Toxin

A known concentration of purified saliva toxins from *Rhipicephalus microplus* was injected intraperitoneal in albino mice and its effects were estimated accordingly.

Determination of Lethality of Salivary Toxin from Rhipicephalus microplus

Albino mice were injected subcutaneously with purified salivary toxins at different concentrations and the LD_{50} was determined in the 24 h period. Deformities such as paralysis and neurotoxic effects have also been reported. Six albino mice were injected with sequential concentrations of salivary toxin to determine LD_{50}. The mortality rate was determined using the Abbot formula. The LD_{50} value was calculated, at which half of the tested animals died. Lethal concentrations for 40-80% LD_{50} were determined using dose-fatality regression curves plotted on the Log Probit method of Spier (1982). Confidence limits are calculated at the 95% probability level.

Albino mice were injected subcutaneously with purified salivary toxins at distinct concentrations and the LD_{50} turned into decided in the range of 24 h length. Deformities which include paralysis and neurotoxic outcomes have additionally been suggested.

Six albino mice had been injected with sequential concentrations of salivary toxin to determine LD_{50}. The mortality rate was decided using the Abbot system. The LD value$_{50}$ was calculated, at which half of the examined animals died. Lethal concentrations for 40-80% of LD_{50} had been decided by the use of dose-fatality regression curves plotted on the log probity technique of Spier (1982). 95% probability is used to calculate confidence limits.

Filtration of Toxins from Freeze-Dried Saliva

After boiling a large volume of 2% (w/v) sodium bicarbonate and 1 mm EDTA (pH 8.0) for 10 min, the cellulose membrane used in the dialysis bags was thoroughly cleaned with distilled water. After that, the membrane was chilled and kept at 4°C. Prior to usage, the membrane was cleaned both inside and out with distilled water. Lyophilized salivary toxin protein was added to a dialysis bag and dialyzed three times against 50 mm (pH 6.9) phosphate buffer in order to eliminate excess salt from the freeze-dried foam of the aqueous toxin protein solution.

Blood Glucose Determination

Changes in blood glucose were measured in accordance with Mendel *et al.* (1954). The serum was deproteinized for this purpose using 5% TCA and 0.1% silver sulfate. For 10 min, the mixture was centrifuged at 10,000 g ×4.5 mL of H_2SO_4 added to 0.50 mL of the deproteinized supernatant and thoroughly mixed. After 6 min of boiling in a water bath, the contents were let to cool to room temperature. At 520 nm, the resultant pink color is detected. 0.5 mL of 5% TCA containing 0.1% silver sulfate and 4.5 mL of H_2SO_4 were used to set up the blank. The glucose level in the serum was reported as mg/100 mL.

Determination of Serum Pyruvic Acid Content

Variations in the concentration of pyruvic acid in serum were ascertained using Friedemann and Haugen (1943). The serum was deproteinized using 5% TCA and 0.10% silver sulfate for this purpose. It was then centrifuged at 10,000× g for 10 min. Subsequently, 0.10 mL of deproteinized serum was mixed with 1.0 mL of dinitrophenyl hydrazine and allowed to react for 15 min at room temperature. A standard solution of diluted pyruvic acid was used for the same process. 3.0 mL of xylene pass air should then be added and the mixture should sit for 2 min. Using a pipette, remove the bottom layer from the reaction mixture once it has solidified. After that, 6.0 mL of 10% sodium carbonate was added and the mixture was once more stirred by aerating through it for 2 min. Once the mixture has had time to settle, transfer 5.0 mL of the water layer and 5.0 mL of 1.5 N NaOH solution into a different test tube. After fully combining, let stand for 10 min. After calibrating the device to zero absorbance, 520 nm was used to measure the absorbance using a blank that contained 5.0 mL of 10% sodium carbonate and 5.0 mL of 1.5 N NaOH. The measurement for serum pyruvic acid was mg/100 mL of serum.

Determination of Serum Uric Acid Content

Changes in serum uric acid levels were ascertained using Folin (1933) cyanide-free method. To achieve this, 1.0 mL of serum and 8.0 mL of distilled water were combined. Next, 10% sodium tungustate solution and 0.50 mL of 0.66 N sulfuric acid were added to each tube. To guarantee full protein precipitation, the contents were

given time to react and were then left for 10 min. Precipitate was disposed of after the contents were filtered. They removed three test tubes. Four milliliters of filtrate should be added to the first tube, followed by four milliliters of the active uric acid standard solution (20 g of uric acid dissolved in 250 mL of distilled water) in the second tube. This process repeats three times. In a flask with 30 mL of distilled water, 1.0 mL of 14% sodium carbonate solution and 1.0 mL of uric acid reagent (10 g of sodium tungstate and 2.0 g of anhydrous disodium phosphate) were dissolved. 50 mL of distilled water and 2.5 mL of concentrated sulfuric acid are gradually added to the second flask and allowed to cool. After that, the sodium phosphate solution was mixed with a diluted sulfuric acid solution and the mixture was heated to reflux for 1 h. After cooling, dilute with 100 mL of distilled water and let sit at room temperature for 15 min. Setting the instrument to zero density with a solution containing only water and reagents, the optical density was measured at 680 nm.

Determination of Serum Cholesterol Level Content

Abell *et al*. (1952) method was used to measure changes in serum cholesterol levels. A 5.0 mL alcoholic potassium hydroxide solution (6.0 mL of 30% KOH solution added to 94 mL of absolute alcohol) was mixed with 0.05 mL of serum to estimate serum cholesterol. After giving the materials a good shake, they were incubated for 55 min at 37°C in a water bath. After allowing it to reach room temperature, thoroughly mix in 10 mL of petroleum ether. Pour in 5.0 mL of water and give it a good stir for a minute. For 5 min, the contents were centrifuged at a slow speed (1,200× g) to produce clear water and petroleum ether layers. Fill a dry test tube with 5.0 mL of petroleum ether, then set it in a water bath set at 60°C. By allowing air to pass through the mixture, the solvent will evaporate. One can make a standard cholesterol solution by dissolving 100 mg of dry cholesterol in up to 250 mL of absolute alcohol. There is 0.4 mg of cholesterol in 1.0 mL of this solution. After thoroughly shaking 5.0 mL of standard cholesterol solution and 0.30 mL of 33% KOH solution, the sample was incubated for 55 min at 37°C in a water bath after the mixture cooled to room temperature, 10 mL of petroleum ether were added and thoroughly mixed. Add 5.0 mL of distilled water and for 1 min, give it a good stir. It was centrifuged for 5 min at a slow speed (122× g) or until the emulsion split into two transparent layers. Place 1-4 mL of the petroleum ether layer into each of the four test tubes and evaporate until completely dry after centrifugation. There is an equivalent of 200, 400, 600 and 800 mg of cholesterol per 100 mL in these standards. To measure cholesterol content in vitro, test tubes are arranged as follows. Four test tubes for the standard containing the dry sample and one empty test tube for the blank were set up, followed by the unknown sample. 2.0 mL of concentrated H_2SO_4 was added to 40.0 mL of cooled acetic anhydride, thoroughly mixed and chilled for 9 min to yield 6.0 mL of modified liebermann-burchard reagent. After bringing the mixture to room temperature, 20 mL of glacial acetic acid was added and placed into each test tube. After shaking them, the test tubes were put back in the water bath. After calibrating the device to read zero density against the blank sample, the optical density was measured at 620 nm after 30 min.

Determination of Total Serum Lipids Content

Folch *et al*. (1956) method was used to estimate changes in total serum lipids. 500 µL of clear serum and a 2:1 v/v mixture of methanol and chloroform were combined for this purpose. The mixture was left to stand at room temperature for 2 h. It was filtered using Whitman paper no. 1. After an hour of re-suspension of the residue in the same volume of the mixture, the supernatant underwent another filtering. 0.6% (w/v) NaCl was added in an equal volume to both filtrates. The above mixture was placed in a separating funnel and allowed to sit at room temperature for 12 h in a dark place. The portion that cannot be sponsored is left unused while the upper solvent layer (chloroform + methanol) is recovered. The bottom layer was kept in a 60°C oven to allow its contents to evaporate. At the conclusion, the total lipid content was measured and reported as mg/100 mL of serum.

Determination of Total Serum Protein Content

Lowry (1951) method was followed for the quantification of serum proteins. 0.3 mL of distilled water was added to 0.20 mL of serum. Next, incorporate 0.50 mL of recently made alkaline copper solution (reagent C). 50.0 mL of Reagent A (2% sodium carbonate in 0.1 N NaOH) and 1.0 mL of reagent B (1% potassium sodium tartrate, 0.5% copper sodium sulfate) are combined to create reagent C. combined at the time of the experiment in a 1:1 ratio. 10 min were spent with the reaction mixture at room temperature. Next, 0.50 mL of the folin-ciacalteu reagent was added. At the time of the experiment, this reagent was diluted 1:2 with distilled water. Well-mixed content is present. A blue hue with a wavelength of 600 nm emerges after 15 min. Standard curves were created using various known BSA (bovine serum albumin) concentrations. The measurement of total serum protein was mg/100 mL of serum.

Determination of Amino Acid Content

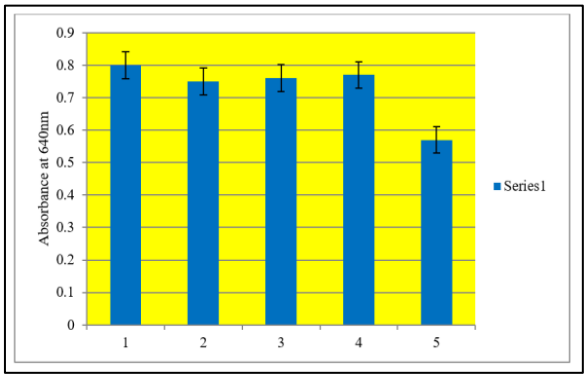
Spies (1957) method was utilized to ascertain alterations in the free amino acid composition of the

albino mice's serum. In a clean glass test tube, 0.10 mL of serum, 0.1 mL of distilled water and 2.0 mL of ninhydrin reagent are added and the mixture is well mixed for this purpose. The ninhydrin reagent was made by dissolving 0.04 g of stannous chloride in 25 mL of citrate buffer (pH 5.0) and 1.0 g of hydrin in 25 mL of absolute ethanol. For 15 min, the reaction mixture was submerged in boiling water. After letting it cool, 2.0 mL of 5.0% ethanol was added. Using a visible spectrophotometer, the color purple is detected at 575 nm (Systronics). A standard curve was drawn with glycine concentrations that were known. The values of free amino acids were reported as mg/100 mL of serum.

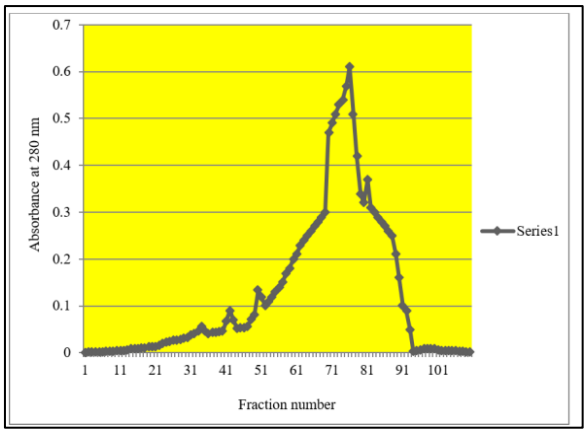
Results

Dissolution of the Toxin from Tick Saliva

Before isolating the toxin secreted by the tick salivary glands, a systemic extract of the tick was prepared; it was homogenized in various solubility buffers. Triton (0.1%) was found to be a better solubilize for *Rhipicephalus microplus* salivary protein than any other substance. Higher protein solubility was achieved in the supernatant compared to the residue, except for TCA (Fig. 1a).



(a)



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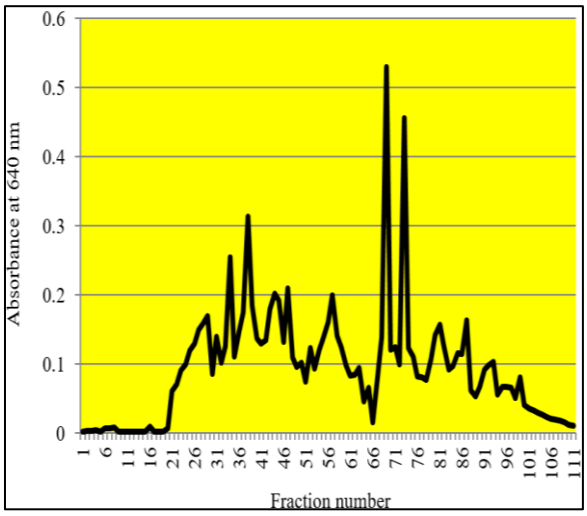

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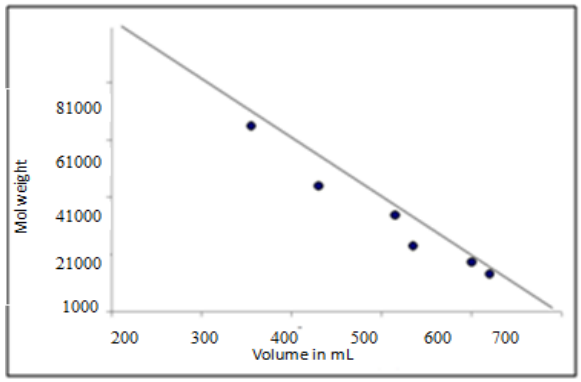

(d)

Fig. 1: (a) Solubilization of *Rhipicephalus microplus* tick proteins in distinct buffers. The absorbance of solubilized protein was taken at 640 nm. Solubilizing buffers on X-axis are (1) Triton ×100 (0.01%), (2) Tris + EDTA (0.1 mm) (3) PBS buffer (4) TCA 5% and (5) Absolute alcohol; (b) Is showing elution pattern of PBS extractable proteins of *Rhipicephalus microplus* tick chromatographed on a Sepharose CL-6B two hundred columns. Absorbance taken at 280 nm; (c) Is showing elution pattern of PBS extractable proteins of *Rhipicephalus microplus* tick chromatographed on a Sepharose CL-6B two hundred column Absorbance taken at 640 nm; (d) For determining the molecular weights of tick saliva toxins/ proteins/peptides proteins of known molecular weight were eluted from the same column Sepharose CL-6B 200 column. Saliva toxins from *Rhipicephalus microplus* were compared with standard proteins of known molecular weight i.e. BSA mol. wt 66, kD, egg albumin mol. wt. 45 kD, pepsin mol. wt. 34,7 kD, trypsinogen mol. wt. 24 kD, beta-lactoglobulin mol. Wt 18.4 kD and lysozyme mol. wt. 14,3 kD. Elution volumes of unknown proteins have been compared with log values at the X-axis for estimation of molecular weights

Purification

Rhipicephalus microplus was homogenized in 5 mL of PBS (pH 7.2) using a glass-glass homogenize. Initially, mL of solubilization buffer in order to isolate and purify the toxins from adult tick saliva. After 30 min of cold centrifugation at 15,000× g rpm and 4°C, the homogenate was gently separated into its supernatant. To separate toxins in saliva, it is loaded into a Sepharose CL-6B 200 column. The tick saliva toxin homogenate's elution pattern revealed five major peaks at 280 nm. Three peaks (31-37, 38-47 and 50-60) appeared immediately after the empty volume of the numbered fractions, while a fourth and fifth peak (67-73) appeared (Fig. 1b). In addition, the Lowry (1951) method was used to determine the amount of tick salivary toxins in each tube. Once more, at 640 nm, two comparable protein peaks were identified (Fig. 1b). Between fractions 50 and 60, there is a minor peak that is the first peak and between fractions 67 and 77, there is a major peak (Fig. 1c). PBS buffer (pH 7.2) was used for the elution of these two peaks. It was determined that 69.21% of the total yield of tick salivary toxins was in the eluted fractions.

Determination of Molecular Weight of Tick Saliva Toxin

The molecular weight of *Rhipicephalus microplus* salivary protein/toxin was determined by Sepharose CL 6B 200 gel column chromatography using standard molecular weight marker proteins I already know. The calibration curve indicated that the molecular weight of the purified salivary toxin protein ranged from 14.3-63 kDa (Fig. 1d). From the standard curve, the molecular weight of the purified salivary toxin protein was obtained in the range of 14-63.0 kDa (Fig. 1d).

Effect of purified toxin from *Rhipicephalus microplus* tick saliva on various biomolecules in mice serum.

In this part, the repercussions of purified saliva toxin are evaluated on different bio-molecules found in the blood serum of albino mice. Alterations in levels of proteins, amino acids, uric acid, cholesterol, pyruvic acid, overall lipids and glucose, had been measured after injection of 40-80% purified saliva toxin of ticks in 24 h LD_{50}.

Serum total protein level was found to decrease significantly ($p<0.005$) up to 86.25-78.02% at 4 h 40-80% respectively in 24 h LD_{50} compared to control. Then, it barely recovered up to 88.51-79.70% at the 8th h compared to the control (Tables 1-2; Fig. 2).

Serum-free amino acid concentrations were found to increase maximally up 97.36% at the 6th h of treatment with 40% of purified tick saliva toxin (24 h LD_{50}), while 80% of the 24 h LD_{50} caused a significant ($p<0.05$) increase of 113.2% at the 6th h. Thereafter, the restoration rate turned 89. 47-113.2% at the 6th h of treatment 8 compared to the control (Tables 1-2; Fig. 3).

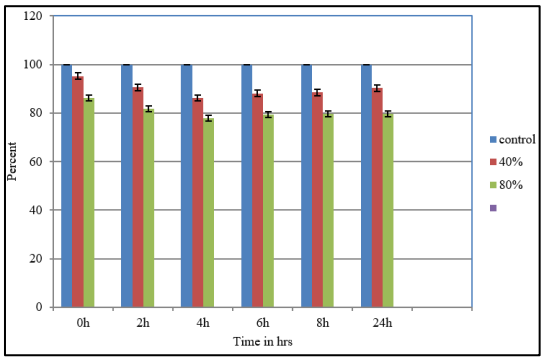

Fig. 2: *In vivo* outcomes of distinct dose of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of serum total protein

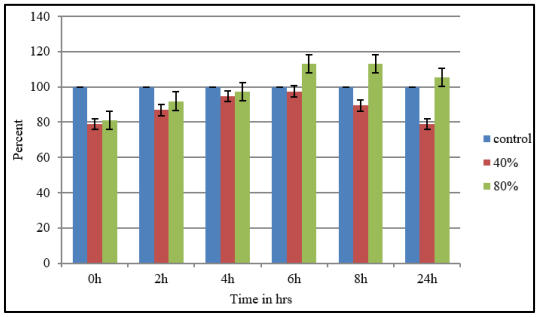

Fig. 3: *In vivo* outcomes of distinct dose of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on the activity of free amino acid

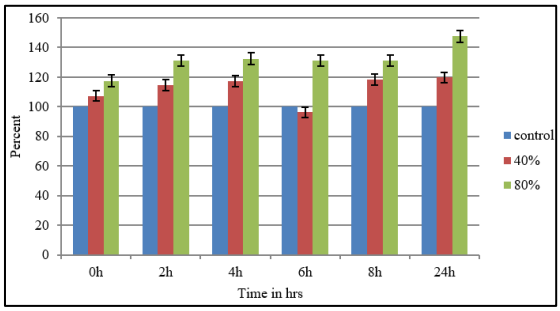

Fig. 4: *In vivo* outcomes of distinct dose of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on activity of serum uric acid

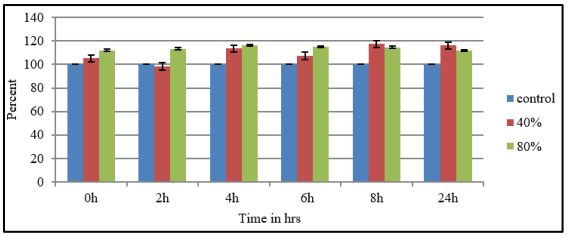

Fig. 5: *In vivo* outcomes of distinct dose of purified saliva toxins of Indian tick, *Rhipicephalus microplus* on the activity of cholesterol

Significant elevation ($p<0.05$) of serum uric acid was found to be 119.8-147.5% after injection of 40-80% of LD_{50} (24 h) of pure salivary toxin from *Rhipicephalus microplus* in comparison to control. Then, this was noted at 118.5-131.3% at the 8th h of toxin treatment compared to the control (Tables 1-2; Fig. 4).

Similarly, a slight increase in serum cholesterol level, i.e., 117.37-114.58%, was observed at the 8th treatment with an LD_{50} of 40-80% compared to the control. Then, at the 24th h of toxin treatment, it was 116.1-111.62% compared to the control (Tables 1-2; Fig. 5).

Serum pyruvic acid concentration was found to increase significantly ($p<0.05$) up to a maximum of 86.44% at the 6th h of treatment with 40%LD_{50} while it was 93.22% at the 6th h of treatment with 80% 24 h LD_{50} in purified salivary toxin compared to control. It was later found to be 81.35-88.13% at the 10th h of treatment with the same dosage compared to the control at a similar dose (Tables 1-2; Fig. 6).

Significant increases ($p<0.05$) were achieved in total serum lipids of 1258 h and 156.5% at 6 h of remedy with forty-eight percent of 24-h LD_{50} in comparison to control. Subsequently, this rate was determined to be 116.7-152.7% on the 10 h of remedy (Tables 1-2; Fig. 7).

Consequently, a significant ($p<0.05$) elevation obtained in serum glucose level was 114.33% at 10 h of remedy with forty and eight percent of 24-h LD_{50} in comparison to control. Later on, it was elevated up to122.66% at 10 h of remedy with eight percent of 24-h LD_{50} and this elevation was sustained up to 8th h (Tables 1-2; Fig. 8).

Effect of purified tick salivary toxin on glycogen levels in the liver, rectus abdominis muscle, gastrocnemius muscle, atrium and ventricle of albino mice effects of tick salivary toxin purified saliva toxins were determined on glycogen contents in the liver, rectus abdominis muscle, gastrocnemius muscle, atrium and ventricle of albino mice. Alterations in glycogen level were assessed after injections of forty and eight percent of 24 h LD_{50} of salivary toxins. The following changes were observed in glycogen levels.

Inside the liver, glycogen concentration decreased substantially ($p<$zero.05) as much as 69.28% after 4 h of 40% of LD_{50} and 54.51% at the 8th h of 80% LD_{50} upon toxin injection tick saliva compared to control. It was elevated up to 88.86-63.72% on the 10th h (Tables 3-4; Fig. 9).

Glycogen level in the rectus abdominis muscle lowered significantly ($p<0.05$) by 71.55% at the 6 h and 62.66% at the 8th h of remedy with forty and eight percent of LD_{50} (24 h) in comparison with control. Then, it recovered up to 86.22-80.88% at the 8th h (Tables 3-4; Fig. 10).

Similarly, glycogen concentration in gastric muscle was found to decrease significantly ($p<0.05$) up to 62.24-47.95% at the 8th h and 40% and 24 h respectively. LD_{50} of purified saliva toxin injection compared to control. Furthermore, the recovery rate was up to 87.75-76.53% at the 10th h of treatment with the same 24 h LD_{50} dose (Tables 3-4; Fig. 11).

Atrial glycogen levels decreased significantly ($p<0.05$) by 58.55% at 6 h of 40-42.79% at 8 h of 80% 24 h LD_{50} of toxicity purified tick saliva, respectively, compared with controls. Then it was 72.97-50.9% in the 24th h (Tables 3-4; Fig. 12).

Similarly, ventricular glycogen concentration decreased significantly to 65.46% at 6 h of 40% 24 h LD_{50} and 58.27% at 8 h of 80% 24 h LD_{50} of tick saliva toxins. Its level at 10 h was 78.5-66.9% compared to the control (Tables 3-4; Fig. 13).

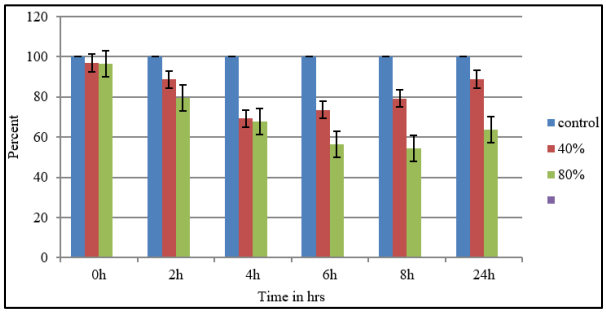

Fig. 6: *In vivo* outcomes of distinct doses of purified saliva toxins of Indian tick, *Rhipicephalus microplus*

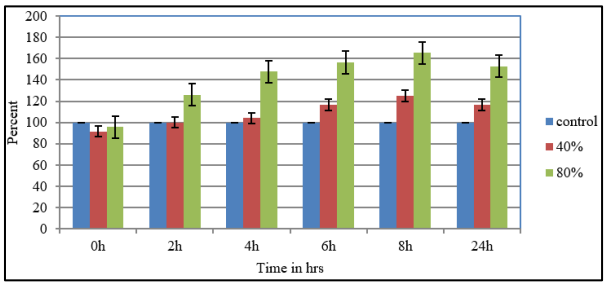

Fig. 7: *In vivo* outcomes of distinct doses of purified saliva toxins of Indian tick *Rhipicephalus microplus*

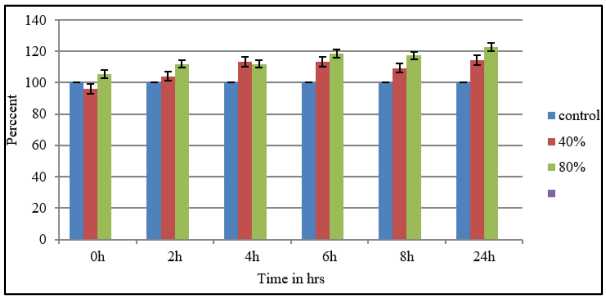

Fig. 8: *In vivo* outcomes of distinct doses of purified saliva toxins of Indian tick *Rhipicephalus microplus*

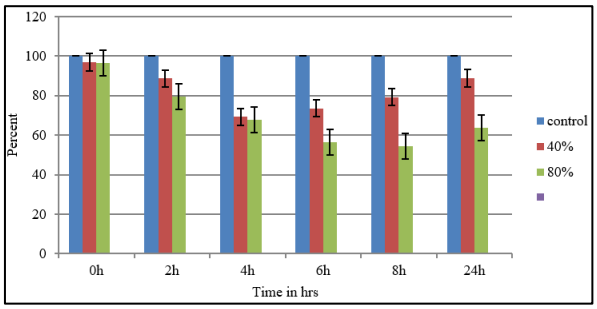

Fig. 9: *In vivo* outcomes of distinct doses of purified saliva toxins of Indian tick, *Rhipicephalus microplus*

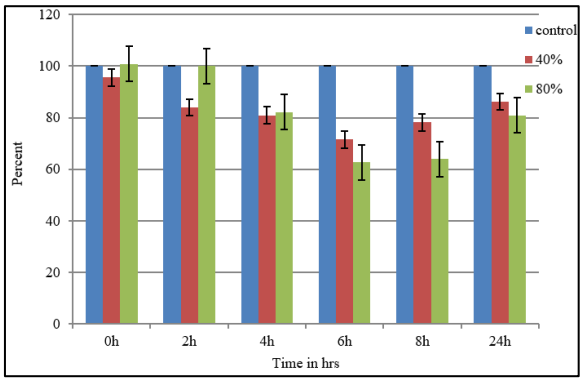

Fig. 10: *In vivo* outcomes of distinct dose of purified saliva toxins of Indian tick, *Rhipicephalus microplus* rectus abdominus glycogen

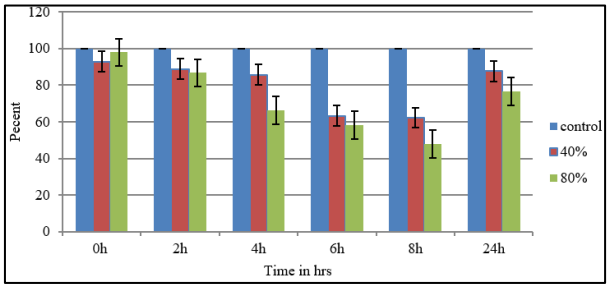

Fig. 11: *In vivo* outcomes of distinct dose of purified saliva toxins of Indian tick, *Rhipicephalus microplus* gastrocnimus glycogen

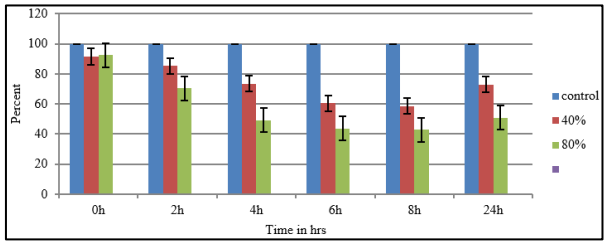

Fig. 12: *In vivo* outcomes of distinct dose of purified saliva toxins of Indian tick, *Rhipicephalus microplus* atria glycogen

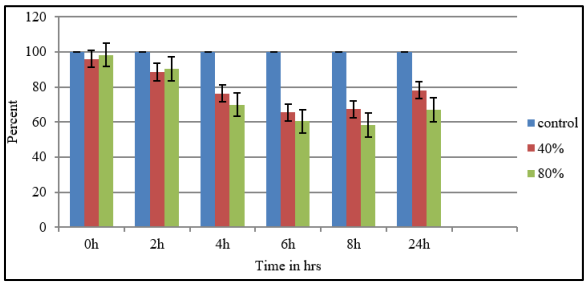

Fig. 13: *In vivo* outcomes of distinct dose of purified saliva toxins of Indian tick, *Rhipicephalus microplus* ventricle glycogen

Discussion

Ticks are parasites that rely on their host's blood for the nutrients they need to meet their physiological needs. They are vectors of various pathogens and transfer them to the blood during transfusion. Tick salivary glands secrete toxins that induce blood feeding and uninterrupted blood supply from hosts (Feng *et al*., 2019). Ticks mix saliva toxins in the blood pool to prevent blood clotting and thereby ensure constant bloodsucking. Long-term blood feeding affects the health and survival of domestic and wild animals worldwide (Blisnick *et al*., 2019). Saliva toxins affect the host's hemostasis and wound-healing processes and destroy the host's immune response. They help create a favorable environment for the survival and spread of many tick-borne pathogens and host-parasite relationships (Blisnick *et al*., 2019). Tick salivary proteins are involved in multiple physiological roles, including oocyte development, protein transport, immunity, antibacterial, anticoagulant and adhesion (Blisnick *et al*., 2019).

Tick saliva is rich in various molecules such as proteins, peptides and lipid derivatives. It also contains two main groups of protease inhibitors, small molecular weight Kunitz inhibitors (18-24 kDa) and serpins and cystatins (Martins *et al*., 2020). Kunitz inhibitors are anti-hemostatic drugs (Abbas *et al*., 2022) and assist ticks to target host homeostasis and immunity (Jmel *et al*., 2023). Protease inhibitors have the ability to inhibit blood clotting, platelet aggregation and vasodilation ultimately promoting successful host trans-differentiation and generating immune responses.

Regular blood feeding meets the metabolic needs of ticks (Blisnick *et al*., 2019). Ticks, mainly *Ixodes scapularis* nymphs, feed on host blood for longer periods of time. To achieve this purpose, they use adhesive biomaterials or cementum to approach the mouthparts to the host's skin and prevent the host from slipping out (Mulenga *et al*., 2022). To feed properly, ticks must overcome or suppress the host's defense mechanisms, including the immune system. To do this, ticks secrete specialized immunomodulatory proteins in their saliva, like

many other blood-sucking parasites (Denisov and Dijkgraaf, 2021). Saliva proteins induce tick immunity and are associated with the development of acquired resistance (Cerný *et al*., 2020). The ticks of the same species differentially express Tick Saliva Proteins (TSPs) when stimulated to start feeding on different hosts (Tirloni *et al*., 2017). These might be suitable candidates for development of a tick vaccine (Cerný *et al*., 2020).

Tick saliva proteins from *Rhipicephalus microplus* notably altered the ranges of numerous bio-molecules after *in vivo* injection of forty percent and eighty percent of 24 h LD50 in albino mice. These have dropped down the level of total serum protein position, by over to 86.25 at the 4th h in albino mice (Tables 1-2; Fig. 2). In contrast serum free amino acid level, was found to elevate significantly up to 97.36% 6 h after toxin injection compared to the control (Tables 1-2; Fig. 3). This suggests that crack saliva toxins also impose strong proteolytic exertion that leads to reduced total serum protein and increased free amino acid concentration (Lima *et al*., 2000). Similar exertion has been reported in social bees (*Polistis infuscatus*), ants (*Ectionburchelli*) (Schmid-Antomarchi *et al*., 1985) and snake venoms (Guitérrez and Lomonate, 1989; Koh *et al*., 2001). They show strong proteolytic exertion and produce high situations of free amino acids. This may be due to the presence of protease enzymes in tick saliva, which strongly act on proteins and peptides and carry out their conversion to free amino acids (Lima *et al*., 2000). This conversion may increase free amino acid levels in toxin-injected albino mice. Similarly, proteinase isolated from snake venom (*Bothrops jararaca* white-tailed pit viper) (Bello *et al*., 2006) showed proteolytic and fibrinolytic activity (Bilo *et al*., 2005).

This notably decreased protein interest due to high proteolysis in mice (Krauze *et al*., 2007). The drop in protein conditions with accelerated transaminase exertion indicates the rallying of free amino acids for the duration of salivary bane induction and strain to satisfy strength needs (Zeba and Khan, 1995). In addition to some non-insect scorpion venoms, additionally they show appreciably decreased protein concentrations due to high proteolysis in mice (Krauze *et al*., 2007). The tick's fatty body plays an essential function in storing and the use of energy.

Additionally, following a 24 h salivary toxin injection, compared to controls, serum uric acid levels increased by as much as 119.8% (Tables 1-2; Fig. 4). This reaction was catalyzed by xanthine dehydrogenase. Extensive exposure to toxins, poisons and toxic substances has been shown to cause hyperuricemia. According to Meneshian and Bulkely (2002), a number of inflammatory cytokines cause xanthine dehydrogenase formation when the body is hypoxic. According to Leyva *et al*. (1998); Quinines *et al*. (1995), renal excretion and endogenous synthesis are also necessary for serum uric acid levels. According to this, when *Rhipicephalus microplus* salivary toxin was administered in tiny doses to experimental mice, there was a tiny increase i.e., (Fig. 6; Tables 1-2) shows that at 8 h, the serum total cholesterol level was 117.37%. The membrane is impacted by toxins found in the saliva of ticks. *In vivo*, tests involving the injection of sub-lethal doses of the toxin revealed severe hemolysis, indicating that cholesterol can be released into the blood as it is a structural component of damaged membranes. Mortal due to salivary mites. Thiol-activated toxins, which are characterized by their ability to form pores in matter, comprise the majority of small molecule toxins. Notably higher ($p<0.05$) i.e., 8 h total serum lipids showed a 125% increase (Tables 1-2; Fig. 7). According to Daisley (1998); Büchler *et al*. (1989), saliva toxins also induce hyperglycemia in rats, which raises serum glucose, total lipids and cholesterol (Daisley, 1998; Büchler *et al*., 1989). The main reason behind this is a breakdown of glycogen and the release of lipid and cholesterol molecules following membrane disruption of these molecules. Another reason for elevated serum cholesterol may be decreased insulin levels (Scheuer and Stezoski, 1970). Therefore, an increase in total serum lipid levels leads to increased oxygen consumption and leads to heart failure in rats. This also suggests a cytotoxic effect releasing more lipids because of membrane disruption. One of the important causes of increased total lipid concentrations also appears to be disruption of carbohydrate metabolism and imposition of cytotoxicity in blood cells (Pothu *et al*., 2019).

Under pathological condition, high lipid level occurs due to the inhibition of Na+ - K+ ATPase activity and sarcolemmal defects in animals (Cuppoletti and Abbott, 1990). However, increased total lipid levels might also elevate the secretion of catecholamine's, glucagon, thyroid hormones and cortisol levels and reduction in insulin secretion (Scheuer and Stejoskins, 1970). The fat body of tick females possesses neutral lipids (Angelo *et al*., 2013). In addition, both cholesterol and other lipids are required by *A. phagocytophilum* for infection and multiplication in human cells (Manzano-Roman *et al*., 2008). PLIN (perilipin) is a major adipocyte lipid droplet-associated phosphoprotein that plays a central role in lipolysis and cholesterol synthesis. The tick's fatty body is vital in storing and utilizing energy.

Tick salivary toxin induces a considerable rise ($p<0$-0.5) in blood glucose level, or 114.33% at the 10 h mark, which has a major impact on serum biochemistry (Tables 1-2; Fig. 8). This may result in a sustained rise in blood glucose levels due to a significant hepatic breakdown of glycogen to glucose and a faster rate of oxidation. Additionally, as a result, during 6 h, the content of pyruvic acid increased to 86.44% (Tables 1-2; Fig. 6). An animal used in an experiment under stress caused by toxins may become

oxygen-deficient and produce lactic acid, much like in an aerobic condition. Upon activation, immune cells rewire metabolic pathways to meet demands for energy and biosynthesis. While regulatory T cells and M2 macrophages predominantly employ the Tricarboxylic Acid (TCA) cycle and have decreased glycolysis, the majority of lymphocytes, including inflammatory M1 macrophages, primarily shift from oxidative phosphorylation to glycolysis. A few "non-metabolic" signaling roles of glycolysis and mitochondrial pathway intermediates have been reported. Carboxylic acids, including citrate and succinate, are involved in immunological response, activating macrophages and modifying proteins post-translationally (Ye *et al*., 2022).

In contrast, glycogen concentration decreased in the liver by 69.28%, in the rectus abdominis muscle by 71.55% and in the gastric muscle decreased by 62.24% after 8 h, while in the myocardium, the glycogen concentration in the atrium was 42, 79, 65.46% in myocardium ventricles after 24 h (Tables 3-4; Fig. 13). This may be due to an increase in use of glucose to eliminate toxic stress hyperglycemia results in increased catecholamine's, glucagon, cortisol and thyroid hormone secretion of and decreased insulin secretion (Scheuer and Stezoski, 1970).

Glycogen serves as a food store and helps the animal's blood glucose levels stay stable. The direct factor in the blood's release of glucose is liver glycogen. On the other hand, after 24 h, the glycogen concentration in the atrium of the myocardium was 42, 79 and 65.46%, respectively. In contrast, the glycogen concentration dropped by 69.28% in the liver, 71.55% in the rectus abdominis muscle and 62.24% in the gastric muscle (Tables 3-4; Fig. 13). This might result from the four times as much glucose being used to get rid of toxic stress. In contrast to the liver, there was less glycogen breakdown in the muscles. Muscle glycogen is not an energy source for sustaining blood glucose levels during fasting; rather, it serves as a local energy source for exercise. Nevertheless, glycogen can also be released to preserve the stiffness and tone when toxins are present in high concentrations. This is due to the fact that lactate, which is produced when muscle glycogen is broken down, is transferred to the liver through hepatic gluconeogenesis. After injecting scorpion (*Mesobuthus tumulus concaves*) venom into experimental animals, similar elevations in blood glucose have been observed (Murthy and Haghanazani, 1999). After injections of a sub-lethal dose of the centipede (*Scolopendera moristans*) toxin into rats, blood glucose levels were found to be elevated and the toxin also causes a persistent decrease in glycogen concentrations in the intestine, liver, skeletal muscle and heart muscle (Mohamed *et al*., 1980). In particular, toxic stress in animals may be the cause of increased blood sugar and a decrease in stored glycogen levels. The animal experiences respiratory tract obstruction and low oxygen levels once poison enters the body.

Animals require more oxygen for catabolism, which can only be compensated by the breakdown of blood sugar and the subsequent increase in pyruvic acid, which converts to acetyl Co A, which produces more energy than in mitochondria. However, glycogenolysis becomes rapid to maintain glucose levels and stored glycogen is broken down (Tunget and Clark, 1993). Similarly, Yousef *et al*. (2003), similar metabolic changes were observed in humans affected by synthetic toxins. Tick saliva toxins inhibit the secretion of insulin, a key enzyme involved in carbohydrate metabolism regulation. In turn, insulin stimulates the oxidation of glucose to produce energy. But however, low blood insulin levels cause slow glucose oxidation leading to the inhibition of lipolysis (Kahn *et al*., 1995). Therefore, increasing glucose levels inhibits lipid utilization and increases serum lipid concentrations in intoxicated rats. At an intravenous dose of 1 µg/mL g/mL, raw saliva toxin also disrupts bone, stomach and myocardial activity and reduces the amplitude of muscle contraction 15 min after injection. In contrast, concentrations between 0.5 and 2.0 g/mL caused a decrease in heart rate (bradycardia) and an increase in P-R interval prolongation (Nabil *et al*., 1998). Specifically, tick saliva toxins block sodium channels in, the heart, skeletal and brain muscles (Datiles *et al*., 2008). Saliva toxins also inactivate Na+ channels and inhibit action potential propagation in rats (Schmid-Antomarchi *et al*., 1985).

In protein meals, the substitution of fat for carbohydrates leads to a higher rise in nitrogen synthesis than does the same substitution in non-protein meals. Thus, the primary effect of substituting fat for carbohydrates in a mixed diet is a reduction in the body's capacity to use protein from the diet. The amount of nitrogen balance that changes when fat is substituted with carbohydrates is the same whether fat is provided in conjunction with or apart from dietary protein. This implies that the phenomena are not caused by the additive impact of feeding fat with protein, but rather by the elimination of carbohydrates from meals containing protein. The mice's nitrogen balance and the quantity of carbohydrates and protein they ate in their diets were correlated linearly.

Temperature is the main factor that affects metabolic activity and the rate of depletion of energy reserves in *Ixodes ricinus*, it also decides host-pathogen interaction and transmission pattern and increases the risk of tick-borne diseases. It was experimentally established in a group of nymphs, males and females of *I. ricinus* by keeping them inside an incubator at temperatures ranging from 5-30°C. Levels of protein, carbohydrate, total lipid, neutral lipid and glycogen were measured in pupae up to 70 days and in adults up to 42 days. In pupae, at day 0, the level of glycogen was

highest, followed by carbohydrates, with relatively low protein and lipid levels. In men, the concentrations of the various metabolites were obtained relatively similarly. In contrast, in women, glycogen and carbohydrate concentrations are relatively low compared to protein and neutral lipid concentrations. Significant exponential decreases in metabolite concentrations of all metabolites were detected over time at all life cycle stages and temperatures. Pupae generally had lower resource depletion rates than adults at all temperatures. The lower threshold for metabolic activity is estimated to be between -10-5°C. The Q 10 value, which describes the thermal sensitivity of metabolic rate, is estimated to be relatively low (1.5 for nymphs, 1.71 for males and 1.63 for females) compared to insect's overlap where they occur, usually about 2.5 (range: 1.5-3). This is considered an adaptation to increase survival during extended periods between feedings.

Conclusion

In this study, a low molecular weight tick saliva toxin was isolated on a sepharose CL 6B 200 gel filtration column. The lyophilized toxin was injected subcutaneously into the above laboratory animal. They significantly affect the levels of various biological molecules in the blood, mainly increasing the utilization of glucose, leading to the breakdown of stored glycogen into glucose. Likewise, higher host protein utilization and greater amino acid release were observed. Tick saliva contains additional protease inhibitors that are variously produced during feeding, many of which have inhibitory effects on coagulation, platelet aggregation, vasodilation and immunity. Tick saliva is a natural reservoir of pathogens, to which antigens or substances of different origins are also added. Ticks have serpins, a group of low molecular weight proteins that secrete saliva. Serpin toxins are used to develop drugs and target molecules to create transmission-blocking vaccines or anti-tick vaccines. Additionally, a better understanding of host-parasite interactions at the molecular level will assist in the generation of new methods to identify a set of antigens that can be used as potential targets for Vaccines.

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Author's Contributions

Nidhi Yadav: Contributed to specific aspects of the experimental work described in this manuscript.

Ravi Kant Upadhyay: Played a central role in conducting the experimental work detailed in this manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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