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Full Length Research Paper

Production of polyclonal antibodies against Indian honeybee (*Apis indica*) venom toxins and its efficacy in reversal of toxic effects

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In this study, the efficiency of polyclonal anti-honeybee venom (HBV) antibody was successfully explored in the form of reversal of toxic effects induced by the venom. Honeybee venom expresses its toxicity not only by allergic reactions but it also causes molecular toxicity by making alteration in enzymes and bio-molecules. To come out from these toxic effects, polyclonal antibodies were generated by immunizing albino mice. Antibody was partially purified by octanoic acid precipitation and by ammonium sulphate treatment. The presence of antibody in the antiserum was confirmed by immuno-double diffusion test. 40% of LD_{50} of bee venom was incubated with 400, 800, and 1200 µg of purified antibody and this incubated mixture was injected into experimental mice. Parallel to this, one set of mice were injected with only 40% of LD_{50} and another injected with only saline which was considered as the control. The venom injected group showed 89.69% decrease in serum protein content while free amino acid, uric acid, cholesterol, pyruvic acid, total lipid and glucose was increased by 112.5, 122.10,102.48,110.0, 125.0 and 107.22% respectively. Subsequently, venom dose also elevated serum ACP, ALP, GPT, GOT, LDH up to 126.92, 128.44, 136.66, 109.09, and 114.24%. Contrarily, it depleted the AchE activity. On the other hand, the group of experimental animals that received 40% of LD_{50} of venom incubated with purified antiserum showed a complete reversal of the above abnormalities in the content of serum bio-molecules and enzymes.

Key words: Honeybee, enzyme, antiserum, toxin.

INTRODUCTION

Bee venom envenomation is a major problem in tropical and subropical regions especially in India, China, Latin, the Middle East and North, central and South Africa. Africanized honeybee, *Apis mellifera* is most commonly involved in stinging incidences throughout the world. However, in India, *A. indica* is the main destructing species, living in large colonies. It causes stinging in mass, which is deadly toxic to the victim (Betten et al., 2006).

Honeybee venom is a complex mixture of various natural chemicals such as alkaloids terpenes, polysaccharides, biogenic amines (Histamine), organic acids (Formic acids) and amino acid, but the major constituents are peptides and proteins (Schmidt, 1986; Blum, 1981). Functionally, honeybee venom toxins are highly active substances (Dotimas and Hider, 1987), which elicits both local and systemic reactions. Initial signs and symptoms include diffuse widespread edema, inflammation of the skin, swelling, headache, weakness and fatigue, and dizziness (Hider, 1988; Sheely, 2003). In heavy honeybee envenomation, most visible symptoms are nausea (Schumacher and Egen, 1995), vomiting (Winston, 1994), diarrhea (Tunget and Clark, 1993) and hypotension (Greenberg et al., 2005) occur. It also make a great trouble in regular physiological machinery such as respiratory, vascular, renal and other important organ system of the body (Park, 2005; Schumacher et al., 1990). Cytotoxic peptides of the venom also damage the storage system of liver by breaking the cells (Wu et al.,

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Abbreviation: TCA, Trichloroacetic acid; **EDTA,** ethylenediaminetetraacetic acid; **GTP,** glutamate pyruvate transaminase; **ACP,** acid phosphatase; **ALP,** alkaline phosphatase; **GOT,** glutamate oxaloacetate transaminase; **LDH,** lactic dehydrogense; **AchE,** acetylcholinesterase.

1998).

Besides, this bee venom bind to ion channels with high affinity and generally display a large array of ion channel targets. It affects voltage gated K^+ channels and do make immune suppression of the body (Devaux et al., 1996) and cause fast death (Okamoto et al., 1995). It also causes a drastic innervation, denervation and alters the expression of the Ca^{++} activated conductance (Schmid-Antomarchi et al., 1985). Bee venom also affects the synaptic functions in the brain and Na^+ and K^+ channels in several cells (Edstrom, 1992).

Therefore, after seeing the causalities caused by honeybee stings in the villages and forest pockets, it was assessed that there must be a prompt action to work on immunotherapy for fast recovery of patient. It was considered as an appropriate method for the treatment of envenomated patients that might be antibody/antisera. It is highly demanded and recommended by the clinicians. It can successfully neutralize the effect of venom toxins in no time. Hence, in this study, venom toxins were isolated and purified from *A. indica* and polyclonal antibodies were raised in albino mice.

MATERIALS AND METHODS

Isolation and purification of honeybee venom

Living honeybees *(Apis indica)* were collected from local forest area. *A. indica* were anesthetized with chloroform, dissected in cold PBS (pH 6.9) and their sting apparatus with glands were taken out from the last segment, were homogenized in a glass-glass and homogenized in 5 ml solubilizing buffer. 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 × *g* rpm for 30 min and proteins were estimated in supernatant according to Lowry et al. (1951).

Proteins were eluted on a sepharose CL-6B 200, a double cavity gel filtration column according to Spier (1982) with sintered disc fitted in the bottom having a height of 1 m in 25 mm diameter. A known volume of toxins/proteins solubilized in PBS (pH 6.8), was loaded in the column and a flow rate between 20-24 ml/h was maintained by a continuous buffer supply 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 were plotted on graph and absorbance was determined at 280 nm using Shimadzu spectrophotometer (UV 2001 PC). Further, absorbance of same fractions was taken at 640 nm after protein estimation by Lowery et al. (1951) method.

Lyophilization of eluted venom proteins

The eluted fractions of venom toxins were pooled and lyophilized to get a desired concentration of venom toxins.

Dialysis of lyophilized venom toxins

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. Membrane was washed again with distilled water inside and out side before use. The lyophilized venom protein was filled in the dialyzing bag and dialyzed against three changes of phosphate buffer (50mM, pH 6.9) to remove the excess of salt from the lyophilized venom protein solution.

Determination of serum glucose

Changes in serum glucose level were measured according to Mendel et al. (1954). For this purpose, blood serum was deproteinized with 5% TCA containing 0.1% silver sulphate. The mixture was centrifuged at 10,000 × *g* for 10 min. In this, 0.50 ml deproteinized supernatant, 4.5 ml of H_2SO_4 was added and mixed thoroughly. Contents were boiled in water bath for 6 min and the mixture was allowed to cool at room temperature. The pink colour obtained was read at 520 nm. The blank was set by using 0.5 ml of 5% TCA containing 0.1% silver sulphate and 4.5 ml of H_2SO_4. The glucose level was expressed as mg/100 ml of blood serum.

Determination of serum pyruic acid

Changes in level of serum pyruvic acid were determined according to the method of Friedeman and Haugen (1943). For this purpose blood serum was deproteinized with 5% TCA containing 0.10% silver sulphate and centrifuged at 10,000 × g for 10 min. Then 1.0 ml of dinitrophenylhydrazine was added to 0.10 ml deproteinized serum to react at room temperature for 15 min. The same procedure was carried out with the dilute pyruvic acid standard solution. Now 3.0 ml of xylene was added, air was passed and the mixture was left for 2 min. After setting the reaction mixture, the lower layer was discarded by means of a pipette. Then 6.0 ml of 10% sodium carbonate was added and mixed again by bubbling air through the mixture for 2 min. After permitting the mixture to settle, 5.0 ml of the aqueous layer was taken into another test tube and 5.0 ml of 1.5N-NaOH solution was added. It was mixed thoroughly and left for 10 min. Absorbance was read at 520 nm after setting the instrument at zero absorbance with the blank containing 5.0 ml of 10% sodium carbonate and 5.0 ml of 1.5N-NaOH. The serum pyruvic acid was measured in terms of mg/100ml blood serum.

Determination of serum uric acid

Changes in serum uric acid level were determined by the cyanide free method of Folin (1933). For this purpose 1.0 ml of serum was taken and to it 8.0 ml of distilled water was added. Then, to each each tube, 0.50 ml of 0.66 N sulphuric acid and 10% sodium tungustate solution were added. Contents were allowed to react and left for 10 min to ensure complete protein precipitation. Contents were filtered and precipitate was discarded. Three test tubes were taken. In the first tube, 4.0 ml of the filtrate was poured, to the second, 4 ml of the working uric acid standard (20 g of uric acid was dissolved into 250 ml of distilled water) was added while 4.0 ml of distilled water was added to the third. 1.0 ml of 14% sodium carbonate solution and 1.0 ml of uric acid reagent (10 g of sodium tungustate and 2.0 g of anhydrous disodium phosphate) were dissolved in a flask containing 30 ml of distilled water. In a second flask, gradually 2.5 ml concentrated sulphuric acid was added to 50 ml of distilled water and it was cooled. Then dilute sulphuric acid solution was added to the sodium phosphate solution and this mixture was refluxed for 1 h. This was then cooled and diluted to 100 ml with distilled water and was left stand for 15 min at room temperature. The optical density was read at 680 nm setting the instrument to zero density with the solution containing only water and the reagent.

Determination of serum cholesterol

Changes in serum cholesterol level were measured according to the method of Abell et al. (1952). For estimation of serum cholesterol, 0.05 ml of serum was added to 5.0 ml of alcoholic potassium hydroxide solution (6.0 ml of 30% KOH solution was added to 94 ml absolute alcohol). Contents were shaken well and incubated in a water bath at 37°C for 55 min. It was allowed to cool at room temperature and then 10 ml of petroleum ether was added and mixed well. 5.0 ml of water was added and was shaken vigorously for 1 min. Contents were centrifuged at slow speed (1200 × g) for 5 min to make the clear layers of petroleum ether and water. 5.0 ml aliquot of the petroleum ether was transferred to a dry test tube and placed in a 60°C water bath. Solvent was evaporated by using stream of air over the solution.

Standard cholesterol solution was prepared by dissolving 100 mg of dry cholesterol in sufficient absolute alcohol to make volume up to 250 ml. This solution contained 0.4 mg cholesterol in 1.0 ml. 5.0 ml sample of the standard cholesterol solution was mixed with 0.30 ml of 33% KOH solution, was shaken well and incubated in water bath at 37°C for 55 min. Contents were cooled at room temperature and to it 10 ml of petroleum ether was added and mixed well. 5.0 ml of distilled water was added and shaken vigorously for 1 min. It was centrifuged at slow speed (122 × g) for 5 min or until the emulsion was broken into two clear layers. After centrifugation, 1, 2, 3 and 4 ml aliquot of the petroleum ether layer were taken into four separate test tubes and was evaporated to make dry. These standards contained the equivalent of 200, 400, 600 and 800 mg of cholesterol/100 ml. Test tubes were arranged for testing the cholesterols contents in the test tubes. An empty test tube (for blank) and four test tubes for standard containing dried sample followed by unknowns were set. 6.0 ml of modified Leibermann-Burchard reagent (2.0 ml of concentrated H_2SO_4 was added to 40.0 ml of chilled acetic anhydride was added, shaken well and kept cold for 9 min. After this, 20 ml of glacial acetic acid was added and the mixture was warmed to room temperature) was added to each test tube. The test tubes were shaken and returned to water bath. After 30 min, optical densities were determined at 620 nm after setting the instrument to read zero density with the blank.

Determination of serum total lipid

Changes in serum total lipid level were estimated according to Floch et al. (1957) method. For this purpose 500 µl of clear serum was mixed with a mixture of chloroform and methanol (2:1 v/v). The mixture was suspended and kept for 2 h at room temperature. It was filtered with Whatmann paper No. 1. The residue was resuspended in the same volume of mixture for 1 h and again the supernatant was filtered. Both filtrates were mixed with equal volume of 0.6% NaCl (w/v). The separatory funnel containing the above mixture was kept in the dark for 12 h at room temperature. The upper layer of solvent (chloroform + methanol) was collected and the unsaponified part was kept unused. The contents of lower layer were allowed to evaporate by keeping it in an oven at 60°C. Total lipid contents were weighted at the end and expressed in mg/100 ml of blood serum.

Determination of serum total protein

Estimation of protein in the serum was carried out by Lowry's method (1951). 0.20 ml of serum was taken and 0.3 ml of distilled water was added to it. Then 0.50 ml of freshly prepared alkaline copper solution (reagent C) was added. Reagent C was prepared by adding 50.0 ml of reagent A (2% sodium carbonate in 0.1 N NaOH) and 1.0 ml of reagent B (1% sodium potassium tartrate, 0.5% copper sulphate mixed in 1:1 ratio at the time of experiment). The reaction mixture was kept for 10 min at room temperature. Then 0.50 ml of Folin-Ciacalteu reagent (diluted 1:2 ratio with distilled water at the time of experiment) was added to it. The contents were mixed well. After 15 min, a blue color was developed which was measured at 600 nm. Standard curve was prepared by using different known concentrations of bovine serum albumin (BSA). The total serum protein was expressed in mg/100ml of blood serum.

Determination of free amino acids

Changes in the level of free amino acids in blood serum of albino mice were determined according to the method of Spies (1957). For this purpose, 0.10 ml of serum was taken in a clean glass test tube, 0.1 ml of distilled water and 2.0 ml of ninhydrin reagent were added to it and the mixture was mixed thoroughly. Ninhydrin reagent was prepared by mixing 1.0 g ninhydrin in 25 ml of absolute ethanol and 0.04 g of stannous chloride in 25 ml of citrate buffer (pH 5.0). The reaction mixture was kept in boiling water for 15 min. Now the contents were allowed to cool and after cooling, 2.0 ml of 5.0% ethanol was added to it. A violet colour was developed which was measured at 575 nm on visible spectrophotometer (Systronics). Standard curve was drawn using the known amount of glycine. The value of free amino acid was expressed as mg/100 ml of serum.

Determination of serum enzymes (*in vivo*)

Determination of acid and alkaline phosphatase enzyme activity

Change in acid and alkaline phophatase activity in blood serum, liver and in gastrocnemius muscle was determined according to the method of Bergmeyer (1967). A 100 mg of gastrocnemius muscle was homogenized in 1.0 ml of 0.9% NaCl solution and centrifuged at 5000 × *g* for 15 min in cold centrifuge. Supernatant was used as enzyme source. In the case of circulating ACP and ALP enzyme assay, blood serum was used as enzyme source For ACP enzyme activity determination. 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 g citric acid, 1.125 g 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 min at 37°C. 5 ml of 4.0 ml of 0.10-N NaOH solution was added to the incubated mixture. For ALP enzyme determination, 0.10 ml of serum was taken in a test tube and 1.0 ml of alkaline buffer substrate was added to it. The alkaline buffer substrate was prepared by addition of 375 mg glycine, 10 mg $MgCl_2.6H_2O$ and 165 mg p-nitrophenyl phosphate sodium salt to 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 min at 37°C. 5.0 ml of 0.02 N NaOH was then added to the incubation mixture. The reaction was stopped due to excess of NaOH. The p-nitrophenol formed as a result of hydrolysis of p-nitrophenyl phosphate gave a yellow colour with NaOH. Optical density was measured at 420 nm. Standard curve was drawn with the help of different concentrations of p-nitrophenol. Enzyme activity was expressed as µ moles of p-nitrophenol formed /30min/mg protein.

Determination of serum glutamate pyruvate transaminase and glutamate oxaloacetate transaminase activities

Changes in serum glutamate pyruvate transaminase (GPT) activity in blood serum, liver and in gastrocnemius muscle was measured

according to the method of Reitman and Frankel (1957). 100 mg of tissue was homogenized in 1.0 ml of 0.25 M chilled sucrose solution in ice bath and centrifuged at 3000 × g for 15 min in cold centrifuge. Supernatant was used as enzyme source. In the case of circulating GPT, 0.10 ml of non-hemolyzed serum was taken and 0.50 ml of GPT substrate (0.292 g of α-ketoglutaric acid and 17.8 g of DL alanine was taken and added 1 N NaOH slowly with mixing until all solids were dissolved and sufficient buffer was added to make the volume 1000ml and adjusted pH 7.4). The buffer solution was prepared by dissolving 13.97 g K_2HPO_4 and 2.69 g KH_2PO_4 in 1000 ml distilled water. For GPT enzyme determination, 0.10 ml of serum was taken and 0.50 ml of GOT substrate was added to it; GOT substrate (0.292 g of α-ketoglutaric acid and 26.6 g 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 make the volume 1000 ml and adjust pH 7.4). Buffer solution was prepared by adding 13.97 g K_2HPO_4 and 2.69 g KH_2PO_4 in 1000 ml distilled water. The contents were mixed well and incubated at 37°C for 1 h. 0.50 ml of 2, 4-dinitrophenyl hydrazine solution (dissolved 0.198 g of 2, 4- dinitrophenyl hydrazine in 85 ml of HCl and added sufficient buffer to make the volume 1000 ml.) was added and contents were left to stand for 15 min at room temperature. Then 5.0 ml of 0.4 N NaOH (1.6 g NaOH dissolved in 100 ml distilled water) was added and mixed well and allowed to stand at room temperature for 20 min. The optical density was read at 505 nm after setting the instrument to zero with the blank. Standard curve was prepared by using oxaloacetic acid as the working standard. The enzyme activity was expressed in units of glutamate pyruvate transaminase activity/ hour/mg protein.

Determination of lactic dehydrogenase

Changes in serum lactic dehydrogease activity in blood serum liver and gastrocnemius muscle were measured by the method of Annon (1984). A 100 mg tissue was homogenized in 1.0 ml of 0.1 M phosphate buffer (pH 7.5) in ice bath and centrifuged at 10000 × g for 30 min in cold centrifuge. Supernatant was used as enzyme source. In the case of blood serum enzyme activity, 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 buffers (10 g of K_2HPO_4 and 0.2 g pyruvic acid were dissolved in enough water to make 1000 ml and one drop of choloroform was added as preservative.) with 0.010 g $NADH_2$. The contents were incubated at 37°C for 45 min. 0.50 ml of 2, 4-dinitrophenyl hydrazine solution (0.2 g of 2, 4- dinitrophenyl hydrazine and 85 ml concentrated HCl made up the volume of 1 L with distilled water) was added and the contents of the mixture were kept at the room temperature. After 20 min, 5.0 ml of 0.4 N NaOH (1.6 g sodium hydroxide was dissolved in 100 ml of distilled water) was mixed and left for 30 min at room temperature. The optical density was measured at 540 nm and converted to lactate dehydrogenase (LDH) unit by means of a special prepared standard curve. Enzyme activity was expressed as µ moles of pyruvate reduced/45min/mg protein.

Determination of acetylcholinesterase

Changes in serum acetylcholinesterase (AchE) activity in blood serum liver and in gastrocnemius muscle was measured according to the method of Ellman et al. (1961). 100 mg tissue was homogeniged in 50 mM phosphate buffer (pH 8) in ice bath and centrifuged at 1000 × g for 30 min in cold centrifuge. Supernatant was used as enzyme source. In the case of the circulating AchE, 0.05 ml of blood serum source was pipetted to a 10 mm path length cuvette. To this 0.10 ml (5×10^{-4} M) of freshly prepared acetylcholinethioiodide 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 3 min at 25°C. Enzyme activity was expressed as µ moles 'SH' hydrolysed per minute per mg protein.

Production of polyclonal antibodies

Choice of animal

Albino mice (*Mus musculus*) that weighted 65±0.015 g were used for the immunization. These animals were reared in the laboratory according to the standard laboratory methods (in 16" X 16" X 16" cages) with proper care, feeding and were provided treatment humanly for nursing.

Antigen

Purified honeybee (*A. indica*) venom toxins were used as the immunogen after mixing with equal amount of Freund's adjuvant.

Preparation of immunogen

Immunogen was prepared by mixing purified *A. indica* venom toxins with an equal amount of complete Freund's adjuvant. Boosting immunogen was prepared by emulsifying the purified bee venom with incomplete Freund's adjuvant. For fine emulsification, emulsion was repeatedly taken in and ejected through syringe by adjusting a fine needle to disburse the water phase.

Dose of immunization

For primary immunization, 100 µl (281.25 µg) venom protein and 100 µl of complete Freund's Adjuvant were mixed well and injected to the animal.

Route of immunization

Immunization was done intraperitoneally. The mice were held in such a position that the abdominal region faced upright and then the needle was inserted into the abdomen to a depth of approximately 6 mm (Figure 3). The immunogen emulsion was released very carefully and slowly. The needle was withdrawn and site of immunization was pinched. Only one injection was given to each mouse.

Boosting

After 7 days of primary immunization, each experimental mouse was provided a booster dose (281. 25 µg) of venom toxins by same route. Similarly, a second booster dose was given to the mice after 21 days of primary immunization.

Bleeding and collection of blood

Animals were scarified and bled after 7 days of the second booster to get the serum. The mice were bled before feeding to obtain clear serum. The blood was collected by cardiac puncture method. For this purpose, the animal was put into a beaker with a wire grid bottom under which chloroform moistened cotton was placed. The top of the beaker was closed by a Petri plate. The chest area of the anesthetized animal was cleaned with alcohol. A fine sterilized

needle attached to a 2.0 ml syringe was inserted between the left third and fourth intercostals muscular space and as close to the sternum as possible. Needle was moved in the direction to the right shoulder and at an angle designated to penetrate the right ventricle of the heart. When the blood appeared in the syringe, it was sucked up.

Freshly drawn blood was collected into a clean glass tube without adding any anti-coagulant. The blood was allowed to clot in cold. The clot was broken very carefully from the side walls and serum was separated in a fresh tube. For the removal of particulate material, it was centrifuged at 10,000 × g for 20 min to get clear antiserum.

Purification of antibodies and storage

Octanoic acid precipitation

Anti-serum was partially purified by octanoic acid precipitation method. For this purpose, one volume of antiserum and two volume of sodium acetate buffer (60 mM, pH 4.0) were added at room temperature. To it 1 ml of n-octanoic acid was added drop wise per 10 ml of original antiserum. Contents were mixed thoroughly for 30 min and centrifuged at 1000 × g for 20 min and supernatant was taken out. The supernatant was dialyzed against appropriate buffer similar to the dialysis of purified venom.

Ammonium sulphate precipitation

Besides octanoic acid precipitation, antiserum was also precipitated by using 1.82 M ammonium sulphate solution. For this purpose, mixture was incubated for 30 min with constant stirring and centrifuged at 3,000 × g for 30 min and precipitate was collected. The precipitate was washed with 40% saturated ammonium sulphate (1.82 M). The suspension was re-centrifuged and process was repeated once more. Precipitate was dissolved in distilled water (approximately 2.0 ml distilled water per 10 ml antiserum) and dialyzed against 150 mM NaCl containing 0.1% sodium azide (w/v). After dialysis, purified antiserum was stored at 4°C.

IgG antibodies were isolated from antiserum by using stepwise octanoic acid and ammonium sulphate precipitation. Purified antibodies were stored at 4°C and were mixed with a bacteriostatic agent and sodium azide to prevent the microbial growth. Antibodies in aqueous aliquots were frozen at 4°C in sterilized plastic tubes.

Detection of antibodies in antiserum

The presence of antibodies in the antiserum was determined by the method of Ouchterlony (1962). 0.1% agar A was poured into clean microscopic glass plate to make a thin film and was allowed to dry. The pre-incubated slide was coated with 1% agarose in phosphate buffer-azide solution. One central and three peripheral wells of 3 mm diameter were made on the agarose-coated slide. 40µl (40 µg) of antiserum was added in the central well while 20 µl (56.25 µg) of antigen (purified bee venom) was loaded in the peripheral wells. This slide was incubated in a humid chamber overnight. After appearance of precipitin band, the glass plate was submerged in 0.15 M NaCl for 2 h to remove non-precipitating protein. The salts were removed from the glass plate by keeping the plate in distilled water. The glass plate was then dried and photographed.

Quantitative determination of antibody in the antiserum

For quantitative determination of antibody in the antiserum, appropriate volumes of antiserum were taken in 1.5 ml Eppendorf tube. This partially purified antiserum contained 4.95 µg/µl proteins. In the first set, varying doses of antibody ranges from 10-160 µl (10, 20, 40, 80, and 160 µl) were taken and fixed volume of antigen (40 µl) was added in all the five different tubes. In the second set, fixed volume of antibody was used (40 µl) and variable doses of antigen range from 10-160 µl were taken. In both sets, Ab and Ag volumes were allowed to be incubated at room temperature for 30 min. After which both sets were spun at 10,000 rpm for 15 min in a centrifuge. Supernatant was separated out and pellet was dissolved in 100 µl of PBS and 10 µl of it was used for protein estimation. From the above assay, PBS soluble pellet was used for iso-propanol treatment to take out non-precipitating material from the tube. Pellet obtained from the re-centrifugation was treated with 100 µl of iso-propanol. Protein content in all the tubes was determined by Lowry's method (1951).

Serotherapy

Efficacy of antivenom was tested in the albino mice. For this purpose, different concentrations (400, 800, and 1200 µg) of purified polyclonal antibody were mixed with 40% of 24 h LD_{50}. This mixture was incubated at 37°C for 2 h and injected in early aged experimental mice having similar body weight of 65±0.015 g each. All the behavioral activities were noted during this period in the mice. All important alterations in bimolecular and enzyme level were determined in the above treated mice after 4 h of treatment.

Experimental protocol for determination of efficacy of *A. indica* anti-venom

The experimental albino mice were divided in the following five groups: Group A: received phosphate buffer only (control group), Group B: received 40% of 24 h LD_{50} of purified *A. indica* venom, Group C: received 40% of 24 h LD_{50} of purified *A. indica* venom pre-incubated with 400 µg of anti-venom Group D: received 40% of 24 h LD_{50} of purified *A. indica* venom pre-incubated with 800 µg of anti-venom and Group E: received 40% of 24 h LD_{50} of purified *A. indica* venom pre-incubated with 1200 µg of anti-venom. After 4 h of the injection, mice were scarified and bled out to obtain serum. These serum samples were analyzed for bio-molecules and enzymes as were determined earlier in this study.

Statistical analysis

Results were expressed as mean ±SE of three replicates for each estimation. The data were analyzed by using one-way ANOVA, statistical probability of $p<0.05$ student's t test and analysis of variance to detect significant changes (Sokal and Rohlf, 1973).

RESULTS

Solubilization of venom proteins

Isolated sting apparatus with venom glands were homogenized and solubilized in different solubilizing buffers, such as Triton X-100 (0.1%), Tris+EDTA (0.1 mM), PBS, 10% TCA and absolute alcohol, among which Triton X-100 (0.1%) was proved to be a good solubilizing agent for the *A. indica* venom protiens than any other solubilizing buffer used. A higher protein solubilzation was obtained in the supernatant than in the residue except TCA (Figure 1a).

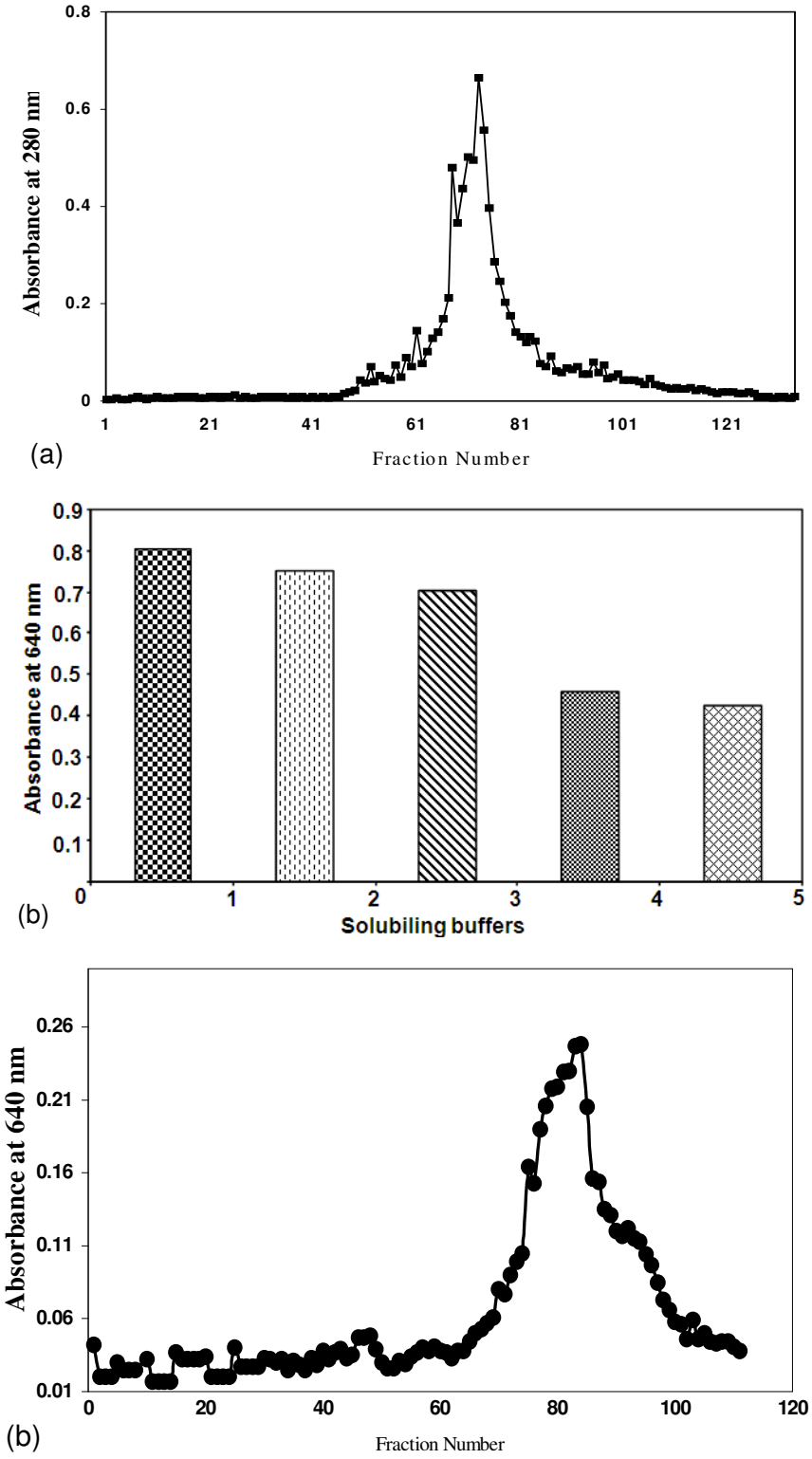

Figure 1. (a), Solubilization of sting gland proteins of *Apia indica* in different buffers. Absorbance of solubilized protein was taken at 640 nm. Solubilizing buffers on X-axis were (1) Triton X 100 (0.1%), (2) Tris + EDTA (0.1 mM) (3) PBS buffer (4) TCA 5% and (5) absolute alcohol; (b), elution pattern of PBS extractable proteins of *A. indica* sting gland chromatographed on a sepharose CL-6B 200 column. Absorbance was taken at 280 nm; (c), elution pattern of PBS extractable proteins of *A. indica* sting gland chromatographed on sepharose CL-6B 200 column. Absorbance was taken at 640 nm.

Purification

For this purpose, venom glands from fresh honeybees, *A. indica* were taken out and homogenized in PBS (pH 6.9) in glass-glass homogenizer. Homogenate was centrifuged in the cold at 4°C for 30 min at 15000 × g and supernatant was gently isolated. It was subjected to loading on a sepharose CL-6B 200 column for separation of venom toxins. Elution pattern of venom gland homogenate exhibited two major peaks at 280 nm; one soon after the void volume from fraction numbers 41-61 while the second peak was present in fractions 81-101 (Figure 1b). Further, concentration of venom proteins was determined in each and every tube by Lowry's method (1951). Again two similar peaks were resolved at 640 nm (Figure 1c). Peak one was a minor one present between 46-51 while the second peak was a major peak located between fraction numbers 60-10. Both peaks were eluted with PBS buffer (pH6.9). The total yield of venom proteins in the eluted fractions was calculated as 69.21%.

Determination of lethality of *A. indica* venom toxins

Lethality of purified *A. indica* venom toxins/proteins was determined both in albino mice and cockroach. Venom proteins from both protein peaks were lyophilized and solubilized in known volume of PBS buffer (pH 6.9). Various serial doses of the solubilized toxins were injected into the albino mice and cockroaches (*Periplaneta americana*) separately for determination of median lethal dose (LD_{50}). The LD_{50} value for purified *A. indica* venom was determined as 8.2+0.07 µg/g of body weight in mice and 4.13+0.21µg/g of body weight in cockroach.

Purification of polyclonal antibodies from antiserum

For this purpose, polyclonal antibodies generated against *A. indica* venom toxins, were partially purified by octanoic acid. It made the anti-venom free of lipoproteins. However, such treatment could not concentrate the antibodies, but remained in solution.

Further, the antiserum was also treated with ammonium sulphate that gently precipitated the antibodies out of the solution. The antibody recovery was found to be 1.0 mg/ml.

Detection of antibodies in antiserum

The presence of antibody was detected by immunodouble diffusion test (Ouchterlony, 1962). For this purpose both antigen (in peripheral wells) and antibodies (in central well) were allowed to diffused radially from their corresponding wells towards each other, therefore, establishing concentration gradients. As their concentration gradients reached to an equivalence zone, a visible cricentric band of precipitation complex of antigen-antibody was formed. This precipitation band represented the formation of antigen-antibody complex (Figure 4).

Quantitative determination of antibody in the antiserum

For quantitative determination of antibody in the antiserum, equivalence zone method was applied. For this purpose, partially purified antiserum that contained 4.95 µg/µl polyclonal antibody was used. In the first set of the experiment, varying concentrations of antibody ranges were used to interact with the fixed volume of antigen, while in the second set of the experiment, fixed volume of antibody and variable doses of antigen were taken. In both the sets, antigen and antibody interaction was allowed to be incubated at room temperature for 30 min. After which both sets were spun at 10,000 rpm for 15 min in the centrifuge. Supernatant was separated out and pellet was dissolved in 200 µl of PBS for protein estimation. Further, PBS soluble pellet was treated with iso-propanol to take out the non-precipitating material from the tube. Protein contents in the tubes were determined by Lowry's method. From the equivalence rule, 2.764 µg antigen/µg antibody was calculated (Figure 2) but after isopropanol treatment of antigen antibody complex, the binding ratio was obtained approximately as 1.01 µg antigen/µg antibody.

Reversal of bimolecular alterations by purified *A. indica* anti-venom

Experimental mice injected with 40% of 24 h LD_{50} of purified *A. indica* venom toxins were pre-incubated with different doses (400, 800, 1200µg) of the purified *A. indica* anti-serum. All metabolic alterations were reversed significantly after 4 h of the treatment.

The elevated serum glucose content was successfully reversed at its normal value as in the control group (100%), while it was merely increased in mice treated with 40% LD_{50}. *A. indica* anti-venom made 100% reversal in the serum pyruvic acid as it was increased up to 110% after the 40% of the LD_{50} venom injection (Table 1). It was also given a significant reversal in serum uric acid up to 101.05% while it was 122.10% in the venom treated mice (Table 1). After antiserum treatment, cholesterol which was marginally elevated in the venom injected mice was also reversed fully at low concentration of the anti-venom (Table 1). The lipid level was reversed up to 101.07% and that obtained in the venom treated mice was 125% (Table 1). In mice treated with only the venom serum protein, the content obtained was 89.69% which

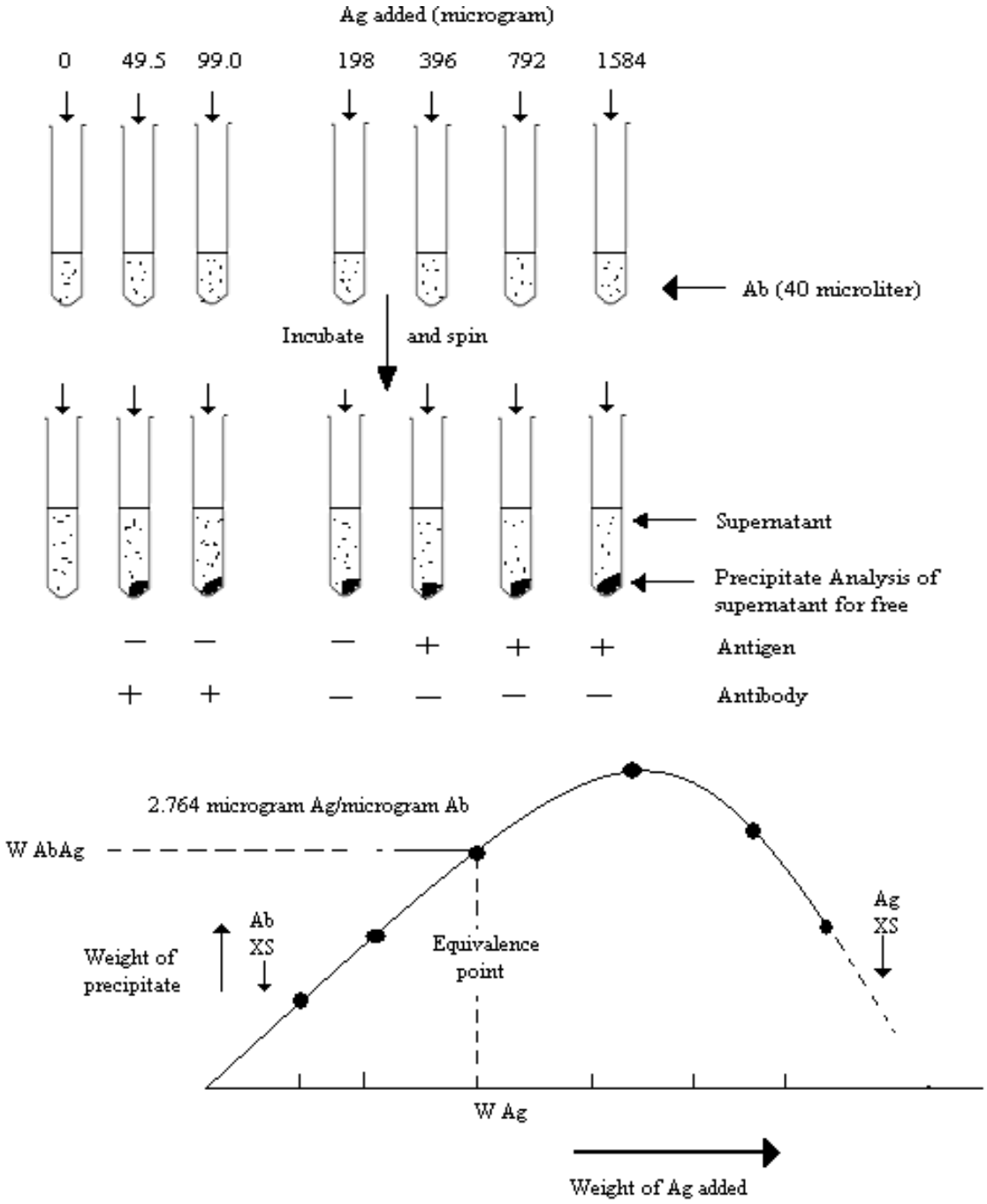

Figure 2. Quantitative precipitin reaction between honeybee venom protein (Ag) and polyclonal anti-honeybee venom antibodies. Increasing amounts of antigen were added to a constant volume of the antibody placed in a number of tubes. After incubation, the tubes were centrifuged and precipitate was weighed. Each supernatant was split into two halves by adding antigen to one and antibody to the other. The presence of reactive antibody/ antigen was calculated on the weight basis per µg antigen binding with per µg antibody from the equivalence where no Ag or Ab was present in the supernatant. Ab X S, antibody excess; Ag X S, antigen excess; WAb Ag, weight of antigen antibody complex; Wag, weight of antigen alone.

was reversed up to 98.96% after the antiserum treatment (Table 1). Similar treatment gave 100% reversal of free amino acid, which was increased to 112.5% in the mice treated with 40% of LD_{50} of the venom (Table 1).

Reversal of enzymatic alterations by purified *A. indica* anti-venom

Anti-serum treatment also successfully normalized the

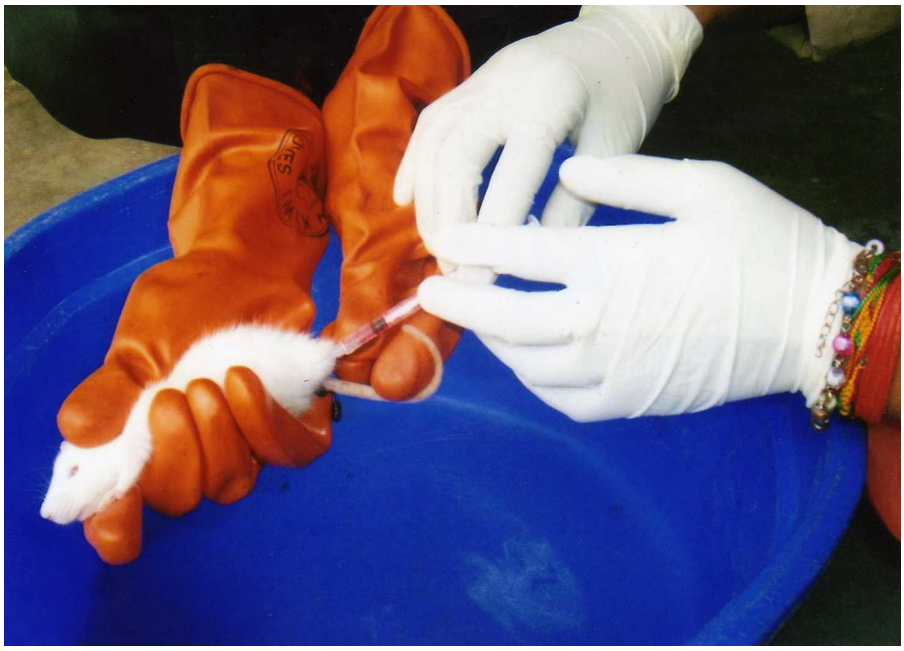

Figure 3. Immunization of albino mice for raising polyclonal antibodies against purified *A. indica* venom toxins.

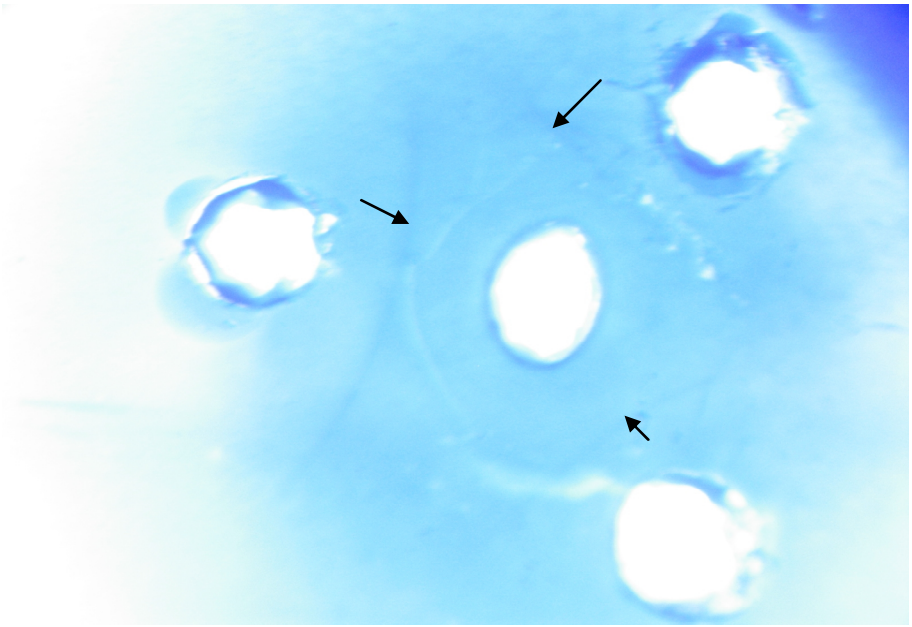

Figure 6. Immunodouble diffusion test (Ouchterlony, 1962) for conformation of antigen antibody interaction. Central well contains antiserum and peripheral antigen.

alteration in serum enzyme just after 4 h. Serum ACP content was obtained as 126.92% after 40% of LD_{50} venom injection, which was get normalized up to 103.84% after 4 h of the anti-venom treatment (Table 2). Similarly, 128.44% elevation was reverse back to 103.66% after anti-venom treatment (Table 2). Also, GPT level was significantly reversed up to 103.33% while it was 136.66% in the venom treated mice (Table 2). A complete reversal was obtained in GPT level, which was obtained as 109.09% in the venom treated animal (Table 2). Similarly, LDH which was elevated up to 114.24% in venom injected mice was successfully reversed up to 100.28% after anti-venom treatment (Table 2). *A. indica* venom showed 72% decrease in the AchE concentration,

Table 1. Reversal of level of certain biomolecules; that is total protein, free amino acids, uric acid cholesterol pyruvic acid total lipid and glucose in blood serum of albino mice after injection with the venom toxins. Before injection, venoms toxins were pre-incubated with purified *A. indica* anti-venom.

Bio-molecule	Group				
	Group A	Group B	Group C	Group D	Group E
Glucose	0.083±0.0024 (100)	0.089*±0.00094 (107.22)	0.085*±0.0016 (102.40)	0.083*v0.0029 (100)	0.083*±0.0045 (100)
Pyruvic acid	0.50±0.037 (100)	0.55*±0.044 (110.00)	0.53*±0.024 (106.00)	0.50*±0.0081 (100)	0.50*±0.035 (100)
Uric acid	0.95±0.036 (100)	1.16*±0.036 (122.10)	1.06*±0.032 (111.57)	0.99*±0.029 (104.21)	0.96*±0.016 (101.05)
Cholesterol	2.82±0.030 (100)	2.89*±0.021 (102.48)	2.85*±0.032 (101.06)	2.83*±0.032 (100.35)	2.82*±0.0047 (100)
Total lipid	0.28±0.012 (100)	0.35*±0.029 (125.00)	0.33*±0.016 (117.85)	0.29*±0.021 (103.57)	0.283*±0.012 (101.07)
Total protein	4.8±0.028 (100)	4.35*±0.041 (89.69)	4.58*±0.020 (94.43)	4.79*±0.024 (98.76)	4.80*±0.020 (98.96)
Free amino acid	0.040±0.032 (100)	0.045*±0.0033 (112.5)	0.043*±0.0016 (107.6)	0.040*±0.0024 (100)	0.040*±0.0008 (100)

Values (mg/100ml blood serum) are mean±SE of three replicates. Values in parantheses indicate per cent level with control taken as 100%. *Significant ($p<0.05$, Student t-test); Group A, without envenomation (control); Group B, after injection of 40% of 24 h LD_{50} of purified venom toxin; Group C, after injection of 40% of 24 h LD_{50} pre-incubated with 400 µg of anti-venom. Group D, after injection of Group C40% of 24 h LD_{50} pre-incubated with 800 µg of anti-venom; Group E: After injection of Group C40% of 24 h LD_{50} pre-incubated with 1200 µg of anti-venom.

which was fully recovered after anti-venom treatment (Table 2).

DISCUSSION

Honeybee attacks in large groups, which cause major casualties to man and his pats. It is a very serious problem for the beekeepers, passerby and children playing near its colony. It suddenly attacks their victims at a little disturbance and discharge a venom by the means of the stinger (Fitzgerald and Flood, 2006; Ciszowski and Mietka-Ciszowska, 2007). It is highly poisonous and lethal for animals and causes sudden death (Brandeburgo, 1990). More specifically, honeybee venom toxins cause cardiorespiratory failure and systemic anaphylactic shock (Przybilla, 1999). Most of the honeybee venom toxins are cytotoxins and cause heavy cell lysis (Wu et al., 1998) and damage the plasma membrane (Hoffman, 1996). Bee venom toxins damage nerve cells (Hider and Dotimas 1987) and show intense myotoxic reaction to humans (Nabil et al., 1998).

However, seeing the severity of the bee sting in patients, there is an urgent need to have an appropriate treatment, which might be more effective and clinically much safer. Therefore, in this investigation, an effort was made to isolate and purify the *A. indica,* venom toxins in the laboratory for the production of polyclonal antibodies in albino mice, which is much efficient to reverse the toxic effects caused by the bee venom successfully.

In the investigation *A. indica* venom proteins/toxins were isolated and purified by gel filtration chromategraphy by using sepharose CL-6B 200 as the gel matrix. These venom proteins were solubilized in different solubilizing buffers such as Triton X-100, PBS (50 mM, pH 6.9), 10% TCA, Tris+EDTA and ethanol. Higher solubilization was obtained in Triton X-100 (0.1%) (Figure 1a). Similarly, Jones et al. (1999) also solubilized the venom proteins isolated from venom glands of Africanized honeybee, *A. mellifera* in phosphate buffer.

The elution pattern of venom proteins/toxins obtained at 280 nm showed two major peaks; one soon after the void volume fractions No. 41-61 while the second peak was present in fractions 81-101 (Figure 1b). Further, protein contents were measured in each tube by following the Lowry's (1951) method, which again resolved two peaks at 640 nm (Figure 1c). The first peak was a minor one present between 46-51 while the second peak was a

Table 2. Reversal of level of certain enzymes; acid phosphatase, alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, lactic dehydrogenase and acetylcholinesterase in blood serum of albino mice after injection with the venom toxins. Before injection of venom, toxins were pre-incubated with purified *A. indica* anti-venom

Bio-molecule	Group A	Group B	Group C	Group D	Group E
ACP	0.26±0.024 (100)	0.33*±0.037 (126.92)	0.30*±0.026 (115.38)	0.28*±0.024 (107.69)	0.27*±0.037 (103.84)
ALP	1.09±0.024 (100)	1.40*±0.048 (128.44)	1.30*±0.044 (119.26)	1.17*±0.020 (107.33)	1.13*±0.021 (103.66)
GPT	0.030±0.0016 (100)	0.041*±0.032 (136.66)	0.036*±0.0037 (120.00)	0.033*±0.0023 (110.00)	0.031*±0.016 (103.33)
GOT	0.33±0.021 (100)	0.36*±0.024 (109.09)	0.35*±0.016 (106.06)	0.33*±0.014 (100)	0.33*±0.048 (100)
LDH	6.95±0.035 (100)	7.94*±0.032 (114.24)	7.02*±0.0024 (101.00)	7.02*±0.033 (101.00)	6.97*±0.030 (100.28)
AchE	0.025±0.0037 (100)	0.018*±0.0048 (72.00)	0.020*±0.0029 (80.00)	0.024*±0.0081 (96.00)	0.025*±0.0048 (100)

Values are mean±SE of three replicates. Values in parantheses 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.Group A, without envenomation (control); Group B, after injection of 40% of 24 h LD_{50} of purified venom toxin; Group C, after injection of 40% of 24 h LD_{50} pre-incubated with 400 µg of anti-venom. Group D, after injection of Group C40% of 24 h LD_{50} pre-incubated with 800 µg of anti-venom; Group E: After injection of Group C40% of 24 h LD_{50} pre-incubated with 1200 µg of anti-venom.

major peak and was located between fraction No. 60-101. Both peaks were eluted with 0.13 M NaCl PBS buffer (pH 6.9). The total yield of protein was calculated as 69.21%. Moreover, molecular weight of the *A. indica* venom was also determined on gel filtration chromatography.

For nullifying the toxic effect of bee venom toxins, polyclonal antibodies were generated in albino mice. For this purpose purified honeybee (*A. indica*) venom toxins were mixed with Freund's adjuvant and injected into the experimental mice intra-peritoneally. After seven days, booster dose was provided. Similarly a 2nd booster was also given to the mice after 21 days (Figure 3). The presence of polyclonal antibodies in the antiserum was detected by the method of Ouchterlony (1962). Due to antigen and antibody interaction, an equivalence zone was formed, which appeared in the form of a visible cricentric band. This precipitation band represented the formation of antigen-antibody complex (Figure 4). Further, polyclonal antibodies were purified by octanoic acid followed by ammonium sulphate precipitation. It was found that the treatment of anti-honeybee venom polyclonal antibodies with octanoic acid removed lipoproteins from the reaction mixture. Further, treatment of the anti-honeybee venom polyclonal antibodies with the ammonium sulphate precipitated the antibodies out of the solution, and concentrated the antibodies in the form of pellet. Similarly, Jones et al. (1999) produced antibody against the whole venom of *A. mellifera* in sheep.

Honeybee venom immunotherapy is an efficient treatment for the bee venom allergy and toxicity. It does not make any side effect (Muller et al, 1992). In response to the venom toxins, B-lymphocytes release a group of immunoglobulins each recognizing different epitopes, which bind to venom toxins (Jeanning et al., 1998; Paull et al., 1978; Kemeny et al., 1983a; 1989b). IgG are the major antibody which are synthesized by plasma B-cells and are much safer to be used in bee venom immunotherapy (Muller et al., 1992). It may be used as the universal safety kit for the cure of toxin envenomation (Lipps and Khan, 2000).

In this investigation, neutralization of honeybee venom toxins was also observed. For this purpose, different concentrations (400, 800, 1200 µg) of polyclonal antibody were mixed and incubated with 40% of 24 h LD_{50}. This pre-incubated mixture was injected into the experimental mice. It was found that all the physiological effects were normalized and anti-venom reversed the oxidative stress and other adverse effects such as muscular paralysis, hypotension and allergic responses in the experimental mice. Further, metabolic and enzyme alterations in the blood serum of the mice were also found reversed after

4 h of antibody treatment. Similarly, efficacy of honeybee anti-venom was also tested by Jones et al. (1999). However, Natu et al. (2006) determined the efficacy of anti-scorpion venom serum in patients stung by scorpion (*Buthus tumulus*) by exploring the data of the reversed metabolic reactions. Similarly, Schumacher et al. (1996) tested the efficacy of honeybee antivenom by giving injections of venom toxins preincubated with antibodies.

Besides mice model, anti- serum is also generated in rabbits, goats, and sheep, which have shown good results (Russel and Lauritzen, 1966). For getting more quantity of antiserum selection of animals for immunization, immunization should be done on the basis of age, weight, resistance and longevity of the animal. However, antigen detoxification or toxoid production requires neutralization of toxins, but at the same time it shows maximum immunogenicity (Khan et al., 1977). Thus the use of adjuvants is considered much effective for the production of antiserum. Similarly, Sjostrom et al. (1994) produced antivenoms in sheep by applying the same method and compared them with equine commercial anti-venom. Normally, sheep present very high tolerance both to Freund's adjuvant and other adjuvants with no local lesions. Therefore, high titers of high-affinity circulating antibodies were quickly developed. Similarlry, Schumaher et al. (1990) mixed bee venom with antibodies from a beekeeper and observed a successful treatment.

More specifically, it was found that; immunotherapy is the best solution to honeybee envenomation which effectively neutralize the effect of venom toxins and allergic patients. Therefore, it can be concluded that the anti-venom produced against honeybee venom has wider clinical and therapeutic application for retardation of all physiological alterations caused by honeybee venom toxins.

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