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COMPARATIVE BIOCHEMICAL STUDIES ON THE POISON GLAND AND POISON SAC OF THE WORKER BEES OF THREE DIFFERENT *APIS* SPECIES (*APIS DORSATA*, *APIS MELLIFERA* AND *APIS FLOREA*)

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ABSTRACT

In Hymenoptera glands associated with the sting apparatus of worker honey bee produce venom which is known to be composed of a wide spectrum of biomolecules ranging from biogenic amines to peptides and proteins. The anatomy of "Venom apparatus" reveals the presence of two important associated exocrine glands i.e. Venom gland and Dufors gland. The secretions of both glands are apocrine and are released into the lumen to be stored in the venom sac. The presence of some exocrine cells in the distal part of venom sac which is otherwise known to only store the component of Venom gland led to the present study. To compare the macromolecules and enzyme activity on extract of Venom gland and Venom sac different biochemical tests were performed in different Apis species. It was observed that there were considerable differences in the composition of Venom gland and Venom sac secretions of different Apis species. The concentration of lipids, proteins, activity of acid phosphatase and hexokinase was found to be more in case of Venom gland of A. dorsata followed by A. mellifera and then by A. florea while cholesterol, glucose, free amino acids, and activity of alkaline phosphatase was more in Venom sac of *A. dorsata* followed by *A. mellifera* and then by *A. florea.* Glycogen was absent in both Venom gland and Venom sac of *Apis* species as confirmed by the absence of glucose-6-phosphatase activity. It is established from the present study that Venom sac also secretes various biochemicals and enzymes which are added to the total Venom.

Keywods : Honeybee, Biochemical, Bee venom, Sting apparatus.

INTRODUCTION

Honey bees have fascinating social structure and advanced societies. Honey bees are classified in the Apini tribe within the subfamily Apinae and family Apidae (Ruttner, 1988). They are part of the large insect order Hymenoptera that includes bees, wasp, ants, and sawflies (Gullan and Cranston, 2000). Apis is the only genus of true honey bees. Oldroyd and Wongsiri (2006) recognize ten Asian species and one western species of honey bees which can be classified in to two groups based upon their nesting behavior. *A.florea, A.dorsata*, builds single comb and open-air nests and these bees are restricted to the Asian tropics and subtropics. *A.florea* is the smallest of all the species of honey bees, while *A.dorsata* has the largest individual body size of all honey bees (Michener, 2000).Among 2nd group *A.mellifera* that nest inside cavities where they build multiple combs. (Michener, 2000; Hepburn and Radloff, 2011). *A. mellifera* is the most commonly domesticated species of honey bees. It probably originated in tropical Africa and spread from there to Northern Europe and East into Asia.

The sting of the species is a modification of the female ovipositor, or egg laying apparatus. It is no longer used to lay eggs but instead serves as a weapon of defense. When a honey bee stings, the barbs on the stinger get stuck in the victim, and the stinger is pulled out of the bee's body. The bee dies shortly after stinging. Queen bees however can sting

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many times and can pull their stinger out of the victim's skin. The major gland with a defensive function is the poison gland. There is a large sac associated with the sting gland (Poison gland) which holds the venom. This gland has been called the acid gland (Snodgrass, 1956). It consist of cells that secret the venom in to the poison sac, which is surrounded by the muscles that pump the venom through the sting (Cruz-Landim and Kitajima, 1996; Bridges, 1977). The composition of bee venom has been a subject of interest for a long time, since venom can trigger serious allergenic reactions in humans. Its active ingrediants have been worked upon by various scientists and found to contain various enzymes, specific toxins, or other bioactive molecules; several of them have been characterized and their primary structures determined by biochemical techniques. Bee venom is haemorrhagic and contains apamin, melittin, phospholipase, hyalurnidase. The venom also contain mineral substances, volatile-organic acids, formic acids and some antibiotics. Venom is one of the products of honey bee, which is an important component in the pharmaceutical industry. It is used in the treatment of various health conditions such as pain (kim *et al*. 2003), cancerous tumours(Russell *et al*.2004), skin diseases, arthritis and rheumatism (putz *et al*.2006).

The aim of the investigation was planned because; recently it has been reported that some cells forming part of the reservoir wall are also secretory in nature (Bridges and Owen, 1964). The paucity of information with respect to these secretory components of the venom gland complex led to the present study.

Objective

To compare the macromolecules, free amino acids, and to assay the activities of certain enzymes (acid phosphatase, alkaline phosphatase, glucose-6-phosphatase and hexokinase) present in the two compartments of the venom apparatus. Also to compare the components among three species of honey bees including *A.dorsata, A.mellifera, A.florea.*

SIGNIFICANCE:

Venom is one of the products of honey bee, which is important component in the pharmaceutical industry. It is used in the treatment of various health conditions such as pain (Kim et al. 2003), cancerous tumours (Russell et al. 2004), skin diseases, arthritis and rheumatism (Putz et al. 2006), and the wide use of apitoxin (honey bee venom) is giving rise to a new branch of medicine referred to as Apitherapy (the medical use of honey bee products). Moreover the presence of some exocrine cells in the distal part of venom sac which is otherwise known to only store the component of Venom gland led to the present study.

MATERIAL AND METHODS

Study material

The samples of sting gland and reservoir of *Apis dorsata* workers taken for the present study were collected from near the nests made on high recesses in the building of Zoology department. The bees descend to this location every year during the winter season.

The samples of sting gland and reservoir of *Apis mellifera* L. workers taken for the present study were collected from colonies maintained by a bee keeper in village "Tee rah"near Chandigarh.

The samples of sting gland and reservoir of *Apis florea* workers taken for the present study were collected from the nests made on branches of bushes, hedges and trees from village Khuda Lahora near P.U., Chandigarh.

Sample collection

A random sample of worker bees was collected from the hive. The sting gland was gently pulled out along with the sting. The sting gland was put on a slide in a drop of saline. The chitinous structures were carefully removed with a needle. The glands and reservoir were separated with the help of a blade. Glands and reservoir were separately homogenized. Seventy glands and seventy reservoirs were pooled in different homogenizing tubes in 1.0 ml of saline and electrically homogenized. Samples S (sting gland) and R (reservoir) were prepared for the glands and reservoir respectively.

Analysis of biochemical parameters

The different macromolecules were estimated by standard methods (glucose by Somogyi-Nelson's method (1945), glycogen by Seifter's method (Seifter *et al.,* 1950), lipids by the method of Fringes and Dunn's (1970), cholesterol by Zalatki's method (Zalatki *et al.,* 1953) and proteins by Lowry's method (Lowry *et al.*, 1951). Amino acid assay was done by paper chromatography (Swarup *et al.,* 1981). Both acid and alkaline phosphatases were estimated by following the method of Bergmeyer (1963), glucose-

6- phosphatase by the method of Freeland and Harper (1959) and hexokinase by the method of Crane and Sols (1953).

RESULTS AND DISCUSSION

Honey bee venom is odourless, clear and water soluble. It is produced by the venom gland and stored in the venom sac. It has been reported that part of the reservoir wall also contain secretory cells. The present studies attempted to quantify and analyse the protein, carbohydrates, lipids, cholesterol, free amino acids and specific enzymatic activities separately in the sting gland and reservoir of the *A. mellifera* workers. The result of various biochemical tests performed on the two compartments of the venom gland are presented in fig 1-5 and table 1-3.

also reported by other workers, these biomolecules were quantified.

Saraf (2005) reported that there is highest amount of protein in the venom apparatus of *Apis dorsata.* Stinging accidents also report that the stings of *A.dorsata* are more painful and toxic than the stings of other species of *Apis*.

It was observed that the protein concentration was more in sting gland than in reservoir of the *Apis* species and the protein concentration were found to be highest in *A.dorsata* followed by *A.mellifera* and than last by *A.florea*.As shown in the fig. 1.(*A.dorsata>A.mellifera>A.florea*)

According to Kreil *et al*. (1980) honey bee venom consisted of several toxic proteins and peptides. The major component being a protein called melittin.

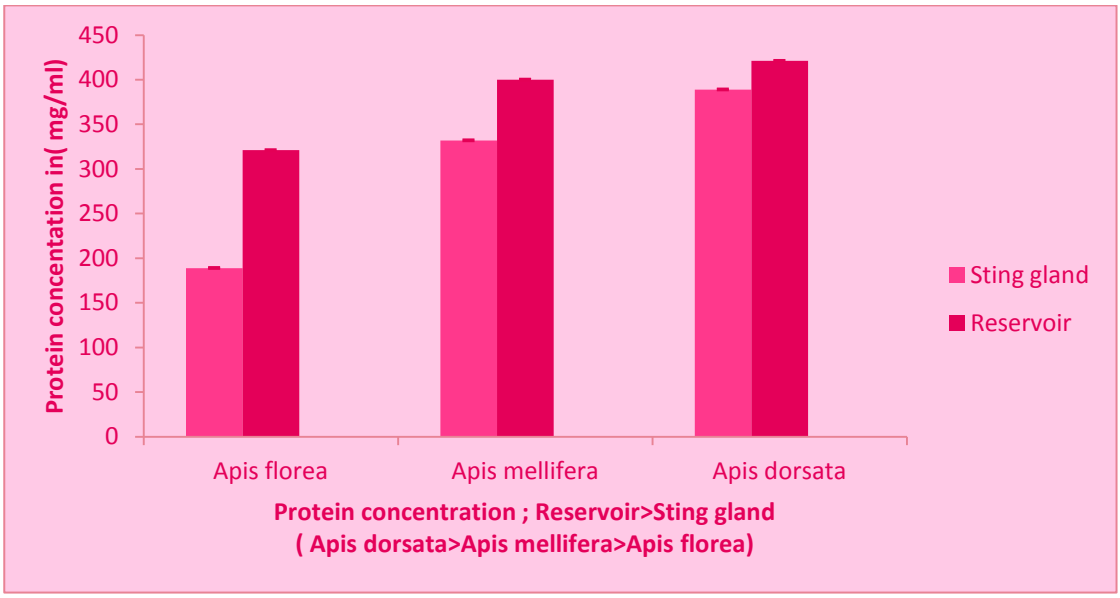

Fig.1. Concentration of protein in sting gland and reservoir.

According to Hider (1988) venom gland in the worker bees become active just after adult emergence and their maximal production is achieved within two or three weeks after the emergence. Venom composition also undergoes some changes during a bee's lifetime, and these changes are believed to occur mainly due to a changing behavior from hive maintenance to food gathering through life. Venom production is also higher during summer months, in which there is a peak of activity in the colony, and when the relatively young individuals are beginning their defence behavior. Since proteins are the major components of hymenopteran venoms as

They reported that melittin was a water soluble toxic peptide. Melittin was the best characterized peptide. It had alkaline characteristics similar to other venom compounds and seemed to be the major component responsible for intense local pain (Habermann, 1972; Edstron, 1992).

Another important peptide in the bee venom was apamin (Dotimas *et al*.,1987;Schmidt,1982). Banks *et al*. (1979) reported that apamin was the smallest neurotoxin in bee venom and was composed of 10 amino acids containing two disulfide bridges. Apamin has long been known as a highly selective inhibitor of the Ca2+ ions activated K+ channels.

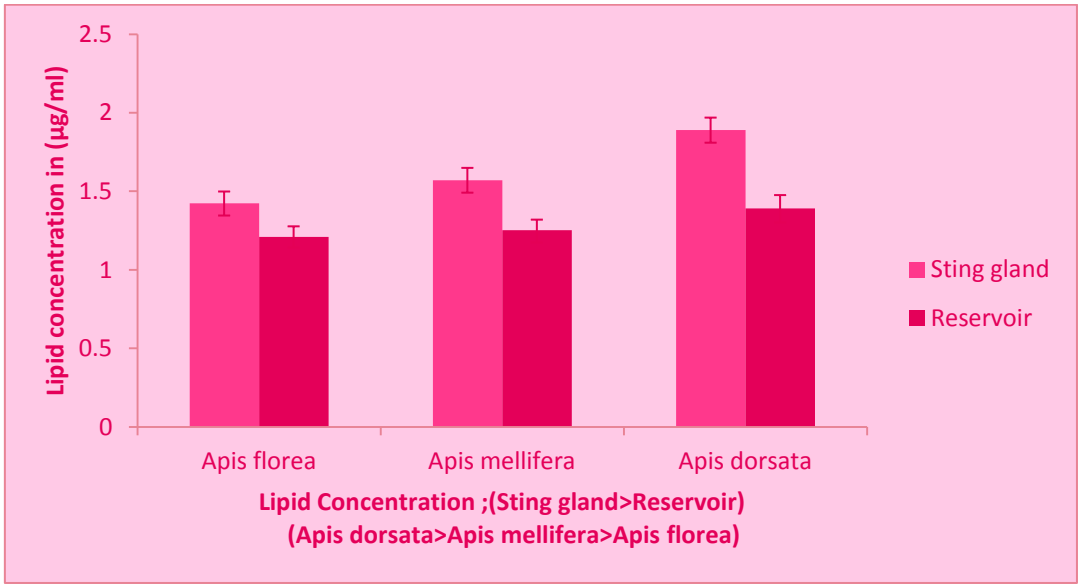

Fig.2. Concentration of lipid in sting gland and reservoir.

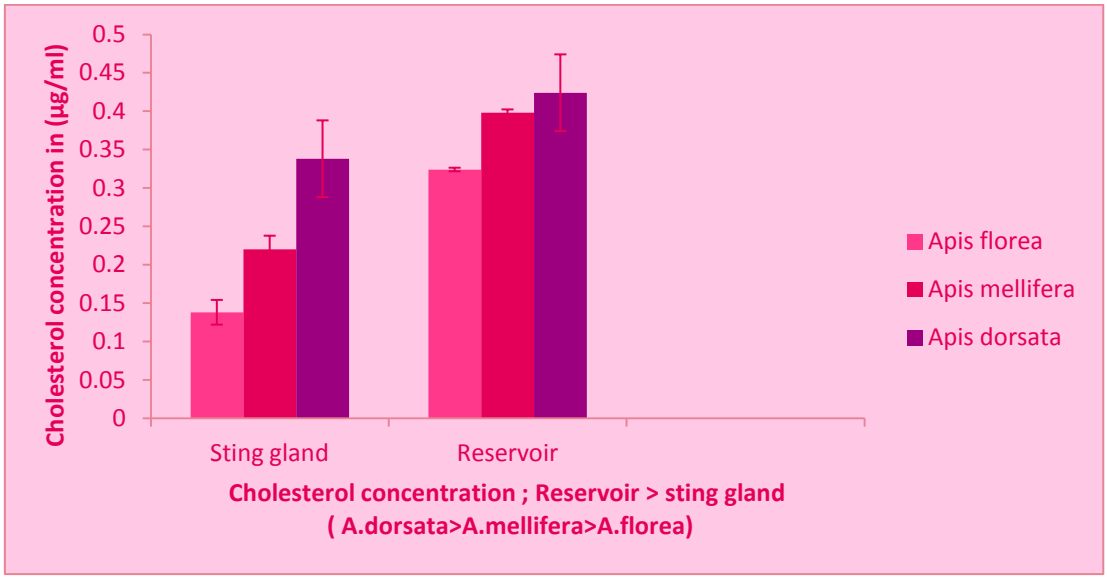

Fig.3. Concentration of Cholesterol in sting gland and reservoir.

According to Crane (1990) 88 percent of venom was water. The glucose, fructose and phospholipid content of venom were similar to those in bee's blood. The remaining 12 percent contained enzymes ,proteins, peptides , physiologically active amines , amino acids , carbohydrates, phospholipids and volatile ingredients.

Of the enzymes detected in venom gland of *Apis* species in the present study, the activity of acid phosphatases, responsible for the removal of phosphate groups of proteins at low pH was found to be more in sting gland than in the reservoir. The activity of the enzyme increases with increase in the substrate concentration and was found to be more in *A.dorsata* followed by *A.mellifera* and last by *A.florea (A.dorsata>A.mellifera>A.florea)* as shown in fig. 5.

Biochemical analyses have shown that, during the active stage, the venom gland of *A.mellifera* secretes a mixture of at least 50 identified

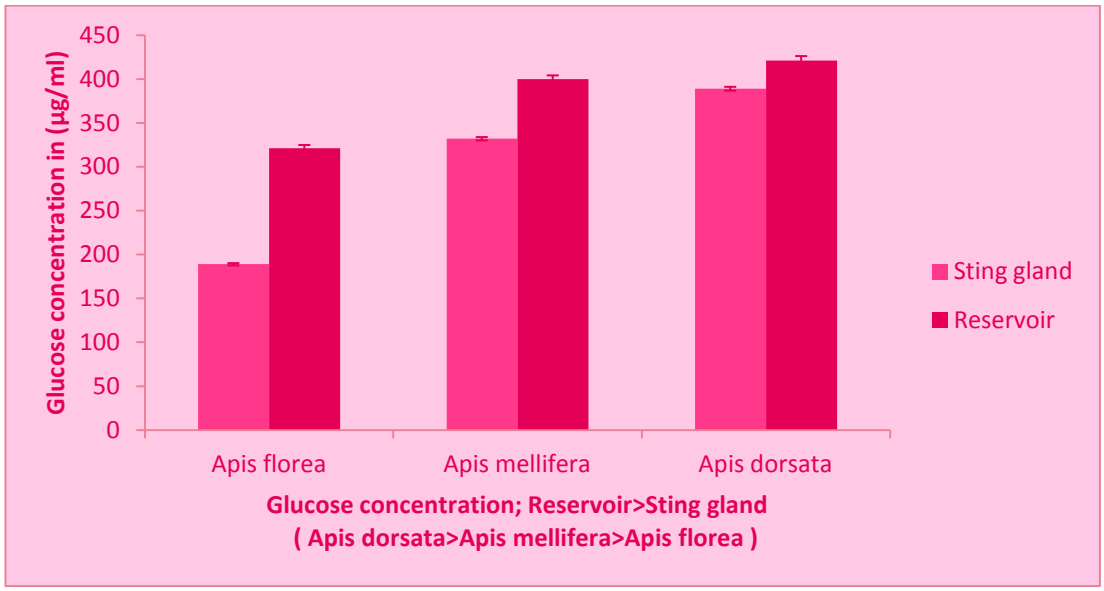

Fig.4. Concentration of Glucose in sting gland and reservoir.

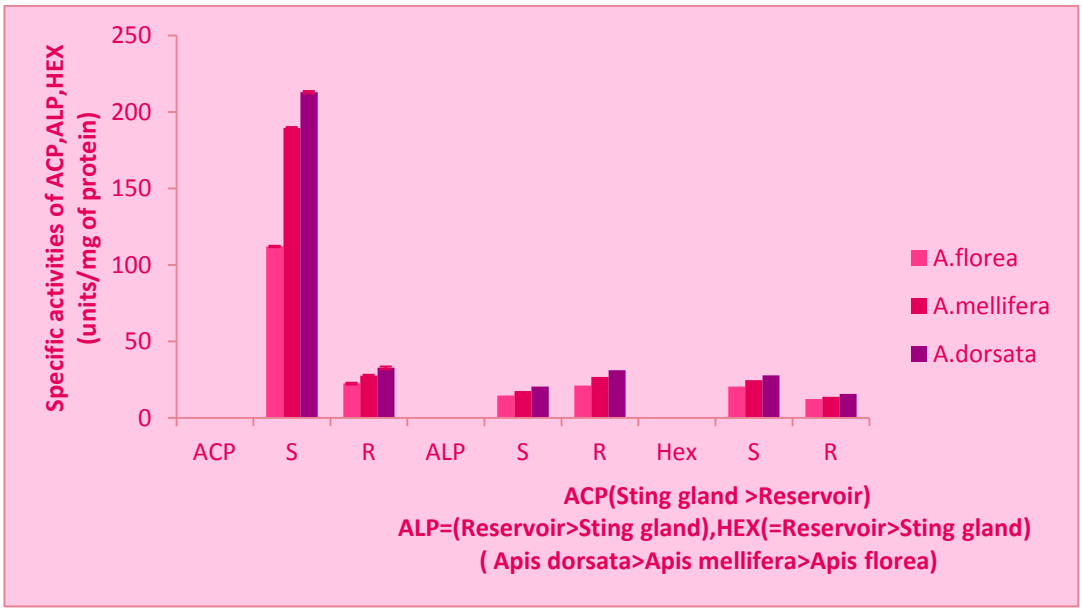

Fig.5. Activities of different enzymes in sting gland and reservoir.

components. Bridges (1977) verified that this mixture includes hyaluronidase, phospholipase A, acid phosphatase , estarases , histamine , dopamine , and noradrenaline , all present at pharmacologically significant concentration. Among these enzymes, acid phosphatase was of special interest given its recognized and important role in the autolysis of tissues (Zakeri *et al*., 1995; Lockshin and Zakeri, 1996; Silva Moraes, 1998) and the fact that glandular regression results in cell death (Cruz-Landim and Silva Moraes, 1973, 1977). The acid phosphatase enzyme (Acp) or phosphomonoestrase was first described by Benton in 1967. Purified samples of this enzyme revealed a glycoprotein nature, the same as phospholipase A2 and hyaluronidase (Barboni, *et al*., 1987). Acid phosphatase is a potent releaser of histamine in human basophils, thus relevant in allergic process to the venom (Whan, *et al.,* 1984).

Therefore the study on the properties of this enzyme has significant importance to the understanding of the venom allergic properties (Barboni, *et al*., 1987). Lima *et al. (*2003) reported that hymenoptera venoms are complex mixtures containing simple organic molecules, proteins, peptides, and other bioactive elements. These compounds are responsible for many toxic or allergic reactions in different organisms, such as local pain, inflammation, itching, irritation and moderate or severe allergic reactions. According to Abreu *et al*. (2009), the highest activity recorded for acid phosphatase, which was cytochemically detected throughout the length of the secretory filament and surrounding the canaliculi of the distal region of the reservoir.

The activity of alkaline phosphatase was found to be more in reservoir than in the sting gland. The activity of the enzyme increases with the increase in substrate concentration and it was found in a order as follow (A.dorsata>A.mellifera>A.florea) as shown in fig.5.

The alkaline phosphatase is responsible for the removal of phosphate group from proteins under conditions of high pH. Zhu *et al*. (2008) detected alkaline phosphatase in the venom apparatus of endoparasitoid wasp, *Pteromlus puparum* L. (Hymenoptera: Pteromalidae).

Glucose-6-phosphatase had been reported to be associated with regulation of the rate of glucose

Table 1.Amino acid identified in the homogenized sample of sting gland and reservoir of *A. mellifera* workers.

S.N.	Sting gland				Reservoir			
	Rf value	Colour	Colour intensity	Amino acid present	Rf value	Colour	Colour intensity	Amino acid present
1	10	Purple grey	++	Unidentified	15	Purple brown	++	DL-Ornithine
2	29	Purple grey	+	Histidine	29	Purple grey	++	Histidine
3	44	Purple grey	++	DL- Alanine	34	Purple light	+	D-Serine
4	55	Purple pink	++	L-Tyrosine	56	Purple pink	+	L-Tyrosine

+=Slightly present, ++ = Moderate, +++ = Abundant, - = Absent.

Table. 2. Amino acid identified in the homogenized sample of sting gland and reservoir of *A.florea* workers.

S.N.	Sting gland				Reservoir			
	Rf value	Colour	Colour intensity	Amino acid present	Rf value	Colour	Colour intensity	Amino acid present
1	30	Purple grey	++	Histidine	33	Purple light	++	Lysine
2	35	Purple light	+++	Serine	48	Purple light	+++	Glycine
3	53	Purple light	++	DL- Aspartic Acid	59	Yellow	++	L-Proline
4	62	Purple	+	DL-Valine	43	Purple light	++	Glutamic Acid
5	-	-	-	-	74	Purple Pink	++	DL-Tryptophan

+=Slightly present, ++ = Moderate, +++ = Abundant, - = Absent.

Table 3.Amino acid identified in the homogenized sample of sting gland and reservoir of *A. dorsata* workers.

S.N.	Sting gland				Reservoir			
	Rf value	Colour	Colour intensity	Amino acid present	Rf value	Colour	Colour intensity	Amino acid present
1	28	Purple grey	++	Histidine	22	Purple brown	++	L-Cystine
2	38	Purple	+++	DL-Arginine	33	Purple grey	+++	L-Lysine
3	51	Purple light	++	DL- Aspartic Acids	48	Purple light	++	Glycine
4	62	Purple	+	DL-Valine	59	Purple pink	++	L-Proline
5	-	-	-	-	66	Purple Pink	++	DL-2-Amino-n-Butyric acid
6	-	-	-	-	78	Brown	+	DL-Tryptophan

+=Slightly present, ++ = Moderate, +++ = Abundant, - = Absent.

dephosphorylation in the muscles of insects (Surhalt *et al*., 1981). The activity of glucose-6-phosphatase was not observed in the sting gland as well as reservoir at any substrate concentration.

Hexokinase is the enzyme that causes phosphorylation of 6 carbon compounds. The activity of hexokinase was observed at different concentrations and the activity was found to increase with increase in substrate concentration. Hexokinase activity was found to be more in the sting gland than the reservoir and it was found in an order as follow (A.dorsata>A.mellifera>A.florea) as shown in fig.5.

It has been reported that the major hymenoptera venom enzyme is the phospholipase A2. It is described as a potent bee venom allergen. It represented about 12 percent of the crude venom and it was extremely alkaline. It has the interesting cleavage property of the main construction block of biological membranes-the phospholipids (phosphatidylcholine,for instance),producing lisophospholipid and long chain anionic fatty acids. It caused pores in the membrane, and consequently, cellular lysis (Schmidt, *et al*., 1986; Hoffman, 1996). Enzymes represent the high molecular weight fraction of the venom (15.0 to 50.0 kDa).

Estimation of glucose in the sting gland and reservoir of *Apis* workers showed higher concentration in reservoir as compared to sting gland and it was found in a order as follow (A.dorsata>A.mellifera>A.florea)as shown in fig.4.

Estimation of lipids in the sting gland and reservoir of *Apis* workers showed small amounts of these to be associated with the venom. The concentration was more in sting gland as comparaed to reservoir, and it was found in a order as follow (A.dorsata>A.mellifera>A.florea)as shown in fig.2.

Estimation of cholesterol in the sting gland and reservoir of *Apis* species workers showed higher concentration in reservoir as compared to sting gland. Cholesterol was perhaps related to the production of steroid based alarm/defence pheromones by the wall of the reservoir in association with the defensive secretion and it was found in a order as follow (A.dorsata>A.mellifera>A.florea)as shown in fig.3.

The major energy reservoirs of the insects are the lipids. Lipids are also the precursors of a variety of hormones and pheromones and form an integral part of membrane structure. Lipids metabolism is essential for growth, reproduction and energy production during extended activity (Arrese and Soulages, 2010). Bee venom has been advocated for the use of rheumatoid arthritis, gout, multiple sclerosis and a variety of other immune disorders including scleroderma and asthma (Cohen *et al., 1942)*. Bee venom also has anticancer activity. Venom from a variety of animals including bees (Liu

et al., 2002), snakes, spiders, scorpions, have the capacity to kill cancer cells. This finding gets supported from the suggestions that the secretory cells of the reservoir probably contribute to the synthesis of steroid pheromones of alarm/warning or defenses secreted along with venom.

CONCLUSION

It was observed that there were considerable differences in the composition of Venom gland and Venom sac secretions of three *Apis* species. Except lipids, proteins, acid phosphates and hexokinase all other contents were found maximum in the Venom sac in order of (*Apis dorsata>Apis mellifera>Apis florea*) because it comprises the secretions of venom gland as well as the secretion of some of the secretory cells of the venom sac which are added to the total venom. Lipids are less in Venom sac because they are utilized by insects in forming some of their steroid hormones (pheromones) and some other physical activities are done by using proteins, and because Venom gland is acidic in nature so acid phosphatase enzyme is more in Venom gland. The greater activity of hexokinase in the poison gland suggests greater secretory activity of the filamentous gland (Venom gland) as compared to the reservoir (Venom sac). Glycogen was absent in both venom gland and Venom sac of three *Apis* species as confirmed by the absence of glucose-6-phosphatase activity. It is established from the present study that Venom sac also secretes various biochemicals and enzymes which are added to the total Venom.

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