QUANTIFICATION AND CHARACTERIZATION OF INDIAN HONEY BEE (*Apis cerana indica* Fab.) VENOM

ALEN JOY

(2019-11-121)

DEPARTMENT OF AGRICULTURAL ENTOMOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695522

KERALA, INDIA

2022

QUANTIFICATION AND CHARACTERIZATION OF INDIAN HONEY BEE (*Apis cerana indica* Fab.) VENOM

by

ALEN JOY

(2019-11-121)

THESIS

Submitted in partial fulfilment of the

requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL ENTOMOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695 522

KERALA, INDIA

2022

DECLARATION

I, hereby declare that this thesis entitled **"QUANTIFICATION AND CHARACTERIZATION OF INDIAN HONEY BEE (*Apis cerana indica* Fab,) VENOM"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellayani
Date:1 4 -02-2022

Alen Joy
(2019-11-121)

CERTIFICATE

Certified that this thesis entitled **"QUANTIFICATION AND CHARACTERIZATION OF INDIAN HONEY BEE (*Apis cerana indica* Fab,) VENOM"** is a record of research work done independently by Mr. Alen Joy (2019-11-121) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Vellayani
14-02-2022

Dr. Amritha V.S.
(Major advisor, Advisory committee)
Associate Professor and Principal Investigator,
AICRP on Honey Bees & Pollinators,
Department of Agricultural Entomology,
College of Agriculture, Vellayani

CERTIFICATE

We, the undersigned members of the advisory committee of Mr. Alen Joy (2019-11-121), a candidate for the degree of **Master of Science in Agriculture** with major in Agricultural Entomology, agree that the thesis entitled "**QUANTIFICATION AND CHARACTERIZATION OF INDIAN HONEY BEE (*Apis cerana indica* Fab.) VENOM**" may be submitted by Mr. Alen Joy, in partial fulfilment of the requirement for the degree.

Dr. Amritha V. S.
(Major Advisor, Advisory Committee)
Associate Professor and
Principal Investigator,
AICRP on Honey Bees & Pollinators,
Department of Agricultural Entomology,
College of Agriculture, Vellayani.

Dr. Anitha N.
(Member, Advisory Committee)
Professor and Head
Department of Agricultural Entomology
College of Agriculture, Vellayani.

Dr. Vijayasree V.
(Member, Advisory Committee)
Assistant Professor,
AICRP on Honey Bees & Pollinators,
Department of Agricultural Entomology,
College of Agriculture, Vellayani.

Dr. Dhalin D.
(Member, Advisory Committee)
Associate Professor
Department of Agricultural Engineering
College of Agriculture, Vellayani.

ACKNOWLEDGEMENTS

As a prelude to my thanks giving, I wish to bow my head before the Almighty God for making me confident and optimistic throughout my journey and enabled me to complete the thesis work successfully on time.

From the inner core of my heart, I wish to express my sincere gratitude to my Major Advisor ***Dr. Amritha V.S.,*** *Associate Professor (Agricultural Entomology), and Principal Investigator, AICRP on Honey bees and Pollinators, Southern Zone, Vellayani Centre, for her guidance, encouragement, precious suggestions, constant care and support, extreme patience and also constructive criticism throughout the course of my research work. This work would not have been possible without her valuable guidance and support. It was her sincerity, dedication and attachment towards me and our work which inspired me deeply to attain a certain level of self-improvement in all aspects. I consider myself extremely fortunate in having the privilege of being able to be guided by her.*

I am grateful to exhibit my inexplicable gratitude to ***Dr. Anitha N.,*** *Professor and Head, Department of Agricultural Entomology, College of Agriculture, Vellayani and a member of my Advisory Committee, for her explicit instructions, affectionate advice, constructive criticism, support, and treasured suggestions rendered during the course of study. I am sincerely grateful for her affectionate encouragement and guidance.*

I am extremely thankful to ***Dr. Vijayasree V.,*** *Assistant Professor (Agricultural Entomology), AICRP on Honey bees and Pollinators, Southern Zone, Vellayani Centre and a member of my Advisory Committee, for her guidance, valuable advice, whole hearted approach, unrelenting support and assistance for the successful completion of my thesis.*

I cordially offer my sincere and heartfelt gratitude to ***Dr. Dhalin. D****, Associate Professor, Department of Agricultural Engineering and member of my Advisory Committee for his timely help and active guidance rendered throughout the study*

I humbly express my gratitude to ***Dr. Ambily Paul, Dr. Berin Pathrose, Dr.Pratheesh P. and Dr. Aparna. B*** *for the help and support rendered in all aspects of my thesis work.*

I humbly express my gratitude to ***Sophisticated Analytical Instrument Facility (SAIF) IIT Bombay*** *and their Technical Superintendent (HR LC-MS, OHR LC-MS)* ***Mrs.Pradnya Nikam*** *for helping me with the chromatographic analysis in my work.*

I take this opportunity to express my heartfelt thanks and respect to all teachers of Department of Agricultural Entomology as well as for their constant encouragement, support, and valuable advice. I also express my gratitude to all non-teaching staff members of our department, ***Prajin chettan, Aswathy chechi, Santhosh chettan, Mani chettan, Surya chechi, Shaji chettan*** *and the non-teaching staff of PRRAL,* ***Vishak sir*** *for their sincere co- operation, timely help and assistance during the field work and throughout my course work.*

I duly acknowledge the encouragement, help and co-operation of my dear Agricultural Entomology and Nematology batch mates, ***Manoj, Jaseel, Akshaja, Neethu, Remya, Subba Rao, Sailaja, Haritha, Neema chechi, Swathy, Azimove, Shabana chechi, Aishwarya chechi, Rupini and Athira***

I am also indebted to express my sincere thanks to my seniors, ***Akhila chechi, Lintu chechi, Hari chettan, and Karthik ettan****, for the support and encouragement rendered throughout my research work.*

A special mention of thanks to my roommates ***Arun, Ashik, Rabeen, Gazal, Akhil*** *and my dearest friends* ***Reshna, Sreelekshmi, Athira, Nadiya, Nayana, Navya***

for their constant technical support and presence in my research.

I also take this opportunity to thank ***Greeshma,*** *for her guidance and efforts towards making my thesis a success.*

A special thanks to ***all my juniors from PG and UG*** *who have helped at different stages of my work.*

Mere word of acknowledgement can never express my love and feeling towards my parents, ***Joy Moses*** *and* ***Premkumari,*** *my sister* ***Aleena,*** *my* ***grandparents*** *for their unconditional love, sacrifices and support bestowed on me and I wish to express my profound indebtedness towards them.*

I once again express my sincere gratitude to all those who helped me in one way or the other in the successful completion of this venture.

Alen Joy.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
@	At the rate of
₹	Rupees
+	Plus
Amp	Ampere
BV	Bee venom
BVC	Bee Venom Collector
CD (0.05)	Critical difference at 5 per cent level
CBRTI	Central Bee Research and Training Institute
CFTRI	Central Food Technology Research Institute
Cm	Centimetre
cm^2	Square centimetre
CRD	Completely Randomised Design
et al.	Co- workers/ co-authors
Fab.	Fabricius
Fig.	Figure
G	Gram
Ha	Hectare
ha^{-1}	Per hectare
HIV	Human Immunodeficiency Virus
HR-LCMS	High Resolution- Liquid Chromatography Mass Spectrometry
Hz	Hertz
KAU	Kerala Agriculture University
Kg	Kilogram
Km^2	Square kilometer
LD_{50}	Median Lethal Dose
M	Meter
Min	Minutes
Mg	Milligram

µm	Micrometer
mm	millimetre
MSL	Mean Sea Level
MCD	Mast Cell Degranulating
NS	Not-significant
º	Degree
°C	Degree Celsius
PAU	Punjab Agricultural University
RH	Relative Humidity
Rs.	Rupees
Sec	Seconds
Sem	Standard Error of means
sp.	Species (singular)
spp.	Species (plural)
viz.,	Namely
V	Volt

Introduction

"Anyone who thinks they're too small to make a difference has never met the honeybee" .

1. INTRODUCTION

Honey bees are eusocial insects living in communities of 10,000 to 20,000 individuals (Hider, 1988) and they are the only class of insects that are beneficial to humans, to such extents. They are obligate social organisms that exist in colonies usually headed by a single reproductive female, the queen which is generally the only egg layer in the colony. A few fertile drones and many thousands of facultative sterile females, the workers are also present in colonies.

Honey bees (*Apis* spp.) are very important for our environment, economy, and health. They are considered as an ideal model for many biological and physiological studies. Domesticated honey bees are reared worldwide, for their products like honey, pollen, beeswax, royal jelly, propolis and bee venom. In addition to that, their importance is increasing due to their role in pollination services and yield enhancement. Garibaldi *et al.* (2013) found that honey bees rank as the most common pollinator, of a single species, for crops worldwide. Even though several products are available from hive, only honey, pollen and wax are commonly utilized while the possibility of other products is still to be explored.

Amongst the hive products, one of the most important product is the bee venom. The prime role of venom with the venom apparatus in honey bee is colony defence. Sting by a single bee is painful enough to deter a vertebrate intruder but has lethal effect on wide range of invertebrates. Even though a bee sting is a painful experience, non-sensitised humans do not suffer complications even after being stung by 10 to 20 bees. Honey bee venom has found to have therapeutic application since ancient times. In recent years, bee venom has been adopted as a remedy for the relief of rheumatic pains. Thus, an interest rose in scientists to study the composition and other properties of bee venom around the 19th century (Dotimas and Hider, 1987)

Bee venom attenuates neuroinflammatory events and is potentially neuroprotective as it has been shown to extend survival against amyotrophic lateral sclerosis in a mouse model (Kim *et al.,* 2013). Similarly, it has been utilized as an optional prescription for Parkinson's disease and patients with inflammatory diseases, such as Rheumatoid Arthritis and osteoarthritis. Several reports have demonstrated the positive effects of BV in arthritic rat models as an anti-inflammatory adjuvant, as it reduces inflammatory edema and polyarthritis (El- Wahed *et al.,* 2018)

Bee venom, a defence tool against predators or intruders, is a complex mixture of peptides, enzymes and other trace components which show extensive biological activities also (Sobral *et al.*, 2016). It contains low molecular mass constituents like melittin, apamin and mast cell degranulating (MCD) peptide and the enzymes phospholipase A2 and hyaluronidase. Besides the small peptides, physiologically active amino acids, phospholipids, amines, sugars, and pheromones are also present (Szokan *et al.,* 1994). It has been used for the treatment of many diseases like arthritis, rheumatism, herpes, etc. (Omar, 2017).

Studies conducted by Benton *et al.* (1963) showed various methods devised and tried for the safe extraction of venom, with minimum damage to both the bees and the collector. Honey bee venom can be safely and easily collected as dried powder using an electric shock instrument, sometimes coupled with stimulating the bees to sting on a glass plate (Rybak *et al.,* 1995).

Indian bee, *Apis cerana indica* Fab. is the common bee species found in southern India with more than 6 lakh bee keepers in Kerala alone. The research works on the safe methods and other aspects of venom collection from Indian bees are wanting. The ideal time and the optimum duration as well as the seasonal variation in the quantity of venom that can be collected have to be studied and hence this research work entitled "Quantification and characterisation of Indian bee honey (*Apis cerana indica* Fab.) venom" was conducted.

Review of Literature

2. REVIEW OF LITERATURE

In India, bees are reared mainly for honey production. Other hive products are not given much importance, even though they are highly valued. Products like royal jelly, pollen, venom and even propolis fetch a higher price than honey. It is mainly the lack of knowledge on the methods of safe collection and storage of these products that prevents bee keepers from exploring these areas. Beekeeping is both an art and a mesmerizing science. In India, beekeeping is practiced both as a full-time occupation and an engaging hobby mainly for honey production. Honey bees are unique gifts to mankind as beekeeping benefits us with the pollination service they provide as well as other hive products.

Bee venom has found its place in the pharmaceutical industry for the treatment of cancer, bursitis, tendonitis, herpes zoster, lyme disease, rheumatoid arthritis, osteoarthritis (Lee and Bae, 2016), HIV, multiple sclerosis, joint diseases and many others (Tilahun *et al.,* 2016). Bee venom is also being used in the cosmetic industry (Han *et al.,* 2016). Due to the increasing usage and applications of bee venom, more research works have to be conducted to study its chemical composition, safe methods of extraction and storage of venom and effects of venom collection on hive activities and post collection.

2.1ASPECTS OF BEEKEEPING

Till now, seven species of *Apis* have been described and out of this, four are present in India: two domesticated species, *viz.*, *Apis mellifera* L. (European honey bee) and *Apis cerana indica* (oriental or the Indian honey bee) two wild species, *viz*., *Apis dorsata* Fab. (giant/rock honey bee) and *Apis florea* Fab. (dwarf honeybee). *A. mellifera* has been introduced to India due to its high resistance to Thai sac brood virus (TSBV) and their suitability to commercial beekeeping (Tej *et al.,* 2017).

Beekeeping of domesticable bees involves their proper management during all the three seasons to achieve a profitable income. Migratory beekeeping can be practised where sufficient flora is not available. In Kerala, beekeeping is mainly done with Indian bee (*A. cerana indica*) and the major honey source is rubber (*Hevea brasiliensis* Muell. Arg.). The extra floral nectaries of rubber produced after the commencement of new flushes serves as rich source of honey during January to April (Padmanabhan, 2003).

2.2 HONEY BEE SPECIES IN INDIA

2.2.1 Rock Bee (*Apis dorsata*)

These are the largest of *Apis* bees found in India, constructing single combs of about 6 feet long and 3 feet breadth. Combs are usually seen in the open on tall buildings, trees and rocks. They are present all over the subcontinent and can construct nest up to an altitude of 2700 m. The main feature of rock bees is that they shift their habitats frequently and abscond their previous hives. According to Mishra (1995), around 50 to 80 kg of honey can be collected from a single rock bee colony per year.

Usually, rock bees construct their hives at a height of about 20 feet from the ground, but cases where hives are seen hanging from branches at just 2 feet from the ground are also observed. *A. dorsata* colonies may be found singly or in groups. The lowest part of the comb is the most energetic area, where the scout bees and foraging bees take off and land. The bees are not domesticated due to their irritable and ferocious nature, peculiar hives and habit of frequently absconding the hives. Due to the aggressive nature, the bees attack any intruders (Ramachandra *et al.,* 2012) and in most cases they chase victims up to 100 m.

2.2.2 Little Bee (*Apis florea*)

Little bee also known as dwarf honeybee is also a wild honeybee species that cannot be domesticated. These bees are smaller in size and less ferocious, compared to rock bees. Palm sized combs are constructed in bushes, twigs, hedges, caves etc. A very peculiar aspect of little bee comb is that, the top of the combs encircles the twigs on which the combs are constructed, while the rock bee combs are constructed on the underside of the branches. Unlike rock bees, the amount of honey produced by little bees are very low, producing only 500 g of honey per hive a year. These bees also have the habit of absconding the hive, hence they are non- rearable. Attempts have been made to rear these bees in India, with partial success (Mishra, 1995). These bees are not found in hills above an altitude of 450m MSL. Like rock bees, little bees also construct single vertical combs (Hepburn and Radloff, 2011). These bees are attractively coloured with red to brown colouration between white bands. These bees are excellent pollinators due to their small size and show protective behavior against predators.

2.2.3 Indian Bee (*Apis cerana*)

Indian honeybee also known as Eastern honeybee is a common bee species in India. Until the introduction of Italian bees, this was the only domesticated *Apis* species in India. These bees construct multiple parallel combs in dark places such as logs, tree openings, pots, etc. These bees are non- aggressive and rarely abscond the hives, hence show the possibility of rearing. *A. cerana* was classified by Ruttner (1988) into sub species, based on living habitats and genetic diversity; of these *Apis cerana indica* and *Apis cerana cerana* occur in India. Now in India, beekeeping with Indian bee is mostly done in South India particularly in Tamil Nadu and Kerala. They produce around 7 to 9 kg of honey per colony per year. These bees are smaller in size than rock bees and Italian bees, but larger than little bees. The Indian honeybees are now domesticated in all parts of the country like Jammu and Kashmir, Uttaranchal, Andhra Pradesh, Himachal Pradesh, Assam. Tamil Nadu, Karnataka, and Kerala (Yadav *et al.,* 2017).

2.2.4 Italian Bee (*Apis mellifera*)

A sub species of *A. mellifera, A. mellifera ligustica* was introduced to India from Europe during the second half of 20^{th} century. The prime reason for introduction was the disappearance of native Indian bee colonies in the country due to the attack of Thai Sacbrood virus. The introduced Italian bees got well adapted to the country due to its rich flora of mustard, safflower, sunflower, moringa, etc. (Tej *et al.,* 2017). These bees are not common in South India due to the high attack of bee-eater birds (*Merops orientalis*) and beewolves or bee-hunter wasps (*Philanthus* spp.) on these bees (Ramachandra *et al.,* 2012).

Similar to Indian bee, they also construct multiple parallel combs in the dark. Italian bees are larger than all bees except rock bees. They do not show swarming tendency and are less aggressive than rock bees. Italian bees possess good honey gathering qualities, yielding about 25 to 40 kg honey per colony per year. The introduction of these bees to India can be called a success as it was able to create good employment opportunities to many people with profitable income and the pollination services provided by them. Even though the introduction caused problems like transmission of pests and diseases, overall, it can surely be called a success (Tej *et al.,* 2017).

2.2.5 Stingless Bee (*Tetragonula iridipennis* Smith)

Stingless bee also called as Dammer bee belongs to the Meliponinae sub family of Apidae. They are the smallest of honey-yielding bees with size less than 5 mm. It consists of two genera *Melipona* and *Trigona.* As the name implies, these bees do not sting as their stings are vestigial and protect their colony using their mandibles (Michener, 2000). These bees can be domesticated and they construct combs which are tree-branch shaped, in tree logs, wooden boxes and clay pots, where they construct using wax or cerumen (a mixture of wax and propolis). The honey yield per colony is very less, about 300 to 400g (KAU, 2016) only. The stingless bee honey is highly valued due to its medicinal properties.

2.3 SCOPE OF BEEKEEPING

In India, the major portion of honey comes from rock bee, *A. dorsata* (Sivaram *et al.,* 1993). In North Indian states like Punjab, Uttar Pradesh, West Bengal, Bihar, Jammu & Kashmir, Haryana, Himachal Pradesh, beekeeping is mainly practiced with *A. mellifera*. According to Sivaram and Anita, (2000) commercial exploitation of bees for honey production in India is very less, even though India has an appreciably high forest cover of 678,333 km^2.

The scope for production and marketing of other bee products, besides honey, like pollen, royal jelly, propolis, beeswax and venom add to the financial stability and diversification of apiculture. In addition to this, sale of bee colonies, rearing and sale of pedigree queen bees also provide additional income (Sivaram, 2012). Demand of honey bees for increasing crop production by pollination is also increasing in the country. This profession offers a great scope in generating employment besides a good livelihood. Apart from direct employment to the beekeepers, there would be need for good artisans, hive manufactures, apicultural equipment and machinery manufactures, transport system for irrigation of colonies, traders, product quality experts, packers, sellers, raw material dealers, etc. and allied industries. The value addition technologies of PAU, CFTRI and CBRTI have already started helping beekeepers in harvesting rich dividends. This industry has, so far, remained unexplored and offers tremendous scope (Sivaram, 2012).

2.4 THE STINGING APPARATUS IN BEES

Sting in honeybees is the modified ovipositor and hence, only female bees (workers and queen) possess sting and have the capability to produce venom. It is a barbed hollow needle-like structure present at the tip of the worker bee abdomen. An inverted trough enlarged into a basal bulb is created by the fusion of the first valvulae, forming the lancet and the second valvulae, the stylet. Venom produced in the venom gland and other contents of the poison sac are drained into this bulbous structure. Almost all the toxic components appear to be produced from the highly convoluted and branched venom gland (Snodgrass and Morse, 2018). The secretory cells of venom glands lead to numerous canaliculi, which further leads to central canals which in turn drains into the poison sac. The wall of poison sac is cuticular, thick and laminated, enabling it to function ideally as a reservoir.

Normally the sting is kept retracted into the abdomen and is protruded out only for stinging when the bees are irritated. The sting shaft is about 2.0μm in length, consisting of four segments that taper together to form a sharp hollow sting point. The centre of the shaft has the poison canal through which the venom is injected. As mentioned earlier, the sting consists of the stylus and two barbed slides or the lancets, one on either side of the stylus and while stinging, in contrary to the usual thought, the bees do not push the stylet into the host body, but is drawn in by the barbed stylets. The slides move alternately up and down so that the sting is pushed inside by the combined action of the barbs such that, when the barb of one slide has caught and retracts, it pulls the stylus and the other barbed slide into the wound (Pursley, 1973).

2.5 VENOM PRODUCTION

Maximum venom quantity is present in newly emerged queens, in order to facilitate their fight for survival against competing queens (Bachmayer *et al.*, 1972). In newly born worker bees, even though their venom glands are functional, venom production starts only after two to three days, the maximum rate reaching only when the bees are two to three weeks old (Dotimas and Hider, 1987).

The synthesis of different bee venom components does not reach maximum rate simultaneously and these changes in venom content have been suggested to be related

to transition from nursing or housekeeping bees to foraging bees (Owen and Braidwood, 1974). Usually, it is observed that the capacity to regenerate venom is less in older bees denoting the degeneration of venom glands in latter parts of life. Generally, it is observed that the quantity of venom in worker bees is highest during the summer months, when there is peak activity in the hive and the younger bees act as hive guards (Dotimas and Hider, 1987).

2.6 PROPERTIES OF BEE VENOM

Bee venom is yellowish to colourless opalescent liquid that rapidly dries in room temperature, turning into a yellowish- brown crystalline gum like powder. It is easily destroyed by sunlight and higher temperature, but stable at low temperature (Bogdanov, 2016). The water content varies from 55 and 70%. Bee venom possess honey-like odour and has a bitter acidic taste. It is soluble in water and diluted acids, but about 10 % of the components are insoluble in water. Bee venom is insoluble in alcohol and ammonium sulphate also, it contains a number of volatile components that are easily lost during collection (Omar, 2017). Bee venom has an acidic pH of 4.5-5.5 and specific gravity of 1.13.

2.7 VENOM COLLECTION

2.7.1 Methods of Venom Isolation

The main method of venom collection by stimulation with an electric current was first described by Markovic and Mollnar (1954). Venom collection is achieved mainly by two methods; first is a manual method by the dissection of whole stinging apparatus and taking out the venom sac or by applying pressure in the abdominal region and a droplet of venom appears at the end of sting and for larger quantities of venom, electrical milking was developed. (Benton and Morse, 1966). An efficient method for collecting maximum venom with minimum damage to the bees and least risk to collector was long wanted and finally this method of electrical milking was devised and helped safer venom collections.

The device as described by Benton *et al.* (1963) consists of a collection frame over which steel wires are stretched across at 6 mm intervals. The wires are alternately charged to 33 V. A timer breaks the circuit every 3 to 4 seconds and rarely is a bee electrocuted by this apparatus. When a bee comes in contact with the wires, the bees get electrocuted for a small period and are irritated, due to which they start to sting. The

bees also release alarm pheromone that alerts others bees and they also start stinging. The bees sting in between the wires. A sheet of nylon taffeta was placed between two thin sheets of polyethene below the wires through which the shocked bees could sting. The bees sting in between the strands and do not pierce individual strands. Most of the venom gets deposited on the underside of the sheet. A cooling coil was also kept on the device to prevent denaturation of any bee venom components. It has been reported that from 20 hives, over a period of 2 h, about 1g of venom can be collected using this device. The bees are usually aggressive during this collection, hence special clothing is recommended during this technique. Dried venom can be easily scraped from the nylon taffeta.

According to Bogdanov (2016), devices for venom collection has been continuously improved and they mostly consist of four parts.

1) Battery of 9 - 12V and 2 Amp; powered by or directly plugged into the power grid
2) Electrical impulse generation with a frequency of 50 – 1000 Hz, duration of 2-3 sec and pauses of 3 – 6 sec
3) Electrical stimulator – Frame that consists of stretched naked wires, at a distance of less than 6 mm
4) Glass slide/ plate on which venom is collected.

O' Connor *et al.* (1963) said that it is possible to isolate venom from individual bees using a specially designed electrical excitation device. But the process is tedious, labour intensive and requires skilled labours, with being able to work on only 20 insects per hour.

2.7.2 **Rate and Quantity of Venom Delivery from Stings**

Studies conducted by Schumacher *et al.* (1990) on European bees showed that approximately 148μg of venom is delivered by the bees after an elapse of just 16 seconds when stimulated. Another study by Schumacher *et al.* (1994) showed that at least 90% of the contents of venom sac was emptied within 20 seconds and completely within 1 minute. The study conducted both *in-vitro* and *in-vivo* were in agreement suggesting that the bees empty their venom sac as soon as they are irritated. It is interesting to note that considering an LD_{50} value of bee venom (3.5 mg/kg) and an

average venom delivery of 140µg by a single bee, one can assume that a 70kg normal human being would have a 50% risk of death from 1750 bee stings (Schumacher *et al.,* 1994).

2.7.3 Frequency and Duration of Venom Collection

Study by Krivtsov and Lebedev (1995) suggested that less frequent venom collections, that is, 3 to 4 times in a season did not influence hive performance.

Bogdanov (2016) found that repeated 3 h venom collection carried out 3 to 4 times a month do not harm the bees, resulting in a total harvest of 4g dry bee venom from an Italian bee colony.

According to El-Mehdi *et al*. (2018) based on studies conducted in Moroccan conditions, the optimum frequency of bee venom collection is a gap of 10 to 15 days and the optimum duration for which the collector has to be placed in the hive for venom collection is 30 to 60 minutes early in the morning. They collected an average of 30 to 35 mg of dry venom at a period of 30 to 60 min. from Italian bee hive.

Studies conducted by de Graaf *et al.* (2020), showed that the amount of venom collected for a period of few hours of uninterrupted stimulation is less than the venom quantity collected for the same period, but with breaks included. They found the optimum time for stimulation is 30 to 60 min. and the optimal break time is 45 to 90 min. On a four-hour study where the bees are electro-stimulated every 30 min. with a short 1-minute break between them showed peak venom yielding the first 30 minutes with the rate falling exponentially thereafter.

de Graaf *et al*. (2020) suggested that venom collection in the presence of dew or with anticipated rainfall should be avoided and the ambient temperature for collection is above 12^{o}C. These parameters were fixed based on the natural tendencies of the hive colony and were established to reduce any harm to the colony.

2.8 EFFECT OF VENOM COLLECTION ON HIVE ACTIVITY

Bogdanov (2016) found that intensive venom collection for repeated 3 h collection periods, carried out 3 to 4 times a month resulted in reduction of brood area and honey yield about 10 to 15%.

Omar (2017) collected venom from Carnolian and Italian hybrid bee colonies and studied whether the collection affected the brood rearing activities of the hive. The study was conducted during the active periods from February to October of 2010 and

2013. The study concluded that monthly venom collection did not affect the brood rearing activities of the hive. The brood area recorded from the hive in which venom was collected did not show any significant difference with the brood area of hive from which venom was not collected.

de Graaf *et al*. (2020) found that honey production reduced by about 10 % when venom collection was carried out daily and more than 3 to 4 h a day. A break of 2 to 3 days between weekly venom collections, was necessary to maintain good hive health.

2.9 COMPOSITION OF VENOM

The major components are Melittin (50%), Phospholipase A2 (10-12%), Apamin (1-3%), Hyaluronidase (1-3%) and Mast Cell Degranulating (MCD) peptide (1-2%). Minor components like Procamine (1-2%), small peptides (13-15%), Secapin (0.5- 2%), histamines, glucose, fructose, and some pheromones (Dotimas and Hider, 1987) are also present. Bee venom is a complex mixture of proteins, peptides and low molecular components (Bogdanov, 2016). A brief about the major components are as follows:

2.9.1 Melittin

Melittin is a small protein in bee venom consisting of only 26 amino- acids arranged in a subtle manner (Habermann and Jentsch, 1967). Melittin forms α-helical shaped cylinders that are amphiphilic in nature. These helices form tetramers that prevents the action of hydrophobic surface of melittin with water, making tetrameric melittin non-lytic (Hider *et al.,* 1983). Melittin is stored in the venom sac in tetrameric form. When melittin molecule is diluted, the tetramer dissociates into monomer and becomes highly surface active. This results in disruption of phospholipid packaging and cell lysis eventually leading in appreciable pain levels (Dotimas and Hider, 1987).

2.9.2 Phospholipase A2

Phospholipase A2 is an enzyme that destroys phospholipids which is one of the major building blocks of biological membranes. It disrupts the molecular structure of phospholipids interfering with the packaging of membranes and causes pore formation. This helps in penetration of other venom components also into cell. Works by Shipolini *et al.* (1974) has found that bee venom phospholipase is the most active phospholipase than that found in any snake venom or mammalian pancreatic phospholipase.

2.9.3 Apamin

Apamin is a small peptide consisting of 18 amino acids that are tightly cross-linked by two disulphide bonds (Shipolini *et al.*, 1967). Similar to melittin, apamin also from rigid α-helices. Apamin is non-lytic and does not have much activity on mammalian cells. Habermann (1977) found the neurotoxic effect of apamin on the post-synaptic membranes of central nervous system; Jenkinson (1981) found its neurotoxic effect on peripheral nervous system. The neurotoxic nature of apamin due to its high affinity to the specific Ca^{2+} dependent K^{+} channel of the nervous tissue was found by Lazdunski (1983) and due to this neurotoxic nature, apamin blocks the channel in mammals.

2.9.4 Hyaluronidase

Hyaluronic acid is highly viscous polysaccharide solution found between the cells. It is the interstitial ground substance that adheres cells together. Owen (1979) found that hyaluronidase level in worker bees is more than in queen bee and this may be attributed to the frequent encounter of worker bees with mammals unlike the queen bee. Hyaluronidase is a hydrolytic enzyme that breaks this viscous polymer into non-viscous segments consisting of 4 to 6 units (Banks *et al.*, 1983). Hyaluronidase is also called as spreading factor, since it causes gaps between the cells and facilitates the penetration of other bee venom components (Dotimas and Hider, 1987).

2.9.5 Mast Cell Degranulating (MCD) peptide

Mast cells are a type of white blood cell, a part of connective tissue and is rich in histamine and heparin. As the name implies, MCD peptide destroys mast cells present in the body releasing histamines. Histamine release occurs as a result of calcium entering the mast cell which is triggered by immunoglobulins (IgE) in the body (Dotimas and Hider, 1987).

Materials and Methods

3. MATERIALS AND METHODS

The study on quantification and characterization of Indian honey bee (*Apis cerana indica* Fab.) venom was conducted at Department of Agricultural Entomology, College of Agriculture, Vellayani during 2019-2021. The study was conducted with the objective to quantify and characterize Indian honey bee (*A. cerana indica*) venom during different seasons. The materials and methods used for the study are given below:

3.1. PEAK BEE VENOM COLLECTION TIME

The present study was conducted during the active periods from November, 2020 to August, 2021. For this, a total of 36 hives of uniform bee strength maintained at the Indian bee apiary of AICRP on Honey Bees & Pollinators, Department of Agricultural Entomology were selected and labelled (Plate 1.).

3.1.1 Bee Venom Collection

The bee venom was collected from the selected hives from 6 am to 6 pm. Each hour of the collection time in a day was considered as a treatment, thus making three replications for a treatment. The selected hives were not fed for at least two days before the collection made, in order to prevent the undesirable moisture and thereby easy drying of the bee venom.

3.1.2. Extraction of Bee Venom

The bee venom was extracted with the help of a Bee Venom Collector (BVC) (Plate 2.) which was placed carefully between the bottom board and brood chamber of the Indian bee colony (Plate 3.). The device is very slim and handy with dimensions of 310 mm length, 220 mm width and 50 mm height. Conducting wires are stretched across the collection frame at a spacing of 2 mm and glass plate of thickness 4mm is kept below the wires for venom collection. The device works on a 9V battery which is reduced to still smaller pulses of current by ingenious circuit in the device. The collecting frame consists of conducting wires stretched over it and the electric pulses are passed through these wires. Below these wires, a well cleaned glass plate is kept on which the venom is collected. When the worker bees come in contact with the wires,

they get electrocuted and irritated, due to which they start stinging and liquid venom gets deposited on the glass plate below. The circuit is designed in such a way that small pulses are produced and the bees get easily detached from the circuit. The device is also pre-set with an automatic cut-off at 40 min after the device is switched on.

After the extraction is over, the device was taken out from the hive. Then the glass plate was carefully removed from the device in a dark well-ventilated area to facilitate drying and to prevent oxidation of the bee venom. The venom got dried after a few minutes, then light strokes with a camel brush are made over the glass plate to remove pollen dust and other debris that was present (Plate 4.). Once, the venom appeared as thin droplets it was carefully scraped using a blade (Plate 5.). The collected bee venom was weighed and then stored in properly labelled amber coloured bottles at 5^0C.

3.2. OPTIMUM DURATION OF BEE VENOM COLLECTION AND ITS QUANTIFICATION FROM INDIVIDUAL BEE HIVE

The study was conducted in the brood rearing season of 2019. Twenty hives of uniform bee strength were selected and labelled.

3.2.1. Bee Venom Collection

The study was conducted to assess the amount of venom that can be collected from an individual hive and the optimum duration at which maximum venom can be collected with minimum damage to the bees. The design of the experiment was CRD, no. of treatments – three and no. of replications – five. Three-time durations were the treatments: 30 minutes, 40 minutes and 60 minutes after switching-on the bee venom collector. Five replications were maintained for each treatment. Apart from these, five hives were also maintained as control to assess the brood parameters. The bee venom collector was placed at the peak hour recorded in 3.1.

3.2.1.1 Bee venom collection at 30 minutes duration

For the bee venom collection after 30-minutes, once the collector was switched on and kept in the hive, a timer was also set for 30 minutes simultaneously. By the end

Plate 1. Labelled hives taken for venom collection

Plate 2. Bee venom collector with accessories

Plate 3. Mode of placement of BVC in hive

Plate 4. Glass plate immediately after collection.

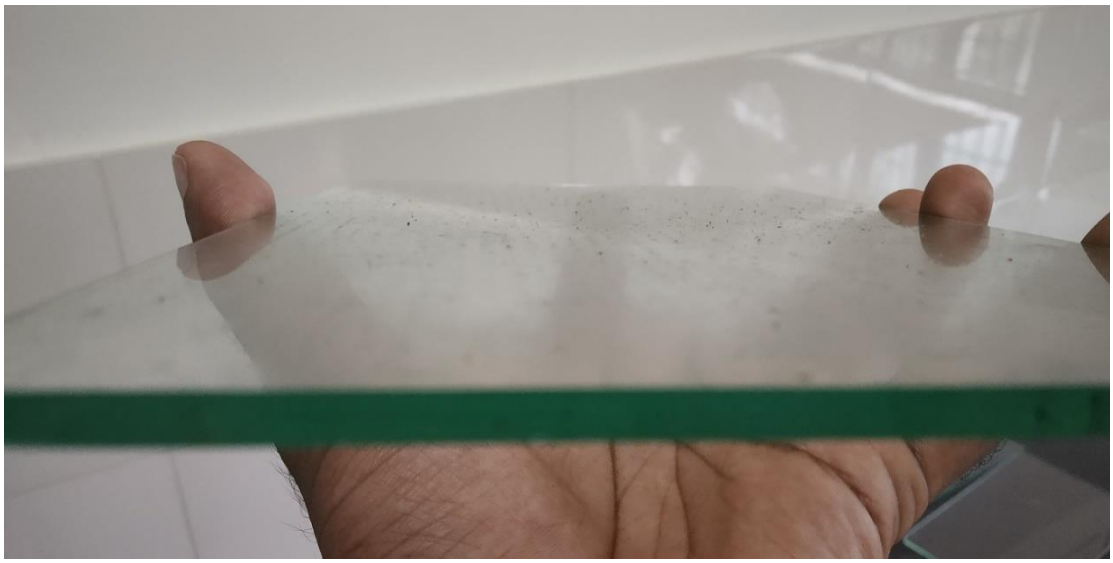


Plate 5. Glass plate after cleaning, with scraped dry bee venom

of 30 minutes, the hive was slightly smoked to calm the bees and the collector was switched off and carefully taken out from the hive.

3.2.1.2 Bee venom collection at 40 minutes duration

As the bee venom collector possess an automatic switch-off mechanism after 40 minutes, no timer was set during this experiment. The collector was safely taken out from the hive once the bees got calm after the device has turned-off.

3.2.1.3 Bee venom collection at 60 minutes duration

For the bee venom collection at 60 minutes duration, after the automatic switch of at 40 minutes the collector was taken out of the hive and was placed again after switching on and setting the alarm for another 20 minutes. After 20 minutes, the hive is lightly smoked to calm the bees and the device is switched-off. The device is taken out carefully once the bees got calm after the device has turned-off.

3.2.2 Extraction of bee venom

The bee venom was extracted as the same procedure as mentioned in 3.1.2.

3.2.3 Measurement of Hive Activity after Venom Collection

The bee mortality, foraging activity of the hives and the brood parameters were assessed both in the treatment as well as the control hives.

3.2.3.1 Mortality of bees

The number of dead bees, if any, remaining in the collection frame soon after the BVC was taken out from the hive after the collection, was recorded to study the bee mortality during venom collection.

3.2.3.2 Assessment of foraging activity

Foraging activity of a hive was studied by recording the number of bees moving in and out of the hive for 5 minutes at the peak foraging hour of the day. The data was recorded daily for a week. Foraging activity was also recorded from the control hives

where venom collection was not done.

3.2.3.3 Assessment of brood parameters

The brood parameters *viz*., brood area, honey and pollen storage were recorded from both the treatment as well as the control hives for a period of one month at weekly intervals. The area was measured using a grid and was recorded in cm^2 (Plate 6).

3.3 SEASONAL VARIATION IN BEE VENOM PRODUCTION

The study was conducted during the brood rearing season (August - December), honey flow season (January - April) and dearth season (May - July) to identify the season at which maximum venom can be harvested from a hive.

3.3.1 Bee venom collection

Hives of uniform strength were selected for the experiment. The design of the experiment was CRD; no. of treatments: three (the three seasons) and no. of replications: five. Five hives were maintained as replications for both the treatments (three season) as well as the control. The collection time as well as the duration of collection were fixed based on the results from 3.1 and 3.2. A single collection was carried out during each season.

3.3.2 Extraction of bee venom

The bee venom was extracted as the same procedure as mentioned in 3.1.2.

3.3.3 Measurement of hive activity after venom collection

The bee mortality and the brood parameters were assessed both in the treatment as well as the control hives as per 3.2.3.

3.3.4. Correlation of weather parameters

Temperature and humidity were recorded while venom collection was carried out, on all the three seasons to assess whether it influenced the venom quantity collected. The data was collected using a data logger outside the hives.

Plate 6. Measurement of brood parameters

3.4. CHARACTERISATION OF BEE VENOM

The bee venom samples collected during the respective seasons were labelled and was subjected to analysis to determine the proportion of major components present in bee venom during different seasons. Five venom samples from the three seasons were selected and analysed. The method employed was High Resolution Liquid Chromatography- Mass Spectrometry (HR LC-MS) – HR LC-MS with database (Plant extract Impurity Profiling and Metabolite Identification). The methodology, in detail is given below.

The abundance was measured using Agilent high resolution liquid chromatography and mass spectrometry model – G6550A with MS Rel. threshold (%)0.010 and MS/MS Rel. threshold (%) 0.010. The column used was ZORBX Eclipse Plus C18, Narrow Bore 2.1 × 150 mm, 5 microns. Ion source was Dual AJS (ESI) and results were obtained on scanning at a negative polarity. The acquisition method was set to be MS – minimum range 120 Dalton (M/Z) and maximum 3000 Dalton (M/Z) with scanning rate each spectrum per second. The source parameters were set as shown in Table 1.

Table 1. Source parameters for HR LC-MS analysis

Parameter	Value
Gas Temperature (0C)	250
Gas flow (l/min)	13
Nebulizer (psig)	35
Sheath gas temperature (0C)	300
Sheath gas flow(l/min)	11

The HiP sampler with model – G4226A was used and the parameters are given in Table 2.

Table 2. HiP sampler parameters in HR LC-MS analysis

Parameter	Value
Auxiliary draw speed (µl/min)	100
Auxiliary eject speed (µl/min)	100
Draw position offset (mm)	0
Wait time after drawing (sec)	2
Sample flush out factor	5
Vial/ well bottom sensing	Yes

The binary pump with model – G42208 was used. The flow rate was set to 0.300 mL/min with synchronized stroke mode. The lower pressure limit was set to zero bar pressure and highest-pressure limit at 1200 bar. Maximum flow ramp up and down was set as 100 mL/min^2. The run time was 30 minutes and the timetable is given in Table 3. and the solvent composition at various time interval shown in Table 4.

Table 3. Solvent composition at different interval

	Time (min)	A	B	Flow	Pressure (bar)
1.	1	95%	5%	0.300 mL/ min	1200
2.	20	0%	100%	0.300 mL/ min	1200
3.	25	0%	100%	0.300 mL/ min	1200
4.	26	95%	5%	0.300 mL/ min	1200
5.	30	95%	5%	0.300 mL/ min	1200

Table 4. Timetable for solvent composition

Sl. No	Channel	Ch. 1 Solv.	Name 1	Ch. 2 Solv.	Selected	Used	Percent
1.	A	100 % Water	0.1% Formic acid in water	100% water	Ch. 1	Yes	95%
2.	B	100 % Acetonitile (ACN)	90% ACN + 10% water + 0.1% Formic acid	100% Acetonitrile	Ch. 1	Yes	5%

The design of experiment for characterization was CRD. No. of treatments: 3; No. of replications: 5. The three seasons were the three treatments and it was carried out in case of both melittin and apamin

Results

4. RESULTS

The results of quantification and characterization of Indian honey bee (*Apiscerana indica* Fab.) venom during different seasons, conducted at Department of Agricultural Entomology, College of Agriculture, Vellayani during 2019-2021 are presented in this chapter.

4.1 PEAK VENOM COLLECTION HOUR OF THE DAY

The result indicated that maximum venom (52.00 mg) was collected at the time from 2 pm to 3 pm which was on par with the venom collected during 8 am to 9 am (35.25 mg), 11 am to 12 noon (36.00 mg), 12 noon to 1 pm (33.00 mg) and 1 pm to 2 pm (48.85 mg) (Table 5.). Lesser venom quantity was obtained at 7 am to 8 am (13.50 mg) which was on par with venom collected at 6 am to 7 am (24.80 mg), 9 am to 10 am (27.50 mg), 10 am to 11 am (20.90 mg), 3 pm to 4 pm (30.25 mg), 4 pm to 5 pm (17.25 mg) and 5 pm to 6 pm (27.00 mg). Maximum venom was collected at around mid-day and lesser quantities at the morning and evening hours

4.2 OPTIMUM DURATION OF BEE VENOM COLLECTION AND ITS QUANTIFICATION FROM INDIVIDUAL BEE HIVE

The results on the optimum duration of bee venom collection and the hive activity are presented in Table 6 to 8.

4.2.1 Optimum Duration of Bee Venom Collection

The results on optimum duration of bee venom collection (Table 6.) indicated that maximum quantity of venom (55.34 mg) was collected at 60 min. duration, which showed significant difference with the venom collected at the other two durations, but the mean bee mortality during this period was very high (5.2 bees). 25.12mg and 34.64 mg of venom was collected at 30 min. and 40 minutes durations respectively, while the mean mortality at 30 minutes and 40 minutes durations were 0.80 bees and 2.00 bees respectively. Ultimately, 30 minutes was selected as the ideal duration for venom collection, as the venom quantity collected at 30 minutes duration was on par with that at 40 minutes and the bee mortality was comparably lower.

4.2.2 Measurement of Hive Activity after Venom Collection

The hive activities like foraging activity and brood parameters were studied after the venom collection to find whether venom collection has affected hive activities.

4.2.2.1 Assessment of foraging activity

The mean foraging activity recorded for five minutes from venom collected and control hives showed no significant difference (Table 7.). In venom collected hives, the number of worker bees moving into the hive ranged from 50.80 to 64.60 while that moving out of the hive ranged from 42.40 to 65.20. In control hives, number of worker bees moving into the hive ranged from 42.00 to 73.60 bees and that moving out ranged from 48.60 to 72.00. This suggested that venom collection, when carried out at spaced intervals did not affect foraging activity of the hive.

4.2.2.2 Assessment of brood parameters

The brood parameters *viz*., honey, pollen and brood storage from venom collected hives showed no significant difference with that of the control hives (Table 8).

In the hives, where venom was collected for 30 min., the mean honey storage area ranged from 441 cm^2 to 582.8 cm^2, pollen storage area from 144.6 cm^2 to 257.2 cm^2, brood storage area from 765.2 cm^2 to 844.8 cm^2 and egg storage area from 206 cm^2 to 273.8 cm^2.

In the hives, where venom was collected for 40 min., the mean honey storage area ranged from 404.8 cm^2 to 572.4 cm^2, pollen area from 125 cm^2 to 377.4 cm^2, brood storage area from 690.3 cm^2 to 832 cm^2 and egg storage area from 118 cm^2 to 357.8 cm^2.

In the hives, where venom was collected for 60 min., the mean honey storage area ranged from 429.8 cm^2 to 558 cm^2, pollen storage area from 238.2 cm^2 to 388 cm^2, brood storage area from 706.2 cm^2 to 934 cm^2 and egg storage area from 118 cm^2 to 357.8 cm^2.

A comparable data was recorded also from the control hives where the mean honey storage area ranged from 495.2 cm^2 to 542 cm^2, pollen storage area from 178.2 cm^2 to 301.4 cm^2, brood storage area from 742.2 cm^2 to 828 cm^2 and egg storage area from 197.2 cm^2 to 300 cm^2.

4.3 SEASONAL VARIATION IN VENOM PRODUCTION

Results on the seasonal variation in the venom production, hive activity as well as the correlation between venom and climatic factors are shown in Table 9 to 11.

4.3.1 Assessment of Seasonal Variation in Venom Production

Studies on the venom quantity during different seasons revealed that highest quantity of venom was collected at honey flow season (55.16 mg) which was statistically on par with the venom collected at dearth season (41.00 mg). Lowest venom quantity (25.12 mg) was collected in the brood rearing season and it differed significantly with the venom quantity collected at the other two seasons (Table 9). With regard to the mortality, highest mortality was observed during the honey flow season (3.2 bees). The mortality at dearth season (1.2 bees) and the brood rearing seasons (0.8 bees) were on par.

4.3.2 Effect of Venom Collection on Brood Parameters at Different Seasons

No significant difference was recorded in the mean values of brood parameters measured from venom collected hives and control hives in any of the three seasons.

In the brood rearing season, the mean value of honey storage area ranged from 441 cm^2 to 582.8 cm^2, pollen storage area from 144.6 cm^2 to 232.4 cm^2, brood storage area from 765.2 cm^2 to 844.8 cm^2 in the venom collected hives and in the control hives, the mean value of honey storage area ranged from 495.2 cm^2 to 542 cm^2, pollen storage area from 178.2 cm^2 to 301.4 cm^2, brood storage area from 742.2 cm^2 to 828 cm^2 and egg storage area from 197.2 cm^2 to 300 cm^2.

In the honey flow season, the mean value of honey storage area ranged from 518.6 cm^2 to 648 cm^2, pollen storage area from 164.8 cm^2 to 396.6 cm^2, brood storage area from 806.4 cm^2 to 947.2 cm^2 and egg storage area from 117.2 cm^2 to 440.6 cm^2 in venom collected hives and in control hives, the mean value of honey storage area ranged from 616 cm^2 to 658.5 cm^2, pollen storage area from 144 cm^2 to 335 cm^2, brood storage area from 818.2 $cm^2$to 941 cm^2 and egg storage area from 196.8 cm^2 to 343.7 cm^2.

In the dearth season, the mean value of honey storage area, pollen storage area, brood storage area and egg storage area ranged from 548.2 cm^2 to 816.4 cm^2, 150.2 cm^2 to 252 cm^2, 567.6 cm^2 to 727.4 cm^2 and 219.2 cm^2 to 281.4 cm^2 respectively in the venom collected hives and in control hives the value ranged from 588.4 cm^2 to 734.6 cm^2, 183.8 cm^2 to 221.4 cm^2, 519.4 cm^2 to 818.2 cm^2 and 155.6 cm^2 to 373.4 cm^2 respectively.

4.3.3 Correlation of weather parameters

Correlation studies on weather parameters (Table 11.) indicated that quantity of

venom showed positive correlation with temperature and negative correlation with humidity on all the three seasons, but the correlation was not significant at 0.001, 0.01 and 0.05 level of significance.

4.4 CHARACTERISATION OF BEE VENOM

The data on abundance of major bee venom components at different seasons are shown in Table. 12.

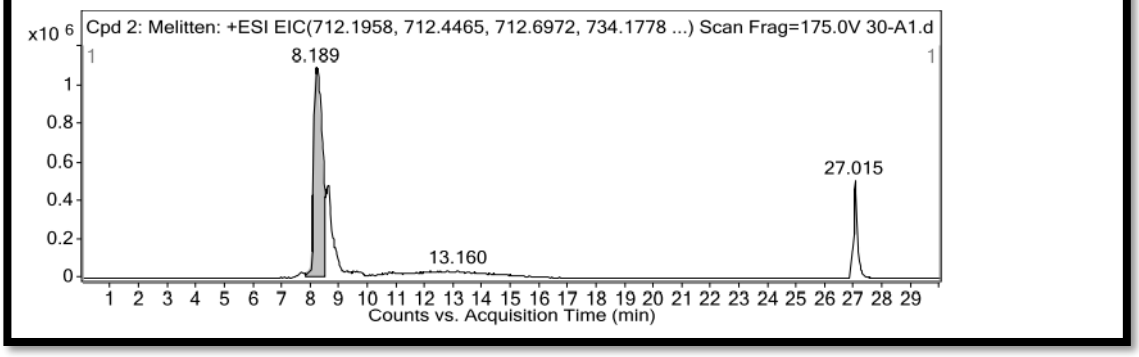
The abundance of major bee venom components was identified by qualitative HR LC-MS analysis. The results revealed that melittin and apamin were the major venom component. Melittin was more abundant (about 8.5 times more) than apamin and the abundance was not significantly influenced by the seasonal difference.

Melittin was most abundant component in the bee venom during the honey flow season (496239.50) followed by the brood rearing season and the dearth season (411586.75 and 355071) whereas apamin showed the most abundance during the death season (11612.25) followed by the honey flow season and the brood rearing season (9405.50 and 7277.24).

The chromatogram of the samples showing peaks at melittin and apamin are shown in Fig.1, Fig.2, and Fig. 3. The retention time for melittin ranged from 8.2 to 8.5 min and for the apamin, it ranged from 3.2 to 3.3 min.

Bee venom components were identified in negative HR LC-MS analysis while in the positive analysis some other compounds were also identified from the scraped venom. The compounds included Megalomicin C2 (587217 abundance), Miserotoxin (297818 abundance), Triethylenemelamine (215374 abundance) and other prominent compounds like oleamide, capsaicin, etc. were also identified. These are clearly not venom components but were found as impurities in the scraped venom. A clean glass plate was always used for collection; hence it can be ensured that these impurities were brought by the bees into the glass plate either as pollen or honey, which remained in the glass plate even after drying and cleaning of the plate with camel brush before scraping. These compounds were not found in all the samples and in all seasons, but Miserotoxin, oleamide and capsaicin or its metabolites (homocapsaicin, dihydrocapsaicin) were present in all samples.

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 2: Melitten	**Melitten**	712.4441	8.189	Find By Formula	2844.7437



Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 3: Apamine	**Apamine**	507.4758	3.276	Find By Formula	2025.8733

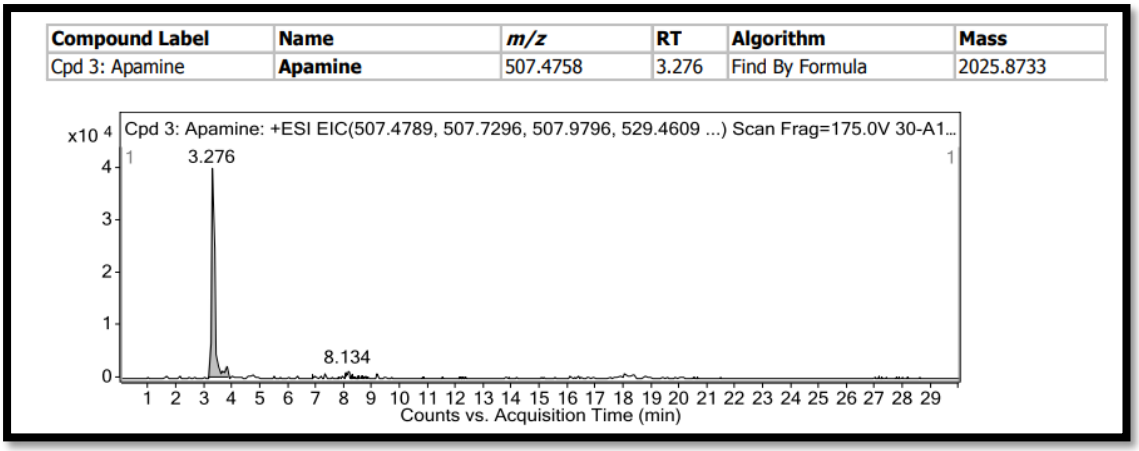


Fig. 1. Chromatogram: Melittin and Apamin peaks in venom sample of brood rearing season

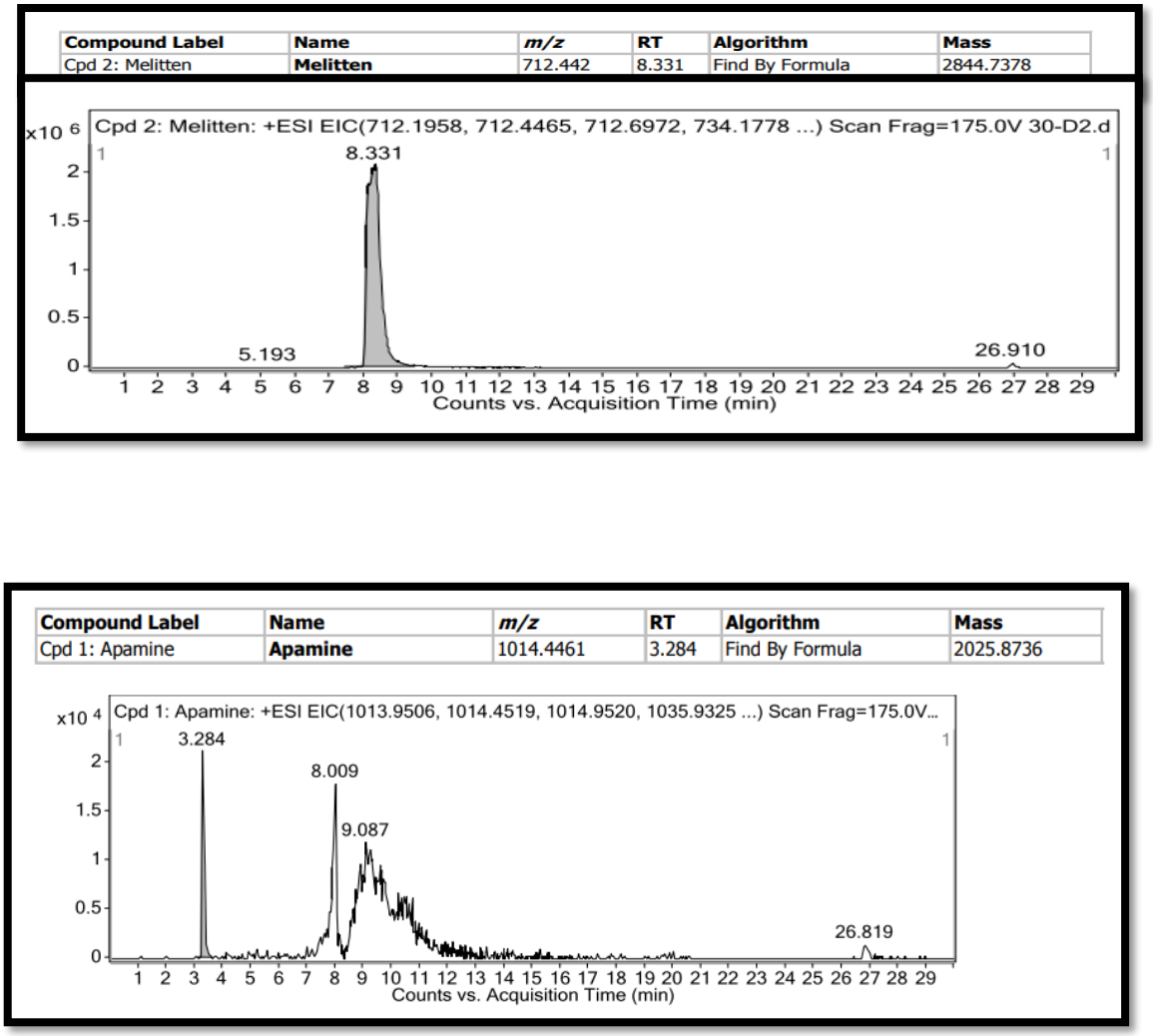
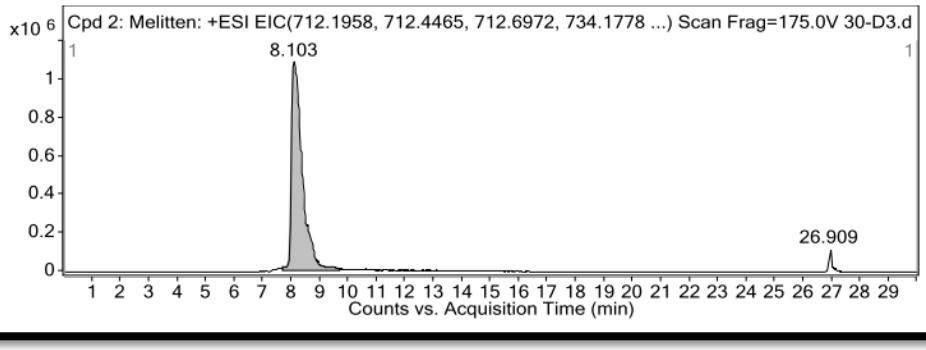


Fig. 2. Chromatogram: Melittin and Apamin peaks in venom sample of honey flow season

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 2: Melitten	**Melitten**	712.4432	8.103	Find By Formula	2844.7396



Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 2: Apamine	**Apamine**	676.6329	3.3	Find By Formula	2025.8721

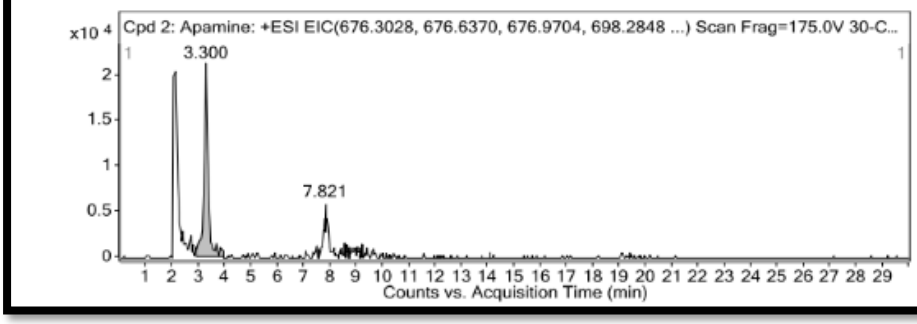


Fig. 3. Chromatogram: Melittin and Apamin peaks in venom sample of dearth season

Table 5. Quantity of venom (mg) collected at different hours of the day

Time of the day	Quantity of venom (mg)
6am – 7am	24.80*^{cd}
7am – 8am	13.50^{d}
8am - 9 am	35.25^{abc}
9am – 10am	27.50^{cd}
10am - 11am	20.90^{cd}
11am – 12noon	36.00^{abc}
12noon- 1pm	33.00^{abc}
1pm – 2pm	48.85^{ab}
2 pm - 3pm	52.00^{a}
3pm – 4pm	30.25^{bcd}
4pm - 5pm	17.25^{cd}
5pm – 6pm	27.00^{cd}
SEm(±)	6.32
CD 0.05	19.489

*Mean of three replications

Table 6. Quantity of venom collected and mortality at different durations.

Duration	Quantity of venom (mg)	Mortality (Number/hive)
30 minutes	25.12*[b]	0.80*[b]
40 minutes	34.64[b]	2.00[b]
60 minutes	55.34[a]	5.20[a]
SEm(±)	4.06	0.44
CD 0.05	12.51	1.355

*Mean of five values

Table 7. Foraging activity in hives at weekly intervals

Week	Returning foragers		Outgoing foragers	
	Venom collected	**Control**	**Venom collected**	**Control**
Week 1	53.40*	42.00	51.40	49.60
Week 2	64.60	49.20	59.80	48.60
Week 3	50.80	71.20	61.80	70.60
Week 4	55.60	73.60	65.20	72.00
t value	0.192		0.657	
P value	0.852 (NS)		0.529 (NS)	

*Mean of four observations

NS- the means are not significantly different

Table 8. Brood parameters in venom collected and control hives at different durations of venom collection

Duration of collection	Week	Mean value of parameters			
		Honey (cm^2)	Pollen (cm^2)	Brood (cm^2)	Egg (cm^2)
30 minutes	**Week 1**	515.8*	246.4	765.2	247
	Week 2	441	144.6	844.8	220.5
	Week 3	`582.8	232.4	797.2	206
	Week 4	517.2	257.2	816.2	273.8
t value		0.210	0.581	1.274	0.921
P value		0.840(NS)	0.582(NS)	0.249(NS)	0.582(NS)
40 minutes	**Week 1**	572.4	125	714	118
	Week 2	517.6	226.6	740.8	238.6
	Week 3	547.2	290.2	832	189.8
	Week 4	404.8	377.4	690.3	357.8
t value		0.079	0.227	0.777	0.645
P value		0.939(NS)	0.827(NS)	0.466(NS)	0.542(NS)
60 minutes	**Week 1**	467.4	240	706.2	118.6
	Week 2	429.8	246	903.5	292.5
	Week 3	558	238.2	837.5	268
	Week 4	465.5	388	934	256.5
t value		1.368	0.819	1.335	0.615
P value		0.220(NS)	0.444(NS)	0.230(NS)	0.561(NS)
Control	**Week 1**	542	178.2	828	197.2
	Week 2	495.2	228	775.6	280.2
	Week 3	539.4	301.4	746.2	300
	Week 4	507.4	257.8	742.2	269.2

*Mean of five observations

NS- the means are not significantly different

Table 9. Quantity of venom obtained and mortality during different seasons

Season	Quantity of venom (mg)	Mortality
Brood rearing season (Sept - Dec)	25.12*[b]	0.8*[b]
Honey flow season (Jan - April)	55.16[a]	3.2[a]
Dearth season (May-Aug)	41.00[a]	1.2[b]
CD 0.05	13.606	1.797
SEm(±)	4.416	0.583

*****Mean of five replications

Table 10. Brood parameters in hives during different seasons at weekly interval

Season	Week	Honey (cm^2)		Pollen (cm^2)		Brood (cm^2)		Egg (cm^2)	
		Venom collected	**Control**	**Venom collected**	**Control**	**Venom collected**	**Control**	**Venom collected**	**Control**
Brood Rearing season	**Week 1**	515.8*	542*	246.4*	178.2*	765.2*	828*	247*	197.2*
	Week 2	441	495.2	144.6	228	844.8	775.6	220.5	280.2
	Week 3	`582.8	539.4	232.4	301.4	797.2	746.2	206	300
	Week 4	517.2	507.4	257.2	257.8	816.2	742.2	273.8	269.2
		t value- 0.210; P value- 0.840(NS)		t value- 0.581; P value- 0.582(NS)		t value- 1.274; P value- 0.249(NS)		t value- 0.921; P value- 0.392(NS)	
Honey Flow season	**Week 1**	585.8	653.2	396.6	144	947.2	818.2	191.8	196.8
	Week 2	518.6	616	164.8	182.2	806.4	895.3	117.2	316.5
	Week 3	528.6	658.5	261.6	244.2	905.4	893.8	440.6	334.2
	Week 4	648	625	221.4	335	922.6	941	373.4	343.7
		t value-2.149; P value-0.075(NS)		t value-0.538; P value-0.609(NS)		t value-0.208; P value-0.842(NS)		t value-0.205; P value-0.844(NS)	
Dearth season	**Week 1**	548.2	648	150.2	221.4	567.6	818.2	281.4	373.4
	Week 2	740.2	734.6	165	197.4	570.6	623.4	220.3	265.6
	Week 3	816.4	642.8	158.2	207	692	553.2	223.8	233.2
	Week 4	568	588.4	252	183.8	727.4	519.4	219.2	155.6
		t value-0.204; P value- 0.844(NS)		t value-0.841; P value-0.432(NS)		t value-0.138; P value-0.894(NS)		t value-0.436; P value- 0.677(NS)	

*Mean of five values; NS-The means are not significantly different

Table 11. Correlation of weather parameters with venom quantity at different seasons

Parameters	Brood rearing season			Honey flow season			Dearth season		
	Venom quantity	**Temperature**	**Humidity**	**Venom quantity**	**Temperature**	**Humidity**	**Venom quantity**	**Temperature**	**Humidity**
Venom quantity	1			1			1		
Temperature	0.72(NS)	1		0.14(NS)	1		0.21(NS)	1	
Humidity	-0.72(NS)	-0.82(NS)	1	-0.16(NS)	0.40(NS)	1	-0.66(NS)	-0.24(NS)	1

NS- The correlation is not significant

Table 12. Abundance of major bee venom components at different seasons

Season	Abundance of components	
	Melittin	**Apamin**
Brood rearing season	411586.75* (628.28)	7277.24 (85.01)
Honey flow season	496239.50 (696.82)	9405.50 (85.08)
Dearth season	355071 (590.20)	11612.25 (87.19)
SEm(±)	61.71	26.31
CD 0.05	NS	NS

*Mean of five replications

The values in parenthesis are square root transformed values

NS- Treatments are not significantly different

Discussion

5. DISCUSSION

The discussion on the results obtained for the study, Quantification and characterization of Indian honey bee (*A. cerana indica*) venom during different seasons conducted at Department of Agricultural Entomology, College of Agriculture, Vellayani are given below:

5.1 PEAK BEE VENOM COLLECTION TIME OF THE DAY

In the present study, maximum venom was collected at 2 pm to 3 pm (52.00 mg) followed by 1 pm to 2 pm (48.85 mg) and the least quantity at 7 am to 8 am (13.50 mg) (Table 5.). While the study conducted by Sanad and Mohanny (2013) revealed that the highest venom was collected at 4 pm to 6 pm followed by 4 am to 6 am. These results can be attributed to the foraging activity of bees. The total number of bees present in a hive vary at different hours of the day depending on various in-colony factors and out-colony factors (Abou-Shaara, 2014). The worker bees present in a colony at a time can vary due to the foraging activity which is dependent on factors like available flora, sunshine, temperature, humidity, etc. This may in turn decide the number of bees which comes into contact with bee venom collector and thus the quantity of bee venom extracted.

In general, the foraging activity of honey bees start early in the morning and commences by evening. Yucel and Duman (2005), while studying on foraging activity of worker bees on onion found that the bees visited the flowers from 8.15 am to 4.30 pm, with the peak foraging between 11.00 am and 12.00 pm. A much-detailed study by Joshi and Joshi (2010) on pollinators of apple found that worker bees start their foraging activity as early as 6.17 am, but this time is greatly influenced by the region. The foraging activity is also dependent on the plants (pollen and honey source) that are available, and bees spend different times per flower depending on plant species. Study conducted by Sushil *et al.* (2013) found that the time spent per flower by bees on Chinese cabbage, broccoli and kohlrabi was 6.92 sec, 6.50 sec and 5.54 sec respectively.

Apart from the floral source, foraging activity of bees also depends on other factors. The degree of pollen need of the hive affects the foraging activity (Weidenmuller and Tautz, 2002). The presence of diseases or parasites, if any in the hive can result in the increase of time taken by the foragers to return back to the colony or even in the inability to return after foraging, as found by Kralj and Fuchs (2006). Studies by Abou-Shaara *et al.* (2013) found that colony strength and brood rearing activity also affects the foraging activity.

Atmospheric temperature was also found to influence the foraging activity of bees. Foraging activity reduced beyond 43°C (Blazyte-Cereskiene *et al.*, 2010) as well as below 10°C (Joshi and Joshi, 2010). Highest foraging activity was recorded at an ambient temperature of about 20°C in *Apis mellifera*. Abou-Shaara *et al.* (2013) found a significant negative correlation between foraging activity and temperature.

5.2 OPTIMUM DURATION OF BEE VENOM COLLECTION AND ITS QUANTIFICATION FROM INDIVIDUAL BEE HIVE

This study aimed to find the optimum duration for which the BVC has to be placed in the hive to obtain maximum quantity of venom without causing much damage to the bees (Table. 6).

In the present study, it was observed that the quantity of venom collected with the BVC increased with the duration of collection and the mean mortality also showed a simultaneous increase with duration. But a progressive pattern cannot be expected between the quantity of venom and duration, as the venom quantity cannot increase further since all the active worker bees have stinged. In the present investigation, bee mortality was given equal weightage to the venom quantity collected. Even though higher quantity of venom was collected at 40 min. and 60 min. duration, 30 min. was finally selected as the ideal duration as the mean mortality was very low compared to the others.

Nenchev and Stoichev (1997), found that increasing the duration of electrostimulation beyond 60 min. (from 60 to 90 min.) did not yield more venom. They also reported that a collection pattern of 30 min. followed by a 60-min. break and then

a 30-minute collection carried out at fortnightly intervals yielded highest quantity of bee venom and caused minimum damage to the bees. According to de Graaf *et al.* (2020), the quantity of bee venom obtained over a few hours of continuous stimulation is less than the quantity collected over the same time period with interruptions. The best period for electro-stimulation is 30 to 60 min., with a break of 45 to 90 min. between the collections. Electro-stimulation was used in tests for a four-hour period, with short one-minute intervals every 30 min. to collect the venom. As a result, bee venom release decreases exponentially with time, with a peak in the first 30 min. The quantity reduces to 10% of the peaks acquired in the last 30 min.

5.2.1 Assessment of Foraging Activity after Venom Collection

The results showed that venom collection did not affect the foraging activities of the bees (Table 7.). In the present study, venom collection from a single hive was carried out only once, that is, a hive from which venom was collected was not again used for collection. It could be assumed that due to this reason the hive activities were not affected. Bogdanov (2016) found that less frequent venom collections, carried out 3 to 4 times from a hive did not affect the bee performance.

5.2.2 Assessment of Brood Parameters after Venom Collection

This study aimed to identify whether venom collection has affected the brood parameters of the hive and the results are shown in Table. 8. As mentioned in 5.2.1 venom collection was carried out only once from an individual hive and due to this the brood parameters were also unaffected.

According to Zhou *et al.* (2003), venom collection by electrical shocking carried out continuously once in every three days and up to ten times was not beneficial to the bees and the honey yield dropped by 45.64 to 49.90 per cent and that of royal jelly by 46.71 per cent. They also found that collecting venom continuously for 11 days did not affect the bee population, but a population reduction of 12.87 per cent and 28.81 per cent was observed after continuous collection for 23 and 35 days.

Studies conducted by Sanad and Mohanny (2013), where venom collection was carried out every three days from March 2012 to November 2012 at South Valley University, Quenna, Egypt, it was found that the brood parameters showed considerable decrease in the venom collected hives by 18.1 per cent during the month of July followed by 16.9 per cent reduction in the month of August. Least reduction in brood area was observed during November. Bogdanov (2016) found that repeated three-hour collection periods performed 3 to 4 times a month can decrease brood production and honey yield by 10 per cent. Another study by de Graaf *et al.* (2020) found that 10 per cent reduction in honey yield can occur when venom collections are carried out daily and more than three to four hours per day. They also suggested a break of two to three days was necessary at every week.

Omar (2017) collected venom from hives with a gap of ten days between subsequent collections and then studied the brood rearing activities of the hive. The experiment was conducted for a period of nine months over two years and found that venom collection when carried out at spaced intervals did not affect the brood rearing activities of the hive.

Argena *et al.* (2021) conducted a study where venom collection was carried out in *A. mellifera* hives with a gap of six and twenty-four days between subsequent collections and then the brood area were studied. The results of both the treatments showed that venom collection did not affect the brood development and the productivity of honey from the hives.

5.3 SEASONAL VARIATION IN VENOM PRODUCTION

Studies on the assessment of seasonal change in bee venom quantity (Table 9.) revealed that maximum quantity of scraped venom (mg) was collected in the honey flow season (Jan - April) followed by dearth season (May - Aug) and brood rearing season (Sept - Dec) (Fig. 4). A serious problem faced during the venom collection at honey flow season and dearth season was the unavoidable presence of honey over the glass plate. During these seasons, the brood chamber is filled with honey and the worker bees are busy gathering honey and hence honey was always present

over the glass plate during venom collection. Honey present over the plate would not dry due to its hygroscopic nature and this in turn added to the weight of scraped venom. Little or no honey was present in the glass plate at brood rearing season. Despite all this, it can never be concluded that honey alone added to the weight of scraped venom, as qualitative HR LC-MS analysis revealed the greater abundance of melittin and apamin (major bee venom components) during honey flow and dearth season.

According to study by Khodairy and Omar (2003) in Egypt, significant variation in venom quantities collected was recorded at different periods of active season and they reported that amount of venom collected in June was greater, compared to that collected during May and July.

Based on the studies by Rybak *et al.* (1995) at Pulawy, Poland, mid- July was the best time for collection and better results were obtained when the collection frame was kept on the upper part of the hive. Argena *et al.* (2021) collected bee venom for different seasons over two years (2016 and 2017) using a BVC which was placed as a frame in the super chamber. The results showed that venom production was high during the spring, decreased in the summer and then increased again during the winter, but the influence of climatic factors on venom quantity was found to be not significant.

5.3.1 Effect of Venom Collection on Brood Parameters at Different Seasons

This study was conducted to identify whether venom collection has affected the brood parameters in any season and the results are presented in Table. 10. Here also, it was found that venom collection did not affect the brood parameters in any season.

5.3.2 Correlation of Weather Parameters

A correlation analysis was made between quantity of venom and weather parameters, *viz.* temperature and humidity on all the seasons and the data obtained is shown in Table. 11. A positive correlation was observed between venom quantity and temperature, while a negative correlation between venom quantity and humidity was recorded during all the seasons, but both the correlations were not significant. It showed that the prevailing conditions of temperature and humidity did not influence the

quantity of venom that can be collected from a hive.

It the present study, temperature ranged only from 30°C to 32°C in the brood rearing season, 31°C to 32°C in the honey flow season and 27.5°C to 31°C in the dearth season, which was not a prominent temperature difference and hence cannot be linked to the venom quantity collected. Honey bees always try to keep an ambient hive temperature of around 33°C to 36°C (Petz *et al.*, 2004), precisely at 34.5 ± 1.5°C (Jones *et al.*, 2005) by various mechanisms. It was noted that the temperature recorded during all these seasons was only slightly below this ambient temperature and hence the effort taken by bees during all these periods can be assumed to be the same. Thus, external temperature did not show a significant correlation with the venom collected.

The relative humidity (RH) values, ranged from 63% to 75% in brood rearing season, 65% to 74% in the honey flow season and 85% to 88% in the dearth season. It can be observed that on all the seasons, the range of RH was small and hence there was no impact on the hive activities. Abou-Shaara *et al.* (2012) found that RH at 15% to 50% had a negative impact on worker bees, but in the present study, all RH values were well above 50% during the collection periods. The change in RH was not prominent to make an effect on hive activities and hence the venom yield.

5.4 CHARACTERISATION OF BEE VENOM

With regard to the Qualitative HR LC-MS analysis of bee venom, melittin the peptide was identified as the major venom component (Table 12). Another component, Apamin was also identified, whereas the abundance of melittin was about 8.5 times more than that of apamin during all the three seasons (Fig. 5). This is in accordance with the studies by Dotimas and Hider (1987) where the composition of melittin in the venom of *A. mellifera* ranged from 30% to 50% and of Apamin ranged from 1% to 3%.

Report by Hoffman and Jacobson (1984) showed that the venom volume and content can slightly vary within different strains; bumble bee species contain less venom than Italian bees (*A. mellifera*), with the Italian bees releasing up to five times more venom than bumble bees. Hence, the venom composition can vary among the bee species.

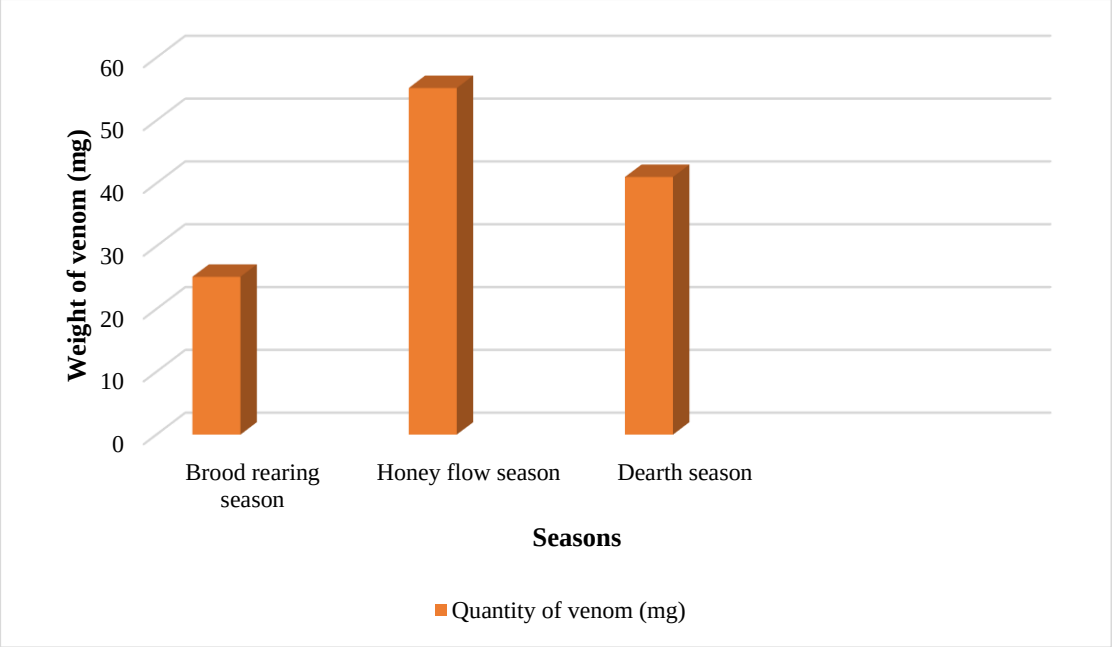


Fig 4. Quantity of venom obtained during different seasons

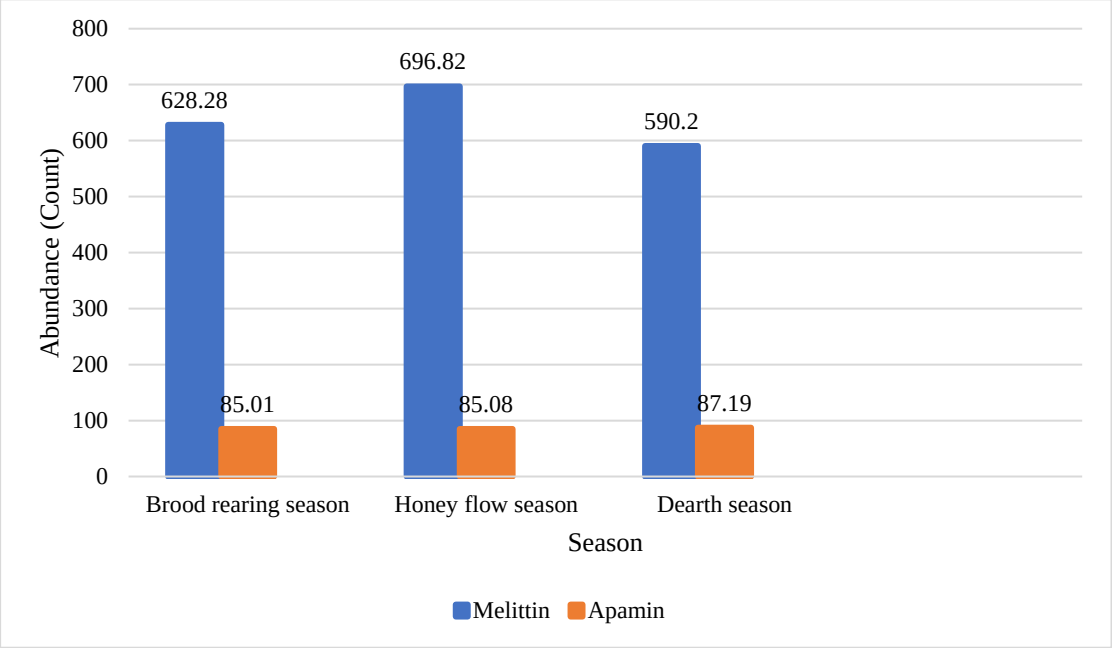


Fig 5. Abundance of bee venom components at different seasons

According to studies conducted by El-Wahed *et al.* (2018) in Europe, apart from the age of a bee, the seasonal variations also influence the bee venom composition. Melittin reached a maximum level in March and May, and declined in January. Furthermore, melittin content changed during summer, peaking at the beginning of June and decreased in August. They also reported that the difference in venom content is related to the change in the honey bee's diet during different seasons.

Summary

6. SUMMARY

The research work entitled “Quantification and characterization of Indian honey bee (*Apis cerana indica* Fab.) venom” was conducted with an objective to quantify and characterize Indian honey bee venom during different seasons. The experiment was conducted from November 2020 to August 2021 at College of Agriculture, Vellayani.

The experiment was carried out in three parts; in the first part, the peak time of venom collection, the optimum duration of venom collection and the quantity of venom that can be obtained from a single hive was studied. In the second part, seasonal variation in venom collection was studied and in the final part, characterization of venom collected during three seasons were carried out. Along with this, bee mortality after venom collection, effect of venom collection on brood parameters and on foraging activity, correlation of venom quantity with temperature and humidity were also studied.

Hives of uniform bee strength maintained in the apiary of AICRP on Honey bees and pollinators were selected to identify the peak time of the day at which maximum bee venom can be collected from a hive. For this, venom was collected from the hives from 6 am to 6 pm for three days. The optimum duration at which maximum venom can be collected from the hive with minimum damage to the bees were also assessed. Venom was collected for three different durations *viz*., 30 min., 40 min. and 60 min. and the quantity of venom collected and mortality was recorded. Seasonal variation was assessed by collecting venom at the peak time and optimum duration on all the three seasons: brood rearing (Sept-Dec), honey flow (Jan-Apr) and dearth (May-Aug). The brood parameters and foraging activity of the hives were also assessed at weekly intervals for a period of one month in order to determine whether the bee venom collection has any impact on these parameters. The venom collected during the three seasons were subjected to characterisation and the proportion of components present in the venom were analysed. Control hives were maintained and the data were subjected to ANOVA and paired t test analysis. The experimental results are summarized below.

Maximum quantity of venom (52.00 mg per hive) was collected at 2 pm to 3 pm and least at 7 am to 8 am (13.50 mg per hive). Data on optimum duration of venom collection showed that maximum quantity of venom (55.34 mg per hive) was collected

at 60 min. duration but the mean mortality recorded at this duration was highest (5.20 bees per hive). Lesser quantity of venom (25.12 mg per hive) was collected at 30 min. duration which was statistically on par with venom collected at 40 min. duration (34.64 mg per hive). The mean mortality at 30 min. duration was lesser (0.80 bees per hive) and was on par with mean mortality at 40 min. (2.00 bees per hive). Hence, the optimum duration for placing the bee venom collector was selected as 30 min., considering the low mortality of bees as compared to that at other durations. Thus, the analysis of data showed that venom quantity and mortality were significantly influenced by seasonal changes.

The number of outgoing and returning foragers of the hives as well as the brood parameters (honey, pollen and brood storage) were studied from venom collected and control hives at weekly intervals up to one month after collection. The means of the data were compared by paired t-test analysis and found that the values did not show significant difference from both the hives suggesting that venom collection did not affect the foraging activities of bees as well as the brood parameters.

Correlation analysis of venom quantity with temperature and humidity at different seasons were studied. The data showed that the quantity of bee venom collected had a non-significant positive correlation with temperature and negative correlation with humidity.

Characterisation of bee venom samples revealed that Melittin and Apamin were identified as the major components, with melittin showing maximum abundance on all the three seasons. Maximum abundance of melittin was observed in the honey flow season (496239.50) followed by the brood rearing season and the dearth season whereas apamin was most abundant during the dearth season (11612.25) followed by honey flow and brood rearing season. No significant difference was recorded in the abundance of both melittin and apamin among the three seasons. The retention time for detection of melittin ranged from 8.2 min to 8.5 and that of apamin ranged from 3.2 to 3.3 min. Apart from venom components, other impurities like megalomicin C2, miserotoxin, capsaicin, etc. were also identified from scraped venom.

Thus, in the present study, highest quantity of bee venom was collected at 2 pm to 3 pm and the optimum duration for collection was 30 min., considering the bee mortality

factors. Studies on the seasonal variation revealed that significantly high bee venom was collected during the honey flow season, while no significant variation was observed in the foraging activity of bees as well as the brood parameters during the three seasons. Characterisation of the bee venom revealed that melittin and apamin were the major components, of which melittin was 8.5 times abundant than that of apamin with no significant variation among the seasons.

References

7. REFERENCES

Abou-Shaara, H.F. 2012. Notes on water collection by honey bees. *Bee World.* 89: 50-51.

Abou-Shaara, H.F., Al-Ghamdi, A.A., and Mohamed, A.A. 2013. Honey bee colonies performance enhance by newly modified beehives. *J. Api. Sci.* 57: 45–57.

Abou-Shaara, H.F. 2014. The foraging behaviour of bees. *Veterinarni Medicinia.* 59(1): 1-10.

Argena, N., Tananaki., Thrasyvoulou, A., Goras, G., Kanelis, D., and Liolios, V. 2021. Seasonal variation on bee venom collection. The impact on some biological aspects on *Apis mellifera. J. Hellenic Vet. Med. Soc.* 72(2): 2861-2868.

Bachmayer, H., Kreil, G., and Suchanek, G. 1972. Synthesis of promelittin and melittin in the venom gland of queen and worker bees: patterns observed during maturation. *J. Insect Physio.* 18(8):1515-1521.

Banks, B.E.C., Dempsey, C.E., and Barboni, E. 1983. Anti-inflammatory activity in the venom of *Apis mellifera. Toxicon.* 53: 29-32.

Benton, A.W., Morse, R.A., and Stewart, J.B. 1963. Venom collection from honey bees. *Sci.* 142: 228-229.

Benton, A.W. and Morse, R.A. 1966. Collection of the liquid fraction of bee venom. *Nat.* 210: 652-653.

Blazyte-Cereskiene, L., Vaitkeviciene, G., Venskutonyte, S., and Buda, V. 2010. Honey bee foraging in spring oilseed rape crops under high ambient temperature conditions. *Zemdirbyste-Agri.* 97: 61–70.

Bogdanov, S. 2016. Bee Venom: Production, Composition, Quality. https://www.researchgate.net/publication/304012422_Bee_Venom_Production_Composition_Quality

de Graaf, D.C., Brochetto, M.R.B., de Abreu, R.M.M., Blank, S., Bridts, C.H., De Clerck, L.S., Devreese, B., Ebo, D.G., Ferris, T.J., Hagendorens, M.M., and Justo, J.D.L. 2020. Standard methods for Apis mellifera venom research. *J. Apicultural Res.* 3(2):1-31.

Dotimas, E.M. and Hider, R.C. 1987. Honeybee venom, *Bee World.* 68(2): 51-70.

El Mehdi, I., Mustapha, H.,Vilas-Boas, M., and Campos, M. 2018. A double-faced bee venom collector: a new contribution to improve apitoxin collection. *V Congresso Ibérico Apicultura.* 8 (25):69-72

El-Wahed, A.A., Khalifa, S.A.M., Sheikh, B.Y., Farag, M.A., Saeed, A., Larik, F.A., Koca-Caliskan, U., Al-Ajmi, M.A., Hassan, M., Wahabi, H.A., Hegazy, M.F., Algethamikk, A.F., Buttner, S., and El-Seedi, H.R. 2018. Bee venom composition: from chemistry to

biological activity. *Stud. Nat. products Chem.* 60: 460-477.

Garibaldi, L.A., Steffan-Dewenter, I., Winfree, R., Aizen, M.A., Bommarco, R., Cunningham, S.A., Kremen, C., Carvalheiro, L.G., Lawrence D. Harder, Afik, O., Bartomeus, I., Faye Benjamin, F., Boreux, V., Cariveau, D., Chacoff, N.P., Dudenhöffer, J.H., Freitas, B.M., Ghazoul, J., Greenleaf, S., Hipólito, J., Holzschuh, A., Howlett, B., Isaacs, R., Javorek, S.K., Kennedy, C.M., Krewenka, K., Krishnan, S., Mandelik, Y., Mayfield, M.M., Motzke, I., Munyuli, T., Nault, B.A., Otieno, M., Petersen, J., Pisanty, G., Potts, S.G., Rader, R., Ricketts, T. H., Rundlöf, M., Seymour, C.L., Schüepp, C., Szentgyörgyi, H., Taki, H., Tscharntke, T., Vergara, C.H., Viana, B.F., Wanger, T.C., Westphal, C., Williams, N., and Klein. A. M. 2013. Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Sci.* 339: 1608-1611.

Habermann, E. and Jentsch, J. 1967. Sequence analysis of melittin from tryptic and peptic degradation products. *Hoppe-Seyler's Zeitschrift Physiol. Chem.* 348(1): 37-50.

Habermann, E. 1977. Neurotoxicity of apamin and MCD peptide upon central application. *Naunyn-Schmiedebergs Archive Pharmakologie.* 300: 189-191.

Han, S.M., Pak, S.C., Nicholls, Y.M., and Macfarlane, N., 2016 Evaluation of anti-acne property of purified bee venom serum in humans. *J. Cosmet. Dermatol.* 15: 324-329.

Hepburn, H.R. and Radloff, S.E. 2011. *Honeybees of Asia.* Springer, Berlin, 259p

Hider, R.C. 1988. Honeybee venom: A rich source of pharmacologically active peptides. *Endeavour.* 12(2): 60-65.

Hider, R.C., Khader, F., and Tatham, A.S., 1983. Lytic activity of monomeric and oligomeric melittin. *Biochimica Biophysica Acta.* 728: 206-214.

Hoffman, D.R. and Jacobson, R.S. 1984. Allergens in Hymenoptera Venom XII: How much protein is in a Sting? *Ann. Allergy.* 52(4): 276-278.

Jenkinson, D.H. 1981. Peripheral actions of apamin. *Trends Pharmacol. Sci.* 2: 318-320.

Jones, J.C., Helliwell, P., Beekman, M., Maleszka, R., and Oldroyd, B.P. 2005. The effects of rearing temperature on developmental stability and learning and memory in the honey bee (*Apis mellifera*). *J. Comp. Physiol. A.* 191 :1121–1129.

Joshi, N.C. and Joshi, P.C. 2010. Foraging behaviour of *Apis* spp. on apple flowers in a subtropical environment. *New York Sci. J.* 3: 71-76.

KAU [Kerala Agricultural University]. 2016. *Package of Practices Recommendations: Crops* (15th Ed.). Kerala Agricultural University, Thrissur, 393p

Khodairy, M.M. and Omar, M.M. 2003. The relationship between bee venom production by electrical impulses and certain characters of honey bee (*Apis mellifera* L.) colonies.

Assuit J. Agri. Sci. 34(5): 115- 131.

Kim, S.H., Jung,S.Y., Lee,K.W., Lee, S.H., Cai, M., Choi, S.M., and Yang, E. 2013. BMC Complement. Altern. Med. 13: 179-187

Kralj, J. and Fuchs, S. 2006. Parasitic Varroa destructor mites influence flight duration and homing ability of infested *Apis mellifera* foragers. *Apidologie.* 37: 577–587.

Krivtsov, N. and Lebedev, V. 1995. *The Bee Products.* Editing House, Niwa Niwa, Russia. 54p.

Lazdunski, M. 1983. Apamin, a neurotoxin specific for one class of calcium-dependent potassium channels. *Cell Calcium* 4: 421-428.

Lee, G. and Bae, H. 2016. Bee venom phospholipase A2: yesterday's enemy becomes today's friend. *Toxins.* 8: 48.

Markovic, O. and Mollnar, L. 1954. Isolation and determination of honey bee poison. *Chemicke Zvesti.* 8: 80-90.

Michener, C.D. 2000. *The bees of the world.* Johns Hopkins University Press, Baltimore. 972p

Mishra, R.C. 1995. *Honeybees and Their Management in India.* Indian Council of Agricultural Research, New Delhi, 168 pp.

Nenchev, P. and Stoichev, K. 1997. Influence of duration and numbers of electro-stimulations on bees' venom extraction. *Zhiotnov" dni Nauki.* Supplement. 281-283.

O'Connor, R., Rosenbrook, W., and Erikson, R. 1963. Hymenoptera: Pure venom from bees, wasps and hornets. *Sci.* 139: 420.

Omar, R.E.M. 2017. Effect of bee venom collection on the measurement of brood rearing activity of honey bee colony *Apis mellifera* L. *Middle East J. Agric. Res.* 6(2): 409-414.

Owen, M.D. and Braidwood, J.L. 1974. A quantitative and temporal study of histamine and histidine in honey bee (*Apis mellifera* L.) venom. *Canadian J. Zool.* 52(3): 387-392.

Owen, M.D. 1979. Relationship between age and hyaluronidase activity in the venom of queen and worker bees (Apis *mellifera*). *Toxicol.* 17: 94-98.

Padmanabhan, P. 2003. Prospects and problems of beekeeping in Kerala, South India. *J. Palynol.* 39(3): 167-173.

Petz, M., Stabentheiner, A., and Crailsheim, K. 2004. Respiration of individual honeybee larvae in relation to age and ambient temperature. *J. Comp. Physiol. B.* 174: 511-518.

Pursley, R.E. 1973. Stinging Hymenoptera. *American Bee J.* 113: 131-135

Ramachandra, T.V., Subashchandran, M.D., Joshi, N.V., and Balachandran, C. 2012. *ENVIS technical report:* 49, Centre for ecological sciences. Indian Institute of Science,

Bangalore

Ruttner, F. 1988. *Biogeography and taxonomy of honeybees*. Springer. Berlin. 292p

Rybak, M., Muszyńska, J., and Skubida, P. 1995. A technology for bee venom collection. *Pszczelnicze Zeszyty Naukowe.* 39(2): 223-231.

Sanad, R.E. and Mohanny, K.M. 2013. The efficacy of a new modified apparatus for collecting bee venom in relation to some biological aspects of honeybee colonies. *J. Am. Sci.* 9(10): 177-182.

Schumacher, M.J., Schmidt, J.O., Egen, N.B., and Lowry, J.E. 1990. Quantity, analysis and lethality of European and Africanized honey bee venoms. *Amer. J. Trop. Med. Hyg.* 43: 79-86.

Schumacher, M.J., Tveten, M.S., Michael, S., and Egen, N.B. 1994. Rate and Quantity of delivery of venom from honey bee stings. *J. Allergy Clin. Immunology*. 93: 831- 835.

Shipolini, R., Bradbyry, A.F., Callewaert, G.L., and Vernon, C.A. 1967. The structure of apamin. *Chem. Commun.* (14): 679-680.

Shipolini, R.A., Doonan, S., and Vernon, C.A., 1974. The disulphide bridges of phospholipase A2 from bee venom. *Eur. J. Biochem.* 48(2):477-483.

Sivaram, V. and Anita, M. 2000. Studies on the feasibility of commercial beekeeping in Western Ghat forests for sustainable forest management. *Asian Bee J.* 2(1): 25- 31.

Sivaram, V. 2012. Status, prospects and strategies for development of organic beekeeping in the South Asian Countries. *Division of Apiculture and Biodiversity, Department of Botany, Bangalore University.*

Sivaram, V., Anita, M., and Reddy, C.C. 1993. The preservation and management of wild honeybees for pollination. *Proceedings of the International Symposium on Pollination in Tropics*, 8-13 August 1993, Bangalore, India, pp.251-253.

Snodgrass, R.E. and Morse, R.A. 2018. *Anatomy of the Honey Bee.* Ithaca, Cornell University Press, USA, 406p.

Sobral, F., Samaio, A., Falcao, S., Queiroz, M.J.R., Calhelha, R.C., Vilas-Boas, M., and Ferreira, I.C. 2016. Chemical characterization, antioxidant, anti-inflammatory and cytotoxic properties of bee venom collected in Northeast Portugal. *Food Chem. Toxicol.* 94: 172-177.

Sushil, S.N., Stanley, J., Hedau, N.K., and Bhatt, J.C. 2013. Enhancing seed production of three Brassica vegetables by honey bee pollination in north-western Himalayas of India. *Universal J. Agri. Res.* 1: 49-53.

Szokan, G., Horvath, J., Almas, M., Saftics, G., and Palocz, A. 1994. Liquid chromatographic

analysis and separation of polypeptide components from honey bee venoms. *J. Liquid. Chrom.* 17(16): 3333-3349.

Tej, M.K., Aruna, R., Mishra, G., and Srinivasan, M.R., 2017. Beekeeping in India. In: *Industrial Entomology*. Springer, Singapore, pp. 35-66.

Tilahun, A., Basa, B., Belay, W., and Teshale, A. 2016. Review on medicinal value of honeybee products: apitherapy. *Adv. Biol. Res.* 10: 236-247.

Weidenmuller, A. and Tautz, J. 2002. In-hive behaviour of pollen foragers (*Apis mellifera*) in honey bee colonies under conditions of high and low pollen need. *Ethol.* 108: 205-221.

Yadav, S., Kumar, Y., and Jat, B.L. 2017. Honey bee: diversity, castes and life cycle. In: Omkar, (ed.), *Industrial Entomology*. Springer Nature, Singapore, pp. 5- 33.

Yucel, B. and Duman, I. 2005. Effects of foraging activity of honey bees (*Apis mellifera* L.) on onion (*Allium cepa*) seed production and quality. *Pakist. J. Biol. Sci.* 8: 123-126.

Zhou, B., Zhang, S., Su, C., Zhou, G., Zhou, B.F., Zhang, S.J., Su, C., and Zhou, G.H. 2003. Effect of collection of venom by electric shocking on honeybee population, production of royal jelly and honey. *Acta Agri. Universitatis Jiangxiensis*. 25(1): 141-145.

Abstract

QUANTIFICATION AND CHARACTERIZATION OF INDIAN HONEY BEE (*Apis cerana indica* Fab.) VENOM

by

ALEN JOY

(2019-11-121)

ABSTRACT

Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL ENTOMOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695 522

KERALA, INDIA

2022

ABSTRACT

The research work entitled "Quantification and characterization of Indian honey bee (*Apiscerana indica* Fab.) venom" was carried out at College of Agriculture, Vellayani during the year 2019 to 2021. The objective of the study was quantification and characterization of Indian honey bee venom during different seasons.

Hives of uniform bee strength maintained in the apiary of AICRP on Honey Bees & Pollinators were selected to identify the peak hour of the day at which maximum bee venom can be collected from a hive using a bee venom collector. Venom was collected from hives at different hours starting from 6 am to 6 pm for three days. The optimum duration at which maximum venom can be collected from the hive with minimum damage to the bees were also assessed. Venom was collected for three different durations *viz*., 30 minutes, 40 minutes and 60 minutes and the quantity of venom collected and mortality was recorded. Seasonal variation was assessed by collecting venom at the peak hour and optimum duration on all the three seasons *viz*, brood rearing (September - December), honey flow (January - April) and dearth (May-August). The brood parameters and foraging activity of the hives were also assessed at weekly intervals for a period of one month in order to determine whether the bee venom collection has any impact on these parameters. The venom collected during the three seasons were subjected to characterisation and the proportion of components present in the venom were analysed. Control hives were maintained and the data were subjected to ANOVA and paired t test analysis.

Observations on the venom collection at hourly intervals of a day revealed that maximum quantity of venom was collected at 2 pm to 3 pm (52.00 mg per hive) and least venom was collected at 7 am to 8 am. Statistical analysis of the data on optimum duration for venom collection showed that highest quantity of venom was collected at 60 minutes duration (55.34 mg per hive), but the mean mortality was high (5.20 bees per hive). Venom collected at 40 minutes and 30 minutes were 34.14 mg and 25.12 mg per hive which were on par. The optimum duration for placing the bee venom collector was selected as 30 minutes considering the low mortality of bees (0.80 per hive) as compared to 40 minutes (2.00 per hive). Significant variation was not observed in the brood parameters as well as in the foraging activity of the venom collected and control

hives.

Studies on the seasonal variation in bee venom collected revealed that maximum quantity of venom was collected at honey flow season (55.16 mg per high) followed by dearth season (41.00 mg) and brood rearing season (25.12 mg). Maximum mortality was also recorded at honey flow season followed by dearth season and brood rearing season. Brood parameters as well as the foraging activity of the bees did not vary significantly among the seasons. The quantity of bee venom collected had a non-significant positive correlation with temperature and negative correlation with humidity.

The characterisation of bee venom samples collected during the three seasons were carried out at SAIF, IIT Bombay by HR LC-MS (High Resolution Liquid Chromatography-Mass Spectrometry) with database (Plant extract Impurity Profiling and Metabolite Identification). Melittin and apamin were identified as the major components, with melittin showing maximum abundance on all the three seasons. No significant difference was recorded in the abundance of both melittin and apamin among the three seasons.

Thus, in the present study, highest quantity of bee venom was collected at 2 pm to 3 pm (52.00 mg per hive) and the optimum duration for collection was 30 minutes, considering the bee mortality factors. Studies on the seasonal variation revealed that significantly high bee venom was collected during the honey flow season (55.16 mg per hive), while no significant variation was observed in the brood parameters among the seasons. Characterisation of the bee venom revealed that melittin and apamin were the major components, of which melittin was 8.5 times abundant than that of apamin with no significant variation among the seasons.

സംഗ്രഹം

ഇന്ത്യൻ തേനീച്ച (എപ്പിസ് സെറാന ഇൻഡിക്ക) വിഷത്തിന്റെ അളവും സ്വഭാവവും നിർണയിക്കുക എന്ന വിഷയത്തിൽ ഒരു ഗവേഷണപഠനം 2019 -2021 കാലഘട്ടത്തിൽ വെള്ളായണി കാർഷിക കോളേജിൽ നടത്തുകയുണ്ടായി. വിവിധ കാലാവസ്ഥകളിൽ ഇന്ത്യൻ തേനീച്ച വിഷത്തിന്റെ അളവിലും സ്വഭാവത്തിലുമുള്ള വ്യത്യാസം നിർണയിക്കുക എന്നതായിരുന്നു പഠനത്തിൻറെ ലക്ഷ്യം.

തേനീച്ചകളിൽ നിന്നും ഒരു ദിവസം ഏറ്റവും കൂടുതൽ വിഷം പുറപ്പെടുവിക്കുന്ന സമയം ഏതാണെന്ന് കണ്ടെത്തുന്നതിനായി ഏകീകൃത തേനീച്ച ശക്തിയുള്ള 36 തേനീച്ചക്കൂടുകൾ തിരഞ്ഞെടുക്കുകയും അതിൽ രാവിലെ 6 മണി മുതൽ വൈകിട്ട് 6 മണി വരെയുള്ള ഓരോ മണിക്കൂറിലും വിഷം ശേഖരിച്ചു. തേനീച്ചകൾക്ക് ഏറ്റവും കുറവ് നാശനഷ്ടം ഉണ്ടാക്കി, പരമാവധി വിഷം ശേഖരിക്കാൻ കഴിയുന്ന അനുയോജ്യമായ കാലയളവായ 30 മിനിറ്റ്, 40 മിനിറ്റ്, 60 മിനിറ്റ് എന്നിങ്ങനെ മൂന്ന് വ്യത്യസ്ത സമയങ്ങളിൽ വിഷം ശേഖരിച്ചു, വിഷത്തിന്റെ അളവും മരണനിരക്കും രേഖപ്പെടുത്തി. തേനീച്ചയുടെ വളർച്ചാ കാലം (സെപ്റ്റംബർ- ഡിസംബർ), തേനുൽപ്പാദന കാലം (ജനുവരി - ഏപ്രിൽ), ക്ഷാമ കാലം (മെയ് - ആഗസ്റ്റ്) എന്നിങ്ങനെ മൂന്ന് കാലങ്ങളിൽ വിഷം ഉത്പാദിപ്പിക്കുന്നതിനുള്ള വ്യതിയാനം വിലയിരുത്തി. തേനീച്ച വിഷ ശേഖരണം നടത്തിയ കൂടുകളിൽ തേനീച്ചകളുടെ പുഴുവളർച്ചയും തേനീച്ചക്കൂടുകളുടെ പ്രവർത്തനവും ആഴ്ചതോറുമുള്ള ഇടവേളകളിൽ ഒരുമാസത്തേക്ക് രേഖപെടുത്തുകയുണ്ടായി. മൂന്ന് സീസണുകളിലായി ശേഖരിച്ച വിഷം സ്വഭാവരൂപീകരണത്തിന് വിധേയമാക്കുകയും

വിഷത്തിൽ അടങ്ങിയിരിക്കുന്ന ഘടകങ്ങളുടെ അനുപാതം വിശകലനം ചെയ്യുകയും ചെയ്തു. കൺട്രോൾ തേനീച്ചക്കൂടുകൾ നിലനിർത്തുകയും ഡാറ്റ ആനോവാ (ANOVA) യ്ക്കും പെയേർഡ് ടി ടെസ്റ്റ് (Paired- T test) വിശകലനത്തിനും വിധേയമാക്കുകയും ചെയ്തു.

ഈ പഠനത്തിൽ, ഉച്ചയ്ക്ക് 2 മുതൽ 3 വരെ (ഒരു ഹൈവിൽ 52.00 മില്ലിഗ്രാം) തേനീച്ചയുടെ ഏറ്റവും ഉയർന്ന അളവിൽ തേനീച്ച വിഷം ശേഖരിക്കുകയും, തേനീച്ച മരണ ഘടകങ്ങൾ കണക്കിലെടുത്ത് ശേഖരിക്കുന്നതിനുള്ള ഏറ്റവും അനുയോജ്യമായ സമയം 30 മിനിറ്റ് ആണെന്ന് കണ്ടെത്തുകയും ചെയ്തു. കാലാനുസൃതമായ വ്യതിയാനത്തെക്കുറിച്ചുള്ള പഠനങ്ങളിൽ, തേനുൽപ്പാദനകാലത്തിൽ (ഒരു ഹൈവിൽ 55.16 മില്ലിഗ്രാം) ഉയർന്ന തേനീച്ച വിഷം ശേഖരിക്കപ്പെട്ടതായി കണ്ടെത്തി, തേനീച്ചവിഷം ശേഖരിക്കുന്നത് കോളനിയിലെ പുഴുവളർച്ചയെയും പ്രവർത്തനങ്ങൾക്കും വ്യത്യാസമൊന്നും ഉണ്ടാക്കുന്നില്ല എന്നും കണ്ടെത്തി. തേനീച്ച വിഷത്തിലെ പ്രധാന ഘടകങ്ങൾ മെലിറ്റിൻ, അപ്പാമിൻ എന്നിവയാണെന്നും, അതിൽ മെലിറ്റിൻറെ അളവ് അപ്പാമിനേക്കാൾ 8.5 മടങ്ങ് കൂടുതലുണ്ടെന്നും ഈ അളവ് സീസൺ അനുസരിച്ച മാറുന്നില്ലെന്നും പഠനങ്ങൾ വ്യക്തമാക്കി.