IN VITRO CHARACTERIZATION AND ANTHELMINTIC POTENTIAL OF HONEYBEE VENOM ON CERTAIN FISH PARASITES

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SUMMARY

Fishes occupy most aquatic habitats on the planet which are associated with both marine and freshwater, together with transient water features habitats, and some migrate between these biomes, which account for half of the diversity of vertebrates. Fishes are an abundant source of proteins, vitamins A, D, K, and other constituents like carbohydrates, Omega-3 fatty acids, and minerals and are very good food for the poor and contribute much to the economy of India (Royal *et. al.,* 2021). Uttar Pradesh has a large potential for aquatic bio resources, provides a significant opportunity for inland aquaculture development and fisheries, and is one of the largest states with the highest population density. In terms of inland fish production, the state comes in fourth place overall, behind West Bengal, Andhra Pradesh, and Bihar, and makes up around 7.3% of all inland fish produced in India. One of the important sister rivers of the River Ganga, Gomti is a significant groundwater-fed Ganga Plain tributary river, coming from the Pilibhit district's Gomath tal. The city Lucknow is the capital of Uttar Pradesh which is situated on the river bank of Gomti and is home to around 265 different species of fish, including carps, clupeids, siluriods, live fish, prawns, and more. These edible fishes harbor several helminth parasites, which cause deterioration in their health, hence their market and nutritive value are affected.

Aristotle coined the term "helminths" for several of the worms that were discovered in animals as parasites (Hugot *et al.,* 2001). Helminths, as parasites in general, do not represent a monophyletic branch but under that term members of phylogenetically not related phyla are included, i.e., Platyhelminthes (“flatworms”) comprising cestodes, monogeneans, and digeneans; Nematoda (“roundworms”), previously placed in the phylum Nemathelminthes (or Aschelminthes); and Acanthocephala (“thorny-headed worms”). Helminths play a key role in the internal parasite infection of fish which leads to economic loss (Rohde, 1993). Parasites also upset the normal reproduction of the host (Faust, 1940). The infection of Parasites either alone or in conjunction with other stress may reduce host weight and reproduction which leads to economic loss interferes with nutrition, metabolism, and secretory function of the alimentary canal damages the nervous system which may lead to gastrointestinal abrasions and facilitate the invasion by opportunistic micro-organisms. Unfavorable environmental conditions contribute to stress which also weakens immunity and opens the pathway to pathogens.

Helminth infections are common among the major parasitic disease in India and other tropical countries. In India, there is increasing protein demand and fish acts as a cheap source of animal protein. It is known for its protein value, high content of essential minerals, and for being low in saturated fats. Hence, to obtain healthy and good quality fish meat, the fish must be free from all types of pathogens like

bacteria, algae, protozoans, helminths, annelids arthropods, and molluscs. Parasites of fish constitute one of the major problems to fish health. Besides the direct losses caused by mortality, parasites have a considerable impact on growth, resistance to other stressing factors, susceptibility to predation, and marketability and pave way for secondary infection.

Trematodes (flukeworms) are small flat leaf-like bodies and have oral and ventral suckers with a blind sac-like gut and acoelomate type of body cavity. They are dorso-ventrally flattened with bilateral symmetry. Most species of trematodes are mostly hermaphroditic (male and female reproductive systems existed on the same individual) but some species of blood flukes are bisexually dimorphic i.e., male and female adults. Nematodes are slightly flattened with cylindrical body cavity with a pseudo coelomate type of body cavity; hence it is called roundworm and kept in a separate phylum Aschelminthes (Nemathelminthes) which is closely related to Platyhelminthes. The external body part is covered with an outer cuticle layer i.e., a noncellular, chemically complex structure that protects different types of digestive juices which are secreted inside the host body, special types of sensory structure anteriorly known as Amphids and posteriorly Phasmids, a thin hypodermis, and musculature. Tapeworms (cestodes) are endoparasites and their adults live in the digestive tract. They have an indirect life cycle, with fish serving both intermediate and final hosts.

Cestodes infect the alimentary tract, muscle, or other internal organs. Tapeworms are hermaphrodites, meaning they have both male and female reproductive systems. Male organs contain one or more tests, cirri, vas deferens, and seminal vesicles, and female organs include a single lobed or unlobed ovary with the connected oviduct and uterus. The genital pore, which is located at the surface aperture of the cup-shaped atrium, is the common external opening for both male and female reproductive system. The adult stage of cestodes are flattened, ribbon-shaped, elongated, and consists of segments called proglottids. Proglottids are capable of regenerating into a new individual. Morphologically, cestodes are divided into a scolex, or head, which bears the organs of attachment such as suckers and hooks, a neck that is the region of segments and proliferation, and a chain of many proglottids called the strobila. Cestodes have an absent digestive system i.e., exchange of nutrients and waste products taking place through the body wall or integuments with acoelomate body cavity. Indian freshwater fishes contain a group of species from the Eucestoda groups Bothriocephalidea, Caryophyllidea, and Proteocephalidea.

In nature, fish parasites are a common occurrence and can educate us a lot about the ecology of host populations (Abdel-Latif *et al.,* 2020; Sures and Nachev, 2022). In aquatic ecology, for instance, distinct populations of the same fish species that cohabit in one location may be recognized using parasite communities (Runghen

et al., 2021). Additionally, parasites have a variety of distinctive traits and life-history strategies that enable them to live inside hosts. Knowing these fascinating aspects of parasite ecology might help us better understand how hosts defend themselves from parasites (Llopis-Belenguer, Blasco-Costa et al. 2020, Timi and Poulin 2020, Hassanine and Al-Hasawi 2021, Runghen, Poulin *et al.,* 2021).

It is best to integrate several forms of information, such as morphological, ultrastructural, genetic, and morphometric aspects, to fully characterize parasites. As a result, parasite biology focuses on how parasites interact with biotic and abiotic components of their macro- and microenvironments (Poulin and Morand, 2000; Bush *et al.,* 2001; Brooks *et al.,* 2006; Behringer *et al.,* 2018; Raj *et al.,* 2019). Because of the extensive pattern of interactions and the large range of factors that affect parasites in a number of ways depending on the factor at play, investigations are frequently tough and complex. Nevertheless, the number of parasites has increased dramatically in recent years (Barber, 2007; Shamsi, 2019; Poulin *et al.,* 2020).

The common parasites of fishes causing the economic loss include the helminth parasites like *Senga* Dollfus (1964), *Diphyllobothrium* (1758), *Lytocestus* Cohn, (1908), *Spinitectus* Fourment, (1883), etc. in India (Malhotra 1988; Jaiswal and Malhotra, 2017). Molecular characterization of host-specific raphidascaridoid worms from the gangetic garfish (Teleostomi: Belonidae) in India. Int. J. Mol. Biol., 2: 1-15.). Several cases of hyper-parasitism of helminth on helminths

believe that hyper-parasitism is the result of intraspecific competition for space, when intensities of parasite infestation are high (Sey and Moravec, 1986).

Fishes are host to many adult helminths' parasites and larval forms, the adult of which occur in amphibians, reptiles, birds, and mammals as well as predatory fish. At present times, there are only some drugs for the treatment of acute and early chronic phase helminths infected fishes. However, neither of these therapeutic meets the following WHO criteria for an optimal drug such as –

(i) Parasitological cure of the acute and chronic phases of the infection

(ii) Effective with a single dose or with few doses

(iii) No side effects or teratogenic effects, among others

These drugs are limited, highly toxic, and rarely beneficial during the chronic phase of diseases. Moreover, these treatments only cure approximately 20% of all fish (Urbina and Docampo, 2003). These restrictions highlight the necessity for developing alternative synthetic or natural compounds that are effective for the fish disease. Honeybee venom is a composition of Melittin, Phospholipase A2, Hyaluronidase, Acidphosphate, small peptides including the neurotoxin Apamin, Histamine, 5-hydroxytryptamine, acetylcholine, dopamine, and norepinephrine (Schmidt *et al.,* 1986). Melittin is among the most represented AMPs (Antimicrobial peptides) (Yeaman and Yount, 2003)

of HBV. AMPs are a component of innate immunity that show a high level of toxicity against bacteria as well as fungi, viruses, metazoans, other parasites, and even cancer cells (Hoskin and Ramamoorthy 2008; Zasloff 2002; Gajski *et al.,* 2013).

Melittin is a highly basic 26- residue peptide that is almost entirely hydrophobic but with a hydrophilic sequence (Lysine- Arginine-lysine-Arginine) near the C- terminus with a 2846.46 of molecular weight and which comprises 40-50 % of *Apis mellifera* dry weight (Haberman 1972). It is water soluble and its aggregation of monometric Melittin to a tetramer is promoted by high salt, high melittin concentration, and high pH (Raghuraman and Chattopadhyay 2007). There is substantial evidence that melittin can permeabilize cell membranes by inducing pore formation and lyse prokaryotic and eukaryotic cells in a non-selective manner (Rahghuraman and Chattopadhyay, 2007; Papo and Shai, 2003). This mechanism of action is responsible for the hemolytic antimicrobial and anti-tumor activity of Melittin. The melittin peptides have been shown to exhibit strong inhibitory activity against the parasitic protozoan (Akuffo et al.1998; Perez-Cordero et al. 2011) and in recent years, the HBV effect studied in some helminth's parasites. HBV in case of trematodes (*Clinostomum complanatum*, metacercaria larva) disrupt tegumental surface, papillae (Rahman et al. 2017). But apart from a report on the anthelmintic potential of HBV on *Schistosoma mansoni* (Mohammed et al.2014), very limited information is available on the anthelmintic effect of HBV. Therefore,

in the present study, the fish parasites will be used as a model to determine the *in vitro* anthelmintic efficacy of HBV.

Fish are one of the most important economic groups because they provide the general population with animal protein, which is severely lacking. Because it is less costly and more frequently available than other sources of animal protein, fish is a popular protein-rich dietary option for a bigger proportion of the Indian population. Freshwater fish have 13.5-25.2% protein (Philibert *et al.,* 2006; Steffens, 2006; Malik *et al.,* 2021). Fish protein is thought to be very accurate in terms of amino acids when compared to other freshwater animals, and it is thought to have strong digestion and growth-promoting properties. There are two basic sources of fish production in India: culture fisheries and capture fisheries (Katiha *et al.,* 2005; Singh, 2015). Freshwater aquaculture, coastal aquaculture, and mariculture, with intermediate culture systems based on culture-based fisheries and enhanced capture fisheries of reservoirs/wetlands, inland capture fisheries of rivers, estuaries, and lakes, and marine capture fisheries of the open sea, are the primary sources of fish production in India (Katiha *et al.,* 2005; Singh, 2015; Mishra *et al.,* 2017; Giri, 2019).

Bee venom is a natural mixture of proteins, enzymes, phospholipids, and volatile compounds that inhibit microbial and cancerous cell growth (Badria et al. 2017). Female honey bee releases bee venom through a sting, a modified form of ovipositor. Based on its

biomolecular nature, honeybee venom is composed of carbohydrates like Glucose, Sucrose (Abusabbah et al. 2016), proteins, and protein derivative peptides (adolapin, apamin, melittin, mast cell degranulating peptides, protamine, protease inhibitor escapin, and tertiapin), proteinaceous enzymes (alpha- glycosidase, hyaluronidase, phospholipase A2, phosphomonoesterase, and lysophospholipase), amino acids (alpha-amino acid and aminobutyric acid), and other biologically active amines (dopamine, histamine, and nor-epinephrine), lipids (phospholipids) and other interconvertible compounds. Among these compounds, melittin comprises 40-50 % dry-weight bee venom having 2846.46 of molecular weight and contains a group of highly basic amino acids sequences, i.e. (GIGAVLKVLTTGLPALISWIKRKRQQ). These 26- residue peptides show almost entirely non-polarity, with a group of polar amino acid sequences such as (Lysine- Arginine-lysine- Arginine) on the C-terminus.

Crude bee venom also contains antimicrobial peptides that provoke innate defense systems that show a high level of toxicity against noncellular viruses, bacteria, fungi, metazoans, other parasites, and even cancerous cells (Adade et al. 2012). Melittin is a water-soluble antimicrobial peptide, and its monomeric structure aggregates to form a tetramer structure in high salt, high melittin concentration, and high pH by strongly suppressing charges (Raghuraman and Chattopadhyay 2007; Leandro *et al.,* 2015; Rady

et al., 2017). There is evidence that melittin can permeabilize cell membranes by causing hole formation and non-selectively lyse bacterial and eukaryotic cells. Cecropin A, Andropin, and Melittin have been reported for their lytic, cell differential effect, inhibition of promastigote, and antileishmanial agents. Honey bee venom (HBV) in case of trematodes (*Clinostomum complanatum*, metacercariae larva) disrupt tegumental surface, papillae. Apart from reports of the antihelmintic potential of HBV on *Schistosoma mansoni*, very few pieces of information are available on the antihelmintic effect of HBV (Rady *et al.,* 2017).

Since ancient times, bee products such as honey, propolis, wax, pollen, and venoms have been considered traditional medicine. Bee venom therapy or Apitherapy has been established as a branch of alternative medicine to treat various diseases such as rheumatism and arthritis (Badria *et al.,* 2017). Many reports suggest multiple benefits of Apitherapy in the health sector, its urgency to investigate the chemical composition, compound structure, and mode of action of honeybee venom on different drug targets. Recently few decades, the separation and identification of various chemical compounds of honeybee venom have been started with the help of other chromatographic and spectroscopic techniques (Hou *et al.,* 2013; Leandro *et al.,* 2015). To date, high-performance liquid chromatography is used to fast purification and separation of major bee venom compounds, which are costly and tedious. The nuclear

magnetic resonance (NMR) technique has been used in pharmaceuticals and chemistry to determine the purity, structure, and molecular weight of biological fluids, toxins, and various organic compounds. Nowadays, these techniques developed as the first choice of researchers to identify and quantify chemical compositions of desired samples matched with a spectral reference library obtained from pure compounds (Ganguly, *et al.,* 2020). Based on repeatability, ^{1}H-NMR profiling is generally more robust than LC-MS profiling. In the present work, we report the metabolite profiling of crude honey bee venom and their quantitative analysis using ^{1}H-NMR.

The current study attempted to determine how much of an influence parasite infection had on fish as a result of environmental changes, as well as how these parasites should be bio-remediated utilizing important biological compounds or derivatives of plant or animal origin, especially the honey bee venom which may be utilized as potential anthelminthic and as a biocontrol agent. So, in the present study, I aim to identify and characterize different components of Honey Bee Venom and its derivatives if so, to use them further as potential biocontrol agents against harmful parasites inhabiting vertebrate hosts. The following particular goal is to assess the existing level of knowledge on the anthelminthic effects of honey bee venom against parasites of the fish spectrum of the Gomti River. The following objectives will be addressed during the proposed investigations- to characterize the honeybee venom of *Apis mellifera*.

To study the anthelmintic effects of HBV on the biotic potential of fish parasites.

The present study focuses on the two main objectives to fulfill the criteria of the proposed work.

(1) To describe the properties of *Apis mellifera* honeybee venom

(2) To study the biotic potential of fish parasites concerning HBV's anthelmintic effects.

Based on this, the following methodologies have been undertaken to accomplish the goals of characterization of Honey Bee Venom. The samples of Honeybee venom were extracted from the apiary setup of the BBAU campus (Zoology department, BBAU Lucknow), where apiculture techniques were set up for experimental purposes on a small level with 10 boxes, from which 8 boxes were selected which contained a good population of bees, nine number of combs in each of them.

The species used for rearing purposes was *Apis mellifera*. On each sampling day, the bee venom samples were collected, by stimulating the bees colony with the pulses of electric current, using the honeybee venom extractor unit patented as 'An Improved Apparatus for collection of honeybee toxin' (Patent No. 282490) sanctioned in favor of the Department of Biotechnology (DBT) and the University of Allahabad to Jaiswal *et al.,* 2017. The non-sticky nature of the glass plate allowed the venom to dry naturally so that it could be collected in a pure, powdered form without any contamination.

The device principally works in an eco-friendly manner, i.e., neither the use of plastic nor the death of the honey bee occurred during venom extraction. The device was kept at an angle of 45°C at the box entrance, where the bees enter their respective hives. The sample characterized in this study was collected for 30 days, with a regular intervals according to seasonal variations. The unit was used twice a day on four selected boxes in rotation, where after 15 minutes, boxes were being changed. Once the bees were getting the shock, the power supply was stopped for 10 seconds to wait for them to recover and stung on the glass plate. After the process, the glass plate was collected, and the venom was scraped with the help of a scraper or blade, and the collected venom in white powdered form was kept inside a microcentrifuge tube on a daily collection basis, sealed with parafilm, and stored for later use in the experimental set up at ≤20℃ to avoid oxidation.

Venom was extracted by use of an improved apparatus, containing a finely wired mesh cage surrounding a single transparent glass pane; on which a power supply of 8-10 volts current was provided by Voltameter and the device is kept at an angle of 45°C at the entrance of the artificial bee hives. As a result, bees use to get disturbed and for sell self-defense to sting, (produce venom by venom glands) on glass, which was then collected and stored at 20 ◌̊C until used. The non-sticky nature of the glathe ss plate allowed the venom to dry naturally so that it could be collected in a pure, powdered form without any contamination. The non-sticky nature of glathe ss plate

allowed the venom to dry naturally so that it could be collected in the pure, powdered form without any contamination. The unit was used twice a day on four selected boxes in rotation, where after 15 minutes boxes were being changed. Once the bees were getting the shock, the power supply was stopped for 10 seconds, to wait for them to recover and sting on the glass plate or the amount of vomit will be more in the collected venom scrape. After that, the glass plate was collected and the venom was scraped with the causes of a scraper or blade the scrape is kept inside an Eanpendrof or glass vile and is sealed with the help of tape kept in fresh Ezer, to avoid oxidation. Samples of honey bee venom were collected from the apiary of the BBAU campus, near the Department of Zoology, where apiculture is going on a small level with around ten boxes, from which four boxes were selected which were having a good population of bees, with eight number of combs in each of them. The variety which is being reared is *Apis mellifera*. Samples were collected by stimulating the bees with electric current pulses, using the 'Honey bee Venom Extractor Unit'.

The 2 mg sample was dissolved in D_2O (33) and vortexed well to record the NMR spectra. To dissolve the sample, sonicated was done to break the large proteins. Centrifugation at 10,000 rpm for 10 minutes at 40°C was used to remove the precipitants. The sonication and centrifugation of the sample were processed repeatedly two times to assure the breakdown of large proteins. From the above-processed solution, an aggregate of 450 µl of the supernatant was utilized in 5

mm NMR tubes (Wilmad Glass, USA) for information obtaining with a coaxial addition containing 0.1% TSP (sodium salt of 3-trimethylsilyl-(2,2,3,3-d4)- propionic corrosive) as an outer reference standard to help metabolite evaluation using the NMR analyser. Deuterium oxide (D2O; as a co-solvent and to give a deuterium field/recurrence lock) and sodium salt of trimethylsilylpropionic corrosive d4 (TSP) was bought from Sigma-Aldrich (Rhode Island, USA).

NMR study was conducted on Bruker Avance-III 800 MHz at 298 K NMR spectrometer running at a proton recurrence of 800.21 MHz. The NMR instrument was outfitted with CryoProbe with protected most extreme angle strength result of 53 G CM^1. The crude NMR information was obtained on Topspin-3.5 (Bruker Biospin Software). For each HBV test, cross over unwinding altered Carr–Purcell–Meiboom–Gill (CPMG). NMR spectra were obtained utilizing the standard Bruker's heartbeat program library grouping (cpmgpr1d) with presaturation of the water top through illuminating it consistently during the reuse delay (R.D.) of 5 s.

The 10µl was mixed in 1ml of HPLC grade Acetonitrile (0.1% formic acid) and then vortex was done. The slimy sample was brought to filter through a 0.2µm membrane polyvinylidene fluoride (PVDF) filter. After filtration, we got 50 µl amount of sample remaining which was further used. The HPLC system which is being used is Systronics SYS LC-138, which was set at 244 nm, systronics autosampler, systronics isocractic pump was used for sample profiling and sampling. The

system operation data acquisition and analysis were controlled and processed by the software Clarify.exe at Cytogene Research Institute Lab. The chromatographic separation was carried out using a HiQSil C-18 HS column (250 mm X 4.6mm, 5µm pore size). The mobile phase was dissolved in acetonitrile (0.1% formic acid), the injection volume used was 20µl and separation temperature was 25°C and the run time was 20 min.

The study of the anthelminthic effects of HBV on biotic potential of fish parasites was conducted by sample collection of fishes, from selected sampling sites, collection of Honeybee Venom and collection of Parasites. The fishes were selected in the present study were- *Channa punctatus* (Common name- Girai) and *Heteropneutes fossilis* (Common name- Singhi). The samples were collected with the help of local fishermen near the Ghaila Bridge of Gomti River. Live fishes were brought in viable condition to the Environmental and Molecular parasitology Laboratory of the Zoology Department. The sample size included were10 fishes per month of each species.

Fishes were dissected for the extraction of endoparasites. Gills, Liver, Gallbladder, and Gastrointestinal tract were examined on regular basis. Parasites collection from Freshwater fishes *C. punctatus* fish species were dissected by bringing them from Specific Sites Gomti U.P. to be brought to the laboratory for examination in the Department of Applied Animal Sciences, BBAU, Lucknow. The standard/body length of fish was measured by using a centimeters scale. The fish was sacrificed by cervical dislocation. After, measuring

and weighing the fish were dissected by an incision through the mid-ventral longitudinal line. The stomach, intestine, gall bladder, and liver were examined separately for endoparasites. The stomach and intestine were split open to dislodge any parasites attached to the epithelial lining. Sometimes, the epithelial layers of the stomach and intestine were scraped with a scalpel or brush to remove the parasite anchored. The collected parasite was kept in a Petri plate and after collecting a few numbers of the parasite from the same species. Just after collection. PBS and HBV (2mg) solution treatment was given to it.

Morphological characterization of helminth parasites was done by simple staining as well as Scanning Electron Microscopy (SEM) for nematodes, cestodes and trematodes. Anthelminthic effects of Honey Bee Venom on Survivability of parasites in fish. The different groups of parasites were first checked for survivability for control in which maximum survivability takes place in Cestodes (56 Hrs) followed by Nematodes and Trematodes, 28 hrs and 14 hrs respectively. The anthelmintic activity of Honeybee Venom was carried out by the standard protocol (Yadav, AK, et.al. 2011; Pandey, J., et.al. 2018). All the working solutions were freshly prepared before the start of the experiment. 10 actively moving, equal-sized worms were placed in wells, of cell culture plates at room temperature (25˚ C–30 ˚C) containing 0.5 mg/mL, 1 mg/mL, and 2 mg/mL, of Honeybee Venom respectively, in PBS. Three replicates were set for each concentration and observations were made at 10 min, 20 min, 30 min, 40 min, 50

min, and 1 Hrs of the time taken to get paralyzed and finally die for all the worms. After each interval of time, the paralyzed worms were placed in PBS for 30 min for the possible recovery of the parasite motility. Molecular analysis for species-level identification was performed by 18S rRNA gene sequencing and phylogenetic analysis.

The current study was pursued to characterize and evaluate the Honey Bee Venom componenets as a potential use in anthelminth of fish parasites. The present study focuses at the characterization of Honey Bee Venom (HBV) through separation techniques like High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR) imaging; and finding out the anthelminthic properties of honey bee venom and its components through survivability assessment of different cestodes on exposure to honey bee venom at different concentration. In addition, to establish the nature of host- parasite relationship and their habitat study, seasonal variations and population dynamics of freshwater fish, as well as the morphological and genetic characterization of parasites have also been done. In order to species specific characterization of parsites, molecular assessment through rDNA sequencing and phylogenetic analysis has been performed to obtain specific data.

Sample collection of bee venom was done by 'Honey bee venom extractor unit' for 6 days straight and amount obtained was 61mg, which gave an average of 10.1mg/day. The chromatogram of RP-HPLC detected 15 peaks, peak number 1,2,3,4,5,6,7,11,12,13,14 were

overlapping except peak number 8, 9, 10 and 15 which were single. The first elute was at highest peak referred to as peak 1, is at a signal of 902.7mAu took a retention time of 2.6 min and having an amount of 23742.8µg/100ml i.e. 23.7mg/100ml and was determined as Apamin, which is one among the main constituent of honey bee venom. Peak number 2 is at a signal of 183mAu, took a retention time of 3.2min and having an amount of 2.9 mg/100ml and the last component which was being eluted was having an peak number 3 which is at signal of 120mAu, took a retention time of 3.6min and the amount was 2.5mg/100ml. The amount of Elute A, which is detected as Apamin in the sample was relatively high as compared to other components of the honey bee venom sample and it took a retention time of 2.6min which relatively higher than the EluteB and EluteC having a retention time difference of 0.62 min and 0.38 min respectively.

Analysis of Seasonal Variation and Population dynamics of helminth parasites in *Channa punctatus* and *Heteropneustes fossilis* was carried out. In the range of freshwater fish *C. punctatus* for the year 2019, the findings of seasonal fluctuation demonstrate a month-wise maximum frequency of helminths parasites during the months of May, followed by April. This is a sign that the summertime is when infections are most prevalent. While the monsoon season exhibits a varied prevalence rate due to shifting water levels and other associated environmental conditions, the month-by-month helminth prevalence data suggests a decreased infestation in the winter season.

The weight-wise infection prevalence statistics by sex, male had a higher prevalence of helminth parasites than females. Infestation rates were 39.82 %, 32.62 %, 28.12, 46.01%, and 33.33% for male and female hosts, respectively, in the weight ranges of 26, 35-45, 45-54, 55-64, and 64-74 gm. Males had a slightly greater mean. In the range of freshwater fish *Heteropneustes fossilis* from Ghaila Bridge during the months of March 2019 to February 2020, the findings of seasonal fluctuation suggest a month-wise maximum frequency of helminths parasites during the months of May, followed by April. This is a sign that the summertime is when infections are most prevalent. While the monsoon season exhibits a varied prevalence rate due to shifting water levels and other associated environmental conditions, the month-by-month helminth prevalence data suggests a decreased infestation in the winter season.

Males had a higher prevalence of helminth parasites than females, according to data on the weight-wise infection prevalence in *H. fossilis* according to sex from March 2019 to February 2020. Infestation rates for male and female hosts, measured in the weight range of 30-35, 34-40, 40-44, 45-50, 50-55, and >60 gm, were 37 %, 33 %, 40%, 16 %, 36%, and 33 %, respectively. Males had a slightly greater mean percentage intensity than females. The largest fish, measured by length (approximately 12–14 cm), had an infection rate of 41 %, according to the results of the length-wise infection prevalence of helminths in *H. fossilis* from March 2019 to February

2020, while the smallest fish, measured roughly between 10 –12 cm, had an infection range of 7.5 %.

The analysis of parasitic prevalence shows a steep rise in infection of freshwater fish *C. punctatus* in the summer season than the other sampled freshwater fish *H. fossilis,* indicative of a species specific host-parasitic interaction due to the fact that *C. punctatus* is a bottom dwelling fish and is more prone to infestation in terms of its habitat, survival rate and sustainability. A weight-wise highest infection prevalence of helminth parasite in males than the females of *C. punctatus*, similar results were observed in *H. fossilis* wherein the mean percentage intensity was slightly higher in males than in females. In order to preserve the consistency of the experimental process, the weight range over the course of the investigation was kept between 31 and 75 gm, which more or less assisted in drawing a conclusion about a sex-specific parasite infestation pattern. While considering a lengthwise infestation pattern of helminth parasite in freshwater fishes, it was surmised that the prevalence of helminths was highest (100%) in the lengthwise largest fish species of *C. punctatus,* when compared to the smallest fish (30 %). In *H. fossilis*, the highest infection rate of 42 % was observed in the largest fish, while the smaller fish showed the lowest infection range of 7.5 %. The range of length of fish was selected between 10 to 20 cm during the course of study to maintain the experimental standards, which helped in concluding a lengthwise parasitic infestation pattern in these two selected fish species, again indicative of the role of physiological

factors like differential body type, size range, in deciding the susceptibility, survivabilitty and sustainability of a host-parasite relationship and the anthelminthic roles of biological compounds.

There was an increasing trend from winter to summer in water temperature in all the sites. The minimum and maximum temperatures recorded in different stations during different seasons ranged from 15 to 27°C. The pH value was the highest during summer and the lowest during autumn. The maximum pH 8 value was recorded during summer and the minimum was pH 6 during autumn. The temperature and pH levels were the most important abiotic factor that affected the parasites at all life cycle stages along with the seasonal variation. The data obtained through these parameters indicate a positive correlation between water temperature, pH levels and parasitic prevalence found in the fishes of the selected site of Gomti River. Hence, it is established that these abiotic factors affect the parasitic prevalence accordingly and contribute broadly in the population dynamics studies of the parasite in fresh water fish.

The charcaterization of HBV reveals the presence several bioactive compounds such as Apitoxins and other metabolites. Lactate is the conjugate base of lactic acid and is a hydroxy monocarboxylic acid anion generated when the carboxy group is deprotonated. It functions as both a human and an *E. coli* metabolite. Propionate is the source of this compound. It is a rac-lactic acid and 2-hydroxypropanoic acid conjugate base. In the apitoxin, lactate was found in the highest concentration (0.0688 mM) of other metabolites.

In comparison, O-Phosphocholine was found in the lowest concentration (0.0002 mM). Phosphocholine is the parent chemical of the phosphocholine family and is the phosphate of choline. It functions as an epitope, hapten, human metabolite, mouse metabolite, and allergy, among other things. It is a choline phosphate conjugate acid. Due to the water peak from the HBV's overlaid spectra, the zone between 4.70 and 5.10 ppm was eliminated to eliminate the leftover water peak. Then, between 1H chemical shift (δ) values 8.5 and 0.8 ppm, the stacked 1H-NMR spectra signal was segmented into various regions (4.0–3.0, and 3.0-0.8) to obtain characteristic fingerprints for the HBV sample to identify and classify the samples based on chemical composition similarity or dissimilarity. The whole overlaid spectra indicated comparable fingerprints for samples in the 8.45–0.8 ppm value range, indicating likely commonalities in the primary chemical composition.

High Performance Liquid Chromatography (HPLC) showed the identification of 45 metabolites of HBV. HBV primarily consists of amino acids, peptides, and analogs with amines, carbohydrates, and carbohydrates conjugates, purine, and pyrimidine nucleosides, phenols, naphthalenes, lipids derivatives, organic acids, and their derivatives.

Apitherapy is a complementary medicine that uses honeybee products, particularly bee venom, to treat various human ailments. The toxin can be injected into the human body manually or directly by bee stings. Bee venom contains peptides and enzymes that can treat

inflammation and central nervous system illnesses like Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis. Furthermore, bee venom has demonstrated remarkable anti-cancer and anti-viral effects, including against the brutal human immunodeficiency virus (HIV). Many researchers have detailed the biological actions of bee venom components and have started preclinical trials to improve the potential use of apitoxin and its constituents as next-generation medications. The broad-spectrum application of honey bee venom may be due to various bioactive compounds present in apitoxin.

Histidine is a semi-vital amino acid present in the apitoxin in L-histidine, and precursor of histamine. Histamine has granulocytic (Mast cell, Basophils), anti-inflammatory, antioxidant, and vasodilator properties. While, low level of histamine causes Rheumatoid Arthritis (R.A.), anemia, and kidney disease. After one hour, oral and intravenous administration of L- Histidine raises the plasma level. Histidine present in HBV can be used as a better source for food supplements.

Putrescine is a low molecular polyamine synthesized by decarboxylation of ornithine and arginine in eukaryotic and prokaryotic cells (28, 29). Generally, increasing the concentration of putrescin in cell culture triggers the hormonal and agnostic role in DNA replication (29). Various experiments were performed on organisms to know the therapeutical effects. In a study conducted on mammals (Transgenic mice), an experimental modal found that a high

concentration of putreisine accumulation causes neural protection in the brain (30).

In some instances, it has been noticed that exhaustion of putrescine inhibits cellular growth and apoptosis. Trimethylglycine, often known as Betaine, is found in animals, plants, and microbes. Betaine has been shown to act as an osmoprotectant and methyl group donor in the body. According to growing research, Betaine appears to have anti-inflammatory properties in various disorders.

Betaine protects sulfur amino acid metabolism from oxidative stress by inhibiting nuclear factor-B activity and activating the NLRP3 inflammasome, regulating energy metabolism, and reducing endoplasmic reticulum stress and apoptosis. As a result, Betaine has therapeutic effects in various human disorders, including obesity, diabetes, cancer, and Alzheimer's disease (32). Motor and sensory problems result from spinal cord injury (SCI) caused by trauma or illness, depending on the lesion's level, severity, and duration. Paralysis of the lower and upper limbs is the most visible symptom of SCI. SCI also causes a loss of bladder and bowel control, which has a negative, long-term impact on the social, psychological, functional, medical, and economic well-being of those afflicted. There is no cure for SCI, nor is there efficient management of its effects.

Although drugs can help with symptoms like muscle spasms, lower urinary tract dysfunction, and hyperreflexia bowel, techniques for repairing spinal injuries and regaining normal limb and organ

function are still being developed. According to growing preclinical and clinical evidence, inosine is a safe, palatable, and effective therapeutic supplement for reducing the cellular effects of nerve injury, oxidative stress, inflammation, hypoxia, and ischemia-reperfusion. Different mammalian organs, particularly the heart, contain the decarboxylated noradrenaline metabolite 3, 4-dihydroxymandelic acid [DHMA, 2-(3,4-dihydroxy phenyl)-2-hydroxyacetic acid]. The antioxidative and radical scavenging properties of DHMA and its physiological function.

The ^{1}H NMR fingerprinting was performed, wherein an untargeted metabolomics analytic study was performed using Nuclear magnetic resonance (NMR) spectroscopy detection of organic compounds in HBV samples. The resonances associated with different metabolites, together with typical 1D 1H CPMG NMR spectra of samples derived from HBV. Metabolites were assigned using Metabominer software and existing databases and literature reports. The following 1D 1H CPMG spectra of HBV samples revealed lipids/lipoprotein fractions (e.g., HDL/LDL, VLDL/triglycerides, etc.), glucose, amino acids, ketone bodies (e.g., acetone), choline metabolites, N-acetyl-glycoproteins, N-acetyl-containing metabolites, and energy metabolism-related compounds.

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identify and classify the samples based on chemical composition similarity or dissimilarity. The whole overlaid spectra indicated comparable fingerprints for samples in the 8.45–0.8 ppm value range, indicating likely commonalities in the primary chemical composition.

The study of anthelminthic effects of Honey Bee Venom on Survivability of parasitic in fish was also performed. The different groups of parasites were first checked for survivability for control in which maximum survivability took place in Cestodes, followed by Nematodes and Trematodes. The criteria selected for survivability of parasites were the time taken to get paralyzed and finally death of all the worms. However, to rule out any other factors of stress, after each interval of time, the paralyzed worms were replenished in PBS to check for possible retrieval of the parasite motility. Infected fish were exposed to three different concentrations of HBV, i.e., 0.5 mg/ml, 1 mg/ml and 2 mg/ml and the survivability observed was lower, moderate to higher respectively in range to these concentrations. According to our findings, it is established that lower dose of HBV, shows a higher range of survivability pattern, while when we increased the concentration of HBV, survivability declined significantly.

The anthelmintic activity of HBV evaluated *in vitro* against *Senga* spp., of *Channa punctutatus* and results revealed that the minimum concentration of HBV i.e. 0.5 mg, 1mg, 2 Mg have efficacy in causing paralysis as well as the death of the worms at all the

tested concentrations. At the conc. of 0.5 mg/ml of HBV the death of worms takes 35+- 1 min, on the conc. of 1mg/ml and 2mg / ml of the HBV caused 20_+ 1 , 12_+ death occurred respectively. The most proficient anthelmintic activity was exhibited at the concentration of 2 mg/mL at least time was taken for paralysis and finally death of the worms. *In vitro* treatment with HBV were investigated by SEM according to procedure described by Abidi *et al.,(*1998) and it was observed that HBV disrupt membrane integrity of Cestod worm. Disruption of Parasite tegument formed a deep fissure on the integument in some places; on the treatment of HBV to the fluke. This also result in sloughing off and defoliation of integument in some regions and a clear view of the inner membranous layer. It was noted that after HBV treatment fluke remained mortal for 4 minutes on that very day.

Scanning Electron Microscopy (SEM)- indicated the effect of Honeybee venom on parasites Integumental disruption at morphological level-V. The morphological integumental SEM study of Cestode show disruption indicative of sever effect of the honey bee venom treatment. A comparative views of Cestode on different resolution at 2mg/ml concenration of HBV show deatails of Erosion of Scolex part by shrinking of surface tegument of cestode, sloughing off and defoliation of segments of cestode, deep fissure and folding's of surface tegument of cestode, bursting or rupture of gravid segment of cestode with release of innumerable eggs in *C. punctatus*.

During SEM, it was observed that Honey Bee Venom (HBV) used, disrupted the parasite tegument, adversely with a shrinking effect. Therefore, this proves one aspect of HBV anthelminthic and anti-parasitic efficacy. Disruption of parasite tegument formed a deep fissure and numerous big and small folding's on the integument, on treatment of HBV to the fluke. This also resulted in sloughing off and defoliation of integument in some regions and rupture of Gravid segment with release of innumerable eggs from it. It was noted that after HBV treatment, fluke remained mortal for 4 minutes in that very day.

For the species level of identification of parasites, molecular analysis has been performed to get a better picture of habitat and biological interaction of the parasites. A single band of high-molecular weight DNA has been observed. A single discrete PCR amplicon band of 1050 bp was observed when resolved on agarose gel. The sample which was labeled as B was found to be *Senga lucknowensis,* that showed high similarity based on nucleotide homology and phylogenetic analysis. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura 1980). The tree with the highest log likelihood (-489.36) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch

lengths measured in the number of substitutions per site. This analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 353 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Tamura, Peterson et al. 2011). The cestode *Senga lucknowensis showed following characteristic features*- anterior region of the body showing scolex with rectangular bothria, mid-region of the body showing body metamerism, and posterior end of the body showing the presence of eggs.

An important biological group that plays a key role in determining the stability of the aquatic ecosystem's balance is represented by fish. Their sustainability is being threatened by parasites, and any alteration to their environment will be a worrying development. Therefore, in light of the detrimental effects of parasitic impact, research into the population dynamics of both parasites and hyperparasites is necessary in order to develop techniques for their bioremediation. The fish provides a significant portion of the protein required for a nourishing and well-balanced diet. Fish tissues or organs may lose micro or macronutrients as a result of any kind of harm or parasite infestation. In the end, fish parsites can seriously harm fish and reduce fish productivity, which has a big economic impact.

In this view, the need of hour is sutainable anthelminthic drugs which may be a cure for these parasites inhabiting fish. However,

natural resources, either animal based or plant based, and their biochemically active metabolites or compounds, are good answers and treatment options against helminths. One such compound is HBV, which is utilized in the present study to explore about its important components and derivatives to be later used as potential anhelminthics. HBV is used as a best medicinal drugs to cure various disease by identification of, its properties and nature of ingredients which the help of several classical and advanced techniques including HPLC, GC-MS and MS/MS. To date NMR spectrophotometer is used as a newly emerging and cost effective techniques to detection of compounds. Apart from this, honey bee venom is used as a drug to cure various diseases. Identification and quantification of metabolites present in apitoxin are necessary because they possess therapeutic characteristics. Numbers of the report are available for characterization of metabolites found in apitoxin using both chromatographic and spectroscopic techniques.

Naturally produced apitoxin has a group of compounds that have biological and physiological effects on other organisms. Honey bee venom is synthesized as by-products in the posterior part of the ovipositor in female bees, modified into venom glands to protect their colony from enemies. Apart from this, honey bee venom is used as a drug to cure various diseases. Identification and quantification of metabolites present in apitoxin are necessary because they possess therapeutic characteristics. Numbers of the report are available for

characterization of metabolites found in apitoxin using both chromatographic and spectroscopic techniques. In the present work, we have used 1D ^{1}H CPMG NMR to record the spectra of metabolites. In contrast, the Metabominer software was used to assign the spectra and quantification of metabolites of apitoxin. Furthermore, this experiment set a milestone for the standardization of biological fluids.

Honey bee venom is a mixture of metabolites that are useful to mankind and needed to explore more and more, so that one can derive all the hidden benefits that it is carrying. Further studies may unlock some of the solutions to the incurable disease, by using its components in the form of drugs. Finally, it can be claimed that parameters like season, sex, and breeding season have an impact on the prevalence and amount of parasites. Fish gonads and weight are important driving factors when it comes to fish parasite infection. All these factors contribute in analysis of survivability against different concentrations of honey bee venom, which will help in deciding its anthelminthic roles in a fish – parasite interaction. Further, studies will be carried out to characterize new metabolites if so, to find their roles as potential helminthic.

Finally, the following points were compiled in the present study:

- The freshwater parasite-infected fish samples (*Channa punctatus* and *Heteropneutes fossilis*) at the Ghalia place of Gomti River from the Lucknow collected.

- The infected fish parasite were identified as belonging to the cestode *Senga* sp., and some other species trematodes.

- Light and Scanning Electron Microscopy confirmed the presence of these parasites groups.

- Molecular investigation for species specific identification of parasites were carried out using 16s-rRNA sequencing and phylogenetic analysis.

- In addition, research on the seasonal fluctuation in fish parasites give insight into the biological niche of these parasites in the ecosystem as well as their major role in environmental influence.

- HPLC based characterization of HBV was done for using its potential as potential anthelminthic was done.

- In this study, a number of compounds have been identified using 1H NMR in which basically the role of two compounds discussed in this work that influenced the biological reaction in organisms after intake direct or indirect from surroundings.

- In the present work, we have used 1D ^{1}H CPMG NMR to record the spectra of metabolites. In contrast, the Metabominer software was used to assign the spectra and quantification of metabolites of apitoxin. Furthermore, this experiment set a milestone for the standardization of biological fluids.