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HISTOPATHOLOGICAL PROTOCOL TO FIND OUT THE MODE OF ACTION OF *FUNGUS BEAUVERIA* BASSIANA (BALSAMO) PREPARATION ON MIDGUT OF FOURTH IN STAR LARVAE OF *ANOPHELES STEPHENSI* (L.)

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Histopathology, *Beauveria bassiana*, Midgut, *Anopheles stephensi*

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ABSTRACT: The effect of many plant extracts and microbial biopesticides on insects' different developmental stages and physiology have been widely studied from time to time, but the effect of microbial biopesticides on the mosquito tissues, especially mid gut tissues, is very few. Since the malaria vector is one of the most important inclusions in human health and mosquito-borne diseases represent a great health threat in tropical and sub-tropical climates, it is quiet logical to know the detailed mode of action of fungus on *Anopheles stephensi* (L.) larvae after assessing the bio toxicity. In the present study, we have mainly emphasized the midgut of Anopheles stephensi (L.) to understand better the larvicidal action of fungal biopesticide *Beauveria bassiana* (Balsamo). After treatment with the fungus *Beauveria bassiana* (Balsamo), the cytopathic effects of the fungus were widespread along the whole anterior and posterior part of the midgut tissues.

INTRODUCTION: Globally, mosquitoes are one of the most life-threatening insects, as they are major vectors for the cause of several diseases [1, 2]. Mosquitoes (Diptera: Culicidae) are zoonotic vectors answerable for numerous infectious diseases such as filariasis, malaria, and arboviruses [3]. These diseases affect health as well as socio-economic development in tropical and subtropical countries. The Anopheles, are competent vectors, and by their biting, the infection can be easily transferred.

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There are approximately 3500 species of mosquitoes grouped into 41 genera, but of the approximately 530 Anopheles species, only 30–40 transmit malaria in nature. Present mosquito control strategies are principally based on synthetic insecticides. Recurrent use of synthetic insecticides for mosquito control has led to the development and spread of insecticide resistance in *Anopheles* sp. populations and is also affecting non-target organisms as well as humans [4, 5].

The goal of mosquito control in malaria should be, to control mosquitoes in a safe, efficient, and cost-effective manner and, while doing so, prevent damage to humans, animals, land and the natural environment. This task can be accomplished through the integrated vector control approach known as Integrated Vector Management. The

Solonum pseudocapsicum extract revealed many compounds *viz*; n-Hexadecanoic acid, 9-Hexadecenoic acid and 9,12-Octadecadienoic acid (Z,Z)-, methyl ester that were responsible for larvicidal and pupicidal properties [6]. The acute toxicity of some bacterial insecticides against fourth-stage larvae of dengue and zika virus vector *Ae. aegypti* [7]. Also, histological changes in mosquitoes targeted with some bacterial insecticides were assessed by light microscopy studies, evaluating the changes in mid-gut epithelial cells following treatment with LC_{50} of Spinosad, *Bacillus thuringiensis* and *B. sphaericus.*

An entomopathogenic fungus can act as a parasite of insects and kills or seriously disables them. The microscopic spores, conidia of fungi habitually attach to the external body surface of insects. Mycelial extract of various fungi has been reported for their larvicidal, cellulolytic and cytotoxic activity [8]. Under permissive conditions of temperature and (usually high) moisture, these spores germinate, grow as hyphae and colonize the insect's cuticle, eventually, they bore through it and reach the insects' body cavity (hemocoel). The fungal hyphae flourish in the host body cavity, as walled hyphae or in the form of protoplasts (depending on the fungus spp.). After some time, the insect is killed by fungal toxins, and new propagules (spores) are formed in/on the insect if environmental conditions are yet again permissive; usually high humidity is required for sporulation.

Since they are considered natural mortality agents and environmentally safe, there is worldwide interest in using and manipulating entomopathogenic fungi for the biological control of insects and other arthropod pests. The fungus *B.bassiana* was chosen for further studies because it is already in use as an agricultural bio-pesticide. In a follow-up experiment using a mouse model of malaria, the investigators demonstrated that exposure to the fungus caused higher mortality rates in malaria-infected mosquitoes and reduced the percentage of surviving mosquitoes carrying sporozoites in their salivary glands. Furthermore, fungus-infected mosquitoes were less likely to take subsequent blood meals than uninfected mosquitoes [9]. Entomopathogenic fungi produce secondary metabolites, which are highly toxic to the all-stages mosquito life cycle [10, 11]. The present study was carried out to establish the larvicidal properties and histopathological effects of *Beauveria bassiana* (Balsamo) on the fourth instar larvae of *Anopheles stephensi* (L.), the vector of malaria.

MATERIALS AND METHODS:

Biological Material Insects (*Anopheles stephensi* (L.): The larvae of Anopheles were collected from fresh and stagnant water in the Udaipur area and raised in the insectariums under control conditions (average temperature 25-30°C and relative humidity of 60-80 %).

The Anopheles larvae were placed in mosquito net-protected breeding tanks. The larvae were fed on yeast powder, and water was changed on alternate days thereafter; the larvae were collected and put in a crate covered with mosquito netting. The emerging pupae were sorted out and kept in a cage of 35 cm× 30 cm× 30 cm size. Developed adults were fed on blood and 10 % glucose solution soaked in cotton pads. Egg rafts were kept in fresh water for the development of larval instars. Screening of entomopathogenic fungus: Conidia of entomopathogenic fungus *Beauveria bassiana* (Balsamo) were obtained from Maharana Pratap University of Agriculture and Technology Udaipur, Rajasthan (India), and sub cultured on Potato Dextrose Agar (PDA) in the laboratory.

Preparation of the entomopathogenic solution: From the fungus colonies aged 8 to 15 days, conidia are detached and placed in a conical flask containing 500-ml of sterile distilled water, which is sealed to keep away from contamination. To let greatest release of the conidia, the flask is stirred for 30 min by adding 2 to 3 drops of Tween 80 reagents. Meanwhile, the spore concentration is evaluated using a hemocytometer. Type of treatment: In order to carry out this study, a dose of $3.5\times6.4\times10^{11}$ conidia /ml has been used with 100 ml of water (dose determined from bioassay test). Fourth instar Larvae of *Anopheles stephensi* (L.) were introduced in the conidia-treated water. Control individuals were distributed in the same manner and counted as the treated individuals. After 72 h of treatment, larvae were observed for their toxicity and pathological symptoms. These infected larvae were taken out and processed by following standard histopathological protocol.

Control larvae were sampled at the same time intervals and were also fixed. Using the microtome 6µ thick sections were cut and stained in Mallory triple stain and Orange G. These treated sections were compared with untreated controlled sections to observe the effect of the fungus on larval midgut.

RESULT: Many histological changes in insects due to the infectivity with entomopathogenic fungi were revealed using light and electron microscopy by many investigators [12, 13]. In the present study, we have mainly emphasized the midgut of Anopheles stephensi (L.) to obtain a better understanding of larvicidal action of fungal biopesticide *Beauveria bassiana* (Balsamo).

Histoarchitecture of Midgut of *Anopheles stephensi* (L.) (Control): Midgut of *Anopheles stephensi* (L.) possesses a well-preserved layer of columnar epithelial cells lined by basal membrane. The epithelial cells towards the lumen are equipped with brush border and regularly placed microvilli towards the lumen. The epithelial cells are protected by a thin sheet of membrane called peritrophic membrane that is generally secreted by the anterior part of midgut. It may be single or multilayered [14,15].

The entire midgut is divided into two regions, *i.e.*, the Anterior midgut and the Posterior midgut.

Anterior Midgut: The anterior midgut included flatter cells with clear cytoplasm extending along with one-third of the midgut. Depending upon the stages of development, the epithelial cells of the anterior midgut are seen with apical swelling, thereby reducing intercellular contacts well developed peritrophic membrane and lumen. On the haemolymph part, epithelial cells are highly bound with basal lamina **Fig. 1: A, B, C**. The entire section is surrounded by well developed cuticular layer. Gastric caeca encircle the anterior midgut.

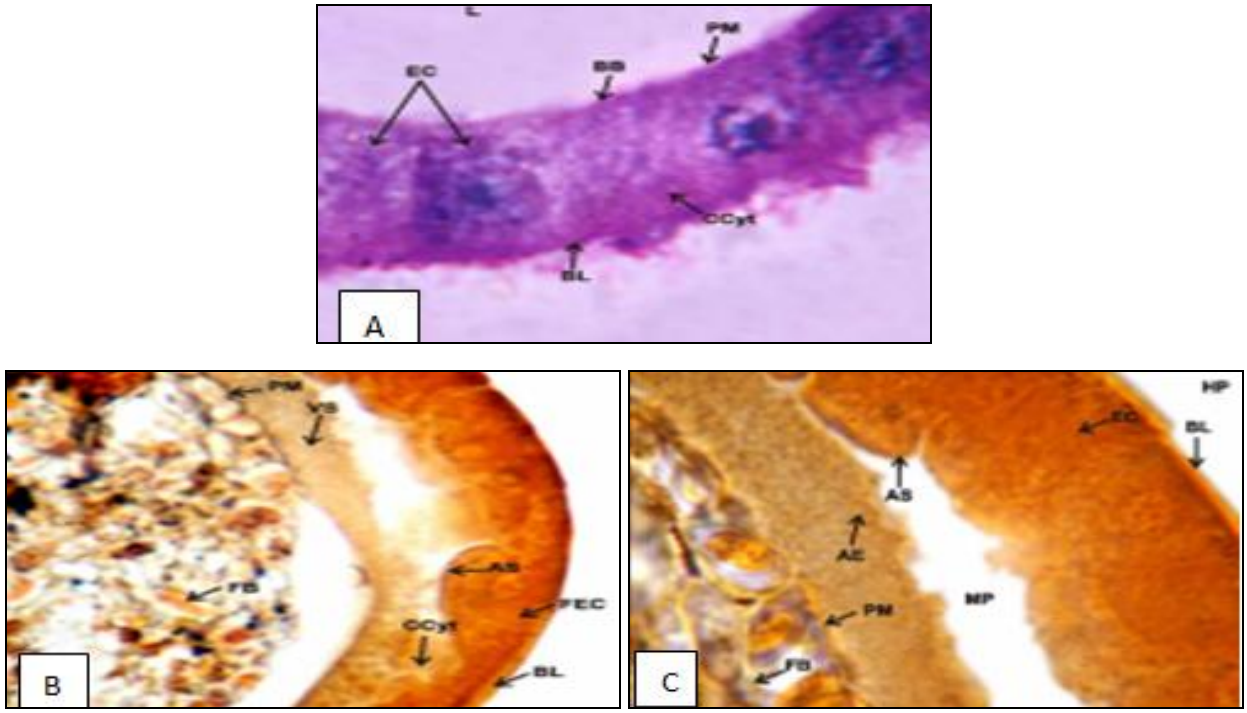

FIG. 1: HISTOARCHITECTURE OF ANTERIOR MIDGUT OF CONTROL FOURTH INSTAR LARVA OF *ANOPHELES STEPHENSI* (L.)

A & B: Enlarged view of few anterior midgut epithelial cells C: Highly magnified view of anterior midgut cells showing apical part of epithelial cells. Food Bolus (FB), Peritrophic Membrane (PM), Apical Extension (AE), Apical Swelling (AS), Midgut Part (MP), Epithelial Cells (EC), Basal Lamina (BL) and Haemolymph Part (HP), Lumen (L), Brush Border (BB), CCyt (Clear Cytoplasm), Vesicular Secretions (VS), Apical Swelling (AS), Flattened Epithelial Cells (FEC) and Basal Lamina (BL) (Stained with Orange G).

Posterior Midgut: Posterior Midgut (Last part) shows dark cells with normal intercellular contacts along the entire lateral plasma membrane, basal lamina, and other structures observed in sections of

control fourth in star larvae. Epithelial cells have well-defined ovoid nuclei at the center of the cells. Well-defined peritrophic membrane and brush border with apical extensions of epithelial cells are seen in normal cross-section **Fig. 2: A, B, C.**

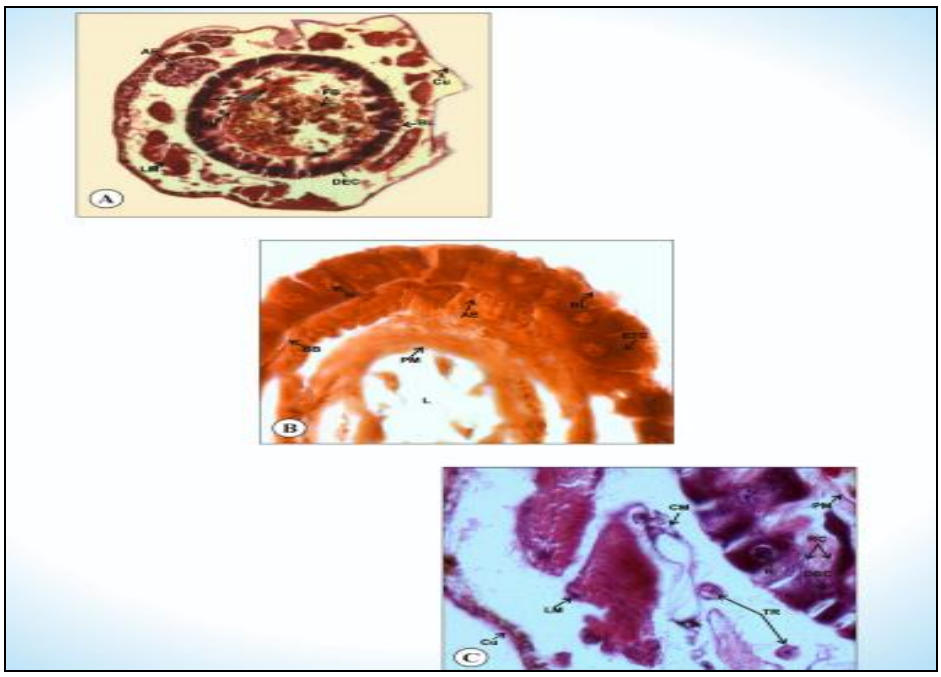

FIG. 2: HISTOARCHITECTURE OF WHOLE BODY SECTION PASSING THROUGH POSTERIOR MIDGUT OF CONTROL FOURTH INSTAR LARVA OF *ANOPHELES STEPHENSI* (L.)

A: Transverse section passing through posterior midgut showing dark-colored midgut epithelial cells with well-defined areas.

B & C: Enlarged view of posterior midgut cells of fourth instar larva showing well-developed nuclei, regenerative cells, and peritrophic membrane of fourth in star larva (×100). Peritrophic Membrane (PM), Regenerative Cells (RC), Dark Epithelial Cells (DEC), large Nucleus (N), Food Bolus (FB), Brush Border (BB), Basal Lamina (BL), Adipose fabric (AF), Lumen (L), Apical Extension (AE), Epithelial Tall Cells (ETC), Tracheole (TR), Circular Muscles (CM), Longitudinal Muscles (LM) and Cuticle (Cu).

Cytopathological Changes in the Midgut: After treatment with fungus *Beauveria bassiana* (Balsamo), the cytopathic effects of fungus was widespread along the whole of anterior and posterior part of midgut tissues **Fig. 3: A, B.**

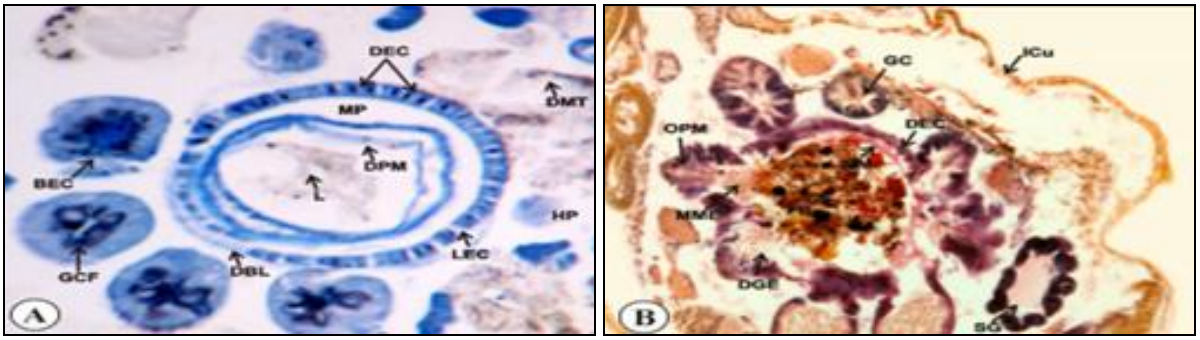

FIG. 3A & B: HISTOARCHITECTURE OF WHOLE BODY SECTION PASSING THROUGH ANTERIOR MIDGUT OF FOURTH INSTAR LARVA OF. *Anopheles stephensi* (L.) treated with LC_{50} of beauveria bassiana (balsamo). Lumen (L), Degenerating Peritrophic Membrane (DPM), Midgut Part (MP), Lysis of epithelial Cells (LEC), Detached Basal lamina (DBL), Bursting of Epithelial Cells (BEC), Gastric Ceacea with Fungal spores (GCF), Degeneration of Muscular tissue (DMT) and Haemolymph Part (HP), Food Bolus (FB), Peritrophic Membrane (PM), Mingling with Midgut Epithelial Cells (MME) with lumen, Degenerating Epithelial Cells (DEC), Damaged gastric Caeca and Epithelial Cells (DGE), Out pockets of Merged Epithelial Cells (OPM), Gastric Caeca (GC), Salivary Gland (SG) and Infected Cuticle (ICu)

Anterior Midgut and Gastric Caeaca: The cell lysis at every level *i.e.*, right from brush border to gut lumen was observed. The epithelial cells of the anterior midgut became small with intense cytoplasmic vacuolization, contrast with those of control and nuclear degeneration also started. Only few cells exhibited brush border and basal lamina, epithelial midgut cells started diminishing intracellularly, and adjacent cell attachments were also lost. The brush border and peritrophic membrane started degenerating and forming space in between compared to control. Epithelial cells of gastric caeca lost their identity, and the whole area from caeca to gut lumen became continuous, showing mixing of all the tissues showing deposition of conidiospores all over the body and lumen. Large vacuolar hypertrophy at the central region of anterior midgut was also observed. Whole epithelial lining degenerated, showing beach of cellular lysis and cytoplasmic rejection could be seen mixing with food bolus at center during advanced stage **Fig. 4 A & B.**

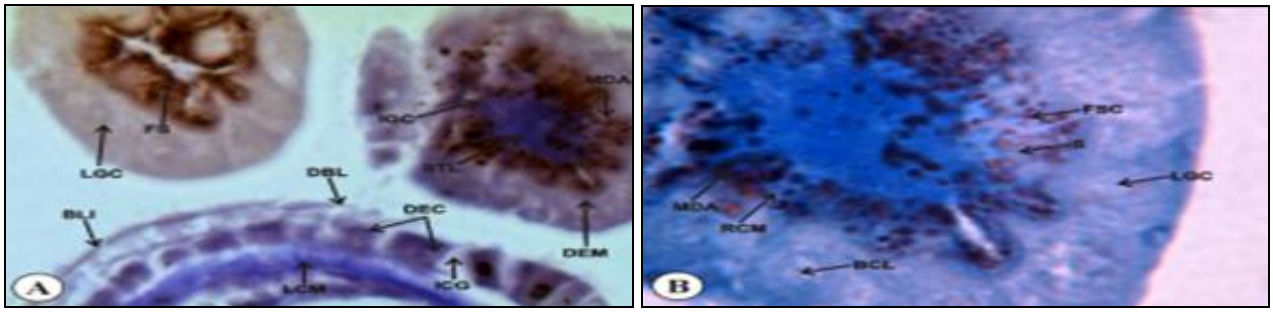

FIG. 4A &B: HISTOARCHITECTURE OF WHOLE-BODY SECTION PASSING THROUGH ANTERIOR MIDGUT SHOWING GASTRIC CAECA OF FOURTH INSTAR LARVA OF *ANOPHELES STEPHENSI* (L.) TREATED WITH. LC_{50} OF *BEAUVERIA BASSIANA*(BALSAMO). Lysed Cell Membrane (LCM), Intercellular Gap (ICG), Disintegrating Epithelial Cells (DEC), Damaged Basal lamina (DBL), Basal Lamina Invasion (BLI), Shrinking of Tissue in Lumen (STL), Infected Gastric Caeca (IGC), development of Melanized Dark Areas (MDA), Lysed Gastric Caecal Cells (LGC) and fungal Spores (FS) Spores (S), Fungal spores are infecting Central part (FSC), Rotten Cellular Mass (RCM), development of Melanized Dark Area (MDA), Beach of Cellular Lysis (BCL) and Completely Lysed Gastric Caeca (LGC).

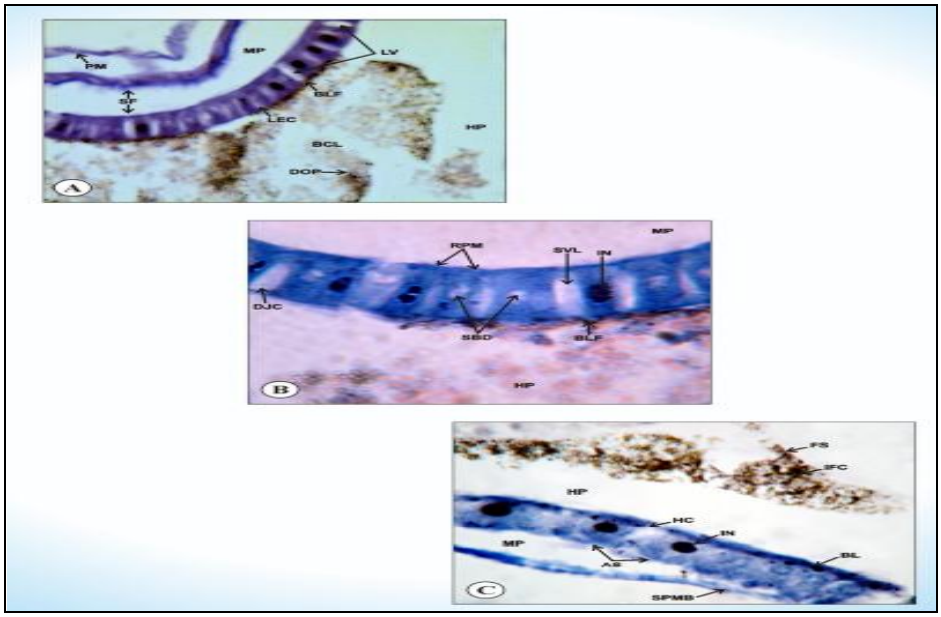

FIG. 5A, B & C: HISTOARCHITECTURE OF WHOLE-BODY SECTION PASSING THROUGH POSTERIOR MIDGUT OF FOURTH INSTAR LARVA OF *ANOPHELES STEPHENSI* (L.) TREATED WITH. LC_{50} OF *BEAUVERIA BASSIANA*. Peritrophic Membrane (PM), Space Formation (SF), Midgut Part (MP), Large Vacuolisation (LV), Lysis of Epithelial cells (LEC), Basal Lamina Loaded with Fungal Spores (BLF), Beach of Cellular Lysis (BCL), Damaged Outer Part (DOP) and Haemocoel Part (HP), Reduced Peritrophic Membrane (RPM), Secretory Vacuoles becoming Large (SVL), infected Nucleus (N), Disruption of Junctional Complexes (DJC), Spherical Bodies due to Disruption of Cytoplasm (SBD), basal lamina Loaded with Fungal Spores (BLF) and Haemocoel Part (HP). Seperating Peritrophic Membrane From Brush Border (SPMB), Midgut Part (MP), Apical Swelling (AS), infected Nucleus (N), Hypertrophied Cells (HC), Basal Lamina (BL), Haemocoel Part (HP), Infected Fat Cells (IFC) and Fungal Spores (FS) (×100).

Major Cytotopathological Effects of the Fungus on Posterior Midgut (Fig 5): The basal lamina revealed deposition of conidiospores above the surface. The large vacuolization in the cells was also prominent. The brush border detached from the apical part of epithelial cells in contrast to control larvae. Hypertrophy and swelling of columnar epithelial cells with large vacuoles and hypertrophied nuclei, conidiospores exhibited heavy deposition on the disintegrating basal lamina. The intra junctional complexes started disintegrating, peritrophic membrane and exudate of epithelial cells, migrated towards lumen. Cells began to swell with vacuolization and disorganized brush border and peritrophic membrane, towards haemolymph side beach of lysed tissues infected with conidiospores were quite significant. The complete cell cytoplasm was flooded with fungal spores giving a spongy appearance. Basal lamina became coarse with invasive conidia; epithelial cells revealed a spongy appearance due to the lysed mass of cells; no intracellular junctions were visible. Hemolymph revealed no identification of any tissues (beach of lysis). It was full of conidiospores, and these spores were seen migrating towards the basal lamina and entering through space into the lumen.

Epithelial cells detached from basal lamina degenerated, and vacuolated nuclei disintegrated by showing atrophied nature. At the last and advanced stage of infection, the midgut layer lost its identity and mixed gut contents with haemolymph, and rejection of cytoplasmic contents towards lumen started. The fungus completely outgrew the different cells. The complete haemolymph area revealed debris of tissues with infected fungal conidiospores, and the midgut area lost its complete identity. Due to dissolution of cells and heavy infection, the midgut area shrank considerably. Whatever lysed infected tissues were left, they could be seen migrating towards lumen. The gut lumen became obliterated with infected lysed remains of alimental flow, brush border and peritrophic membrane. The cells were seen dislodged, sloughed, and detached leaving a lysed mass in the gut lumen.

DISCUSSION: The present study was carried out to establish the larvicidal properties and histopathological effects of *Beauveria bassiana* (Balsamo) on the fourth instar larvae of *Anopheles stephensi*(L.), the vector of malaria. The effect of many plant extracts and microbial biopesticides on insect's different developmental stages and their physiology has been widely studied from time to time, but the effect of microbial biopesticides on the mosquito tissues, especially midgut tissues, are very few. Since the malaria vector is one of the most important inclusions in human health and mosquito-borne diseases represent a great health threat in tropical and sub-tropical climate[16], it is quite logical to know the detailed mode of action of fungus on *Anopheles stephensi* (L.) larvae after assessing the bio toxicity. The *Bacillus licheniformis* exopolysaccharide treatment on 4th instar larvae of *An. stephensi* and *Ae. aegypti* and found different histological changes such as the gastric caeca, muscles shrinkage, damaged and disorganized nerve cord ganglia [17]. Earlier, studied the *B. bassiana* metabolites toxicity on 4th instar larvae of *Cx. pipiens* and observed many histological changes and malformation in the treated larval body and tissues, especially in the adipose cells, cuticles and midgut [18]. Also reported, the midgut cells of tested mosquitoes (4th-instar larvae) had swelling in the gut lumen, reduced intercellular contents, and degeneration of nuclei after treated with *P. daleae* mycelium extract[19].

Insect pathogenic fungi are a positive substitute to chemical control against mosquitoes[20]. Fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* (Vuillemin), commonly found in soil and on terrestrial insects, can also successfully kill mosquitoes [21]. In a previous study, ar-turmerone, a sesquiterpenoid ($C_{15}H_{20}O$) extracted from *C. longa*, has demonstrated significant larvicidal activity against the fourth instar larvae of *Culex pipiens pallens*, with a LC_{50} value of 138.86 ppm [22, 23]. This LC_{50} value is higher than that for organophosphate insecticide Temephos, which is commonly recommended by WHO for the larvae control of a range of mosquito species. However, a number of reports indicate the emergence of mosquito resistance to Temephos [24].

In the present study we examined the bio toxicity of different concentrations of *Beauveria bassiana* (Balsamo) against different developmental stages and established the toxic effects of fungus on insects. Since any pathogen or chemical mode of

action is one of the important aspects of establishing any new drug in the system, we studied the cytopathological effects of fungus on the LC50 treated fourth instar larvae *Anopheles stephensi* (L.). The midgut of the mosquito is able to carry out major physiological processes, *i.e.*, synthesis, secretion, absorption, and transport [25], it was reasonably thought to take up this part to see the exact cytopathological changes in the larval body after exposing them to fungal biopesticides. Further, it is a fact that fungus attaches on chitin [26,] and chitin is one of the basic platforms for insects that makes these tiny creatures on top. It was interesting to see the effect of fungus on extracurricular structures.

The midgut of Dipteran larvae has been subdivided into two different regions with thin characteristic cell type [27]. The anterior midgut has small, flatter cells extending one-third of the midgut and surrounded by gastric caeca. In contrast, the posterior part of the midgut reveals long dark columnar cells with intercellular contacts, normal nuclei, brush border, and peritrophic membrane towards the lumen side and adhesive basement membrane towards haemolymph side [28].

When treated with *Beauveria bassiana* (Balsamo), the larvae developed dramatic changes in the epithelial cells. The cells started showing lesions and degeneration. The apical part started swelling, and distinct cell flow towards lumen could be seen. The microvilli and brush border became feeble and thin and seen dislodged and detached from the place. [29] observed in *Ostrimia nubilalis* the same type of changes and suggested that enzymatic activity due to fungal attack might be the reason of such lytic changes in the tissues. The gastric caeca revealed total degeneration.

The epithelial cells have been destroyed with seriously damaged cellular components of the midgut wall. At places, out pocketing of midgut, thereby mixing of whole contents, was also observed. Effect of Neem products on caecum and gut of *Aedes aegypti* have been observed by [30]. The mosquito larval midgut region that most resembles the caterpillar midgut is like gastric caeca. It has eight caecal chambers in mosquito midguts and may be functionally equivalent to the goblet cells of the caterpillar. Maximum alkalization was observed in anterior part of midgut [31]. The damage of these areas of gastric caecae may be due to the ambient pH for fungal action. Further histopathological studies of gastric caeca were also important because this part of the midgut is directly in contact with the toxic elements [30]. Whereas the normal gastric caeca were with well-developed epithelial cells and nuclei, the treated larvae of *Anopheles stephensi*(L.) revealed the sign of intoxication, slowly proving the fungus toxicity from the anterior part of midgut. The other structure of the anterior midgut revealed the severe sign of fungal intoxication. The vacuolization and hypertrophy of cells increased, and cells of epithelium were lysed completely.

The extreme intoxication caused the bursting of some of the cells into lumen. The midgut is that part of the alimentary canal in which the cells secrete digestive enzymes, absorb nutrients, and play an important role in ion transport [32]. Hence, the damaged cells of the midgut led to the formation of weak larvae, which was well evident from morphogenetic changes. Further, our observations at various levels revealed that damage to different parts of the midgut was sequential.

The changes started first in anterior midgut with lysis of clear epithelial cells, an increase in apical swelling of the cells, and the lysis was further increased was evident from bursting of epithelial cells into the gut lumen, and gastric caeca. Anterior midgut further revealed detachment of basal lamina on one side and peritrophic membrane and brush border towards lumen side. At the same time, partial lysis of midgut cells started. The epithelial junctional complex degenerated.

Cells were hypertrophied, and nuclear lyses were well evident at the end; all cells were completely necrotized with a detachment of cells and epithelial cells along with basal lamina and peritrophic membrane and became an aggregation of lysed mass, thereby losing their complete identities. The mixing of gut contents with haemolymph was further enhanced. The cells became vacuolar, dislodged, sloughed off, and detached from one other, and the midgut was apparently vacuolated in structure. The insect midgut is the site of nutrient uptake and is the first line of defense against pathogens and toxins. It is further the largest

epithelial organ system and is the main site of uptake of ingested ions, water, and nutrients [33].

Further, the midgut is the main target organ for many xenobiotics, including dietary substances and pathogens [34]. This is not surprising owing to the major role of midgut in absorption. Histological studies revealed fungus invasion through midgut anterior part causing spore germination and utilization of alimentary tissues. These resulted in degeneration of epithelial cells and lysis of basal lamina. Presences of fungal spores at the core of gastric caeca were significant evidential reports of fungus attack on midgut cells. Gut invasion by conidia and the initial stage of germination of conidia were observed on *Culex quinquifaciatus* treated on filter paper [20].

Entomopathogenic fungi produce fungal toxins. The midgut is that part of the alimentary canal that secretes digestive enzymes and absorbs nutrition, and fungus infection blocks both of these vital phenomenons of insects. This was evident by the loss of microvilli and brush border in the treated larvae. The well-defined brush border lining in control suggested physiologically active cells involved in both secretion and absorption of food [35]. These features are lost gradually in treated insects. The important peritrophic membrane of the posterior midgut revealed complete damage, and the mixing of gut contents and midgut lysed cellular mass was apparent at the last stage of infection. A peritrophic membrane separates food from midgut epithelium[36,37].

Our observations could observe a slight difference in the mode of attack of fungus in the anterior and posterior midgut. In the anterior region, the cellular damage was not as severe as in the posterior part. Abrasion of peritrophic membrane, the beach of lysed tissues, atrophied cells, and necrotisation were more pronounced in the posterior part of the midgut than the anterior part. The reason may be analyzed in the light of the fact that when contracting the midgut the pathogens are at a critical point of their life cycle. They are present in relatively low numbers since they are present in low numbers as they have not yet had the chance to multiply in insects' bodies. Hence the pathogen may be particularly susceptible at midgut surface, and also that midgut may represent the best opportunity to interfere with the transmission of mosquito-borne diseases[38].Hence the start of the life cycle of fungus after invasion and establishment on tissues were seen by dystrophy of the anterior midgut and the spread of infection from anterior to posterior by the proliferation of fungus and spore formation resulted in severe loss of cellular architecture, that was seen in the posterior midgut. The effect on the two different regions of the midgut clearly assists in the differences in vulnerability between clear and dark cells correlated with their morphofunctional status. Clear cells of anterior mid gut appear to be the first target of intoxication [34]. For our convenience and to observe the entry of fungus and its pathways, we divided the entire body section into two parts *i.e.*, haemolymph part containing cuticle, fat bodies, sections of tracheole, longitudinal and circular muscle fibers and ending at basal lamina from where the midgut starts having midgut epithelium, brush-border microvilli, peritrophic membrane and gut lumen with food bolus. The cuticle of treated larvae also exhibited total loss of architecture. Distinctions between epicuticle, exocuticle, and endocuticle were not evident.

The fungal spores were found to settle in large number on the cuticle. However, no apposporium (mechanical entry) could be seen on the body wall but hyphal body invasion dissolving cuticle and epidermis were apparent. The germination of conidiospore in whole haemolymph area, leaving all tissues lysed as if rotten mass of cells provided substratum for fungal growth. Germination of conidia on integument was similar to that reported for *H.zea* [39] and *Odontotermes obesus* [40].

Entomopathogenic fungi have been shown to produce proteases, chitinases, and lipases to break down the insect cuticle. Once inside the insect, the fungi invade the haemocoel, producing toxins that aid in overcoming the insect’s body before infection of organs begins. Many of these toxins are dipeptides which contain proteases. Toxins are thought to be responsible for host mortality. Sporulating insect cadavers can serve as an inoculum reservoir in periods of adverse conditions. Infected cadavers will not sporulate at low levels of humidity, but the hyphae will emerge from the cadaver and sporulate as humidity levels increase. Conidia's dispersal takes place by wind,

water, or insect movement [41] . Once fungal isolate had successfully penetrated into the haemocoel and transformed into hyphal bodies, there was no recognition by haemolymphopsonins or haemocyte (blood cell) surface receptors and no phagocytosis by circulating haemocytes [42]. However, to sum up, the treatment to mosquito larvae with fungal concentrations with immersion method indicated that spores enter both ways i.e. through the cuticle and through the midgut.

CONCLUSION: Numerous studies have been conducted to examine natural products' bioefficacy against mosquitoes. The application of the entomopathogenic fungus *B. bassiana* on the larvae of the 4th stage of *A. stephensi* induces disturbances in all parts of the body, including the structure of the midgut, observed the effectiveness of this fungus towards the larvae of the mosquito *A. stephensi.* This study revealed the histopathological modification in *A. stephensi* after infection with *B. bassiana* and gave a better understanding of the mode of action of entomopathogenic fungus *B. bassiana* used as bioinsecticide against mosquito larvae. These results recommend the opportunity of carrying out further experiments on a larger scale to confirm the results obtainable in this study.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

REFERENCES:

1. Benelli G and Romano D: Mosquito vectors of Zika virus. Entomol. Gener 2017; 36: 309–318. doi: 10.1127/entomologia/2017/0496
2. Ward M and Benelli G: Avian and simian malaria: do they have a cancer connection? Parasitol Res 2017; 116: 839–845. doi: 10.1007/s00436-016-5352-3
3. Mullen G and Durden L: Medical and veterinary entomology.2nd ed. Academic Press 2009; 637.
4. Riveron JM, Tchouaku M, Mugenzi L, Menze BD, Chiang MC and Wondji CS: Insecticide resistance in malaria vectors: an update at a global scale," in Towards Malaria Elimination: A Leap Forward, eds S. Manguin and V. Dev (London: Intech Open Limited) 2018; 150–175.
5. Liu H, Xie L, Cheng P, Xu J, Huang X, Wang H, Song X, Liu L, Wang H and Kou J: Trends in insecticide resistance in Culex pipiens pallens over 20 years in Shandong, China. Parasites Vectors 2019; 12: 167.
6. Jeyasankar A and Chinnamani T: Larvicidal and pupicidal activities of *Solonum pseudocapsicum* fruits compounds against *Aedes aegypti*, Anopheles stephensi and *Culex quinquefasciatus* (Diptera: Culicidae). J Infect Dis Med Microbiol 2018; 2: 11–16.
7. Mahyoub Jazem A, Al-Ghamdi Khalid M and Barnawi Abdulaziz BM: Toxicological and Histological Effects of Selected Bacterial Biopesticides on *Aedes aegypti* (L.), The Vector of Dengue in Saudi Arabia. Research Journal of Biotechnology 2018; 13: 9.
8. Fahd AA: Larvicidal, ovicidal activities and histopathological alterations induced by Carum copticum (Apiaceae) extract against Culex pipiens (Diptera: Culicidae). Saudi J Biol Sci 2018; 25: 52–56. doi: 10.1016/j.sjbs.2017.02.010
9. Blanford S, Chan BHK, Jenkins N, Sim D, Turner RJ, Read AF and Thomas MB: Fungal pathogen reduces potential for malaria transmission. Science 2005; 308: 1638-1641.
10. Vivekanandhan P, Karthi S, Shivakumar MS and Benelli G: Synergistic effect of entomopathogenic fungus Fusarium oxysporum extract in combination with temephos against three major mosquito vectors. Pathog. Glob. Health 2018; 11237–46.
11. Vivekanandhan P, Swathy K, Kalaimurugan D, Ramachandran M and Yuvaraj A: Larvicidal toxicity of Metarhizium anisopliae metabolites against three mosquito species and non-targeting organisms. PLoS ONE 2020; 15: 0232172.
12. Khaleil ME, Mougith A, Hashem H and Lokma N: Biocontrol Potential of Entomopathogenic Fungus Trichoderma Hamatum against the Cotton Aphid Aphis Gossypii. J Environ Sci Toxicol Food Technol 2016; 10(5): 11-20.
13. Ragavendran C, Mariappan T and Nataraja D: Larvicidal Histopathological Efficacy of Penicillium daleae against Larvae of Culex quinquefasciatus and *Aedes aegypti* Plus Biotoxicity on *Artemia nauplii* a Non-target Aquatic Organism. Front. Pharmacol 2017; 8: 1-14.
14. Lehane MJ: The formation and histochemical structure of the peritrophic membrane in the stable fly, *Stomoxys calcitrans*. J Insect Physiol 1976; 22: 1551-57.
15. Lehane MJ: Biology of Blood-Sucking Insects. London: Chapman & Hall 1991; 288.
16. Ravindra J, Eapen A and Kar I: Evaluation of repellent action of neem oil against the filarial vector Culex quinquefasciatus Diptera: Culicidae. Indian Journal of Malariology 2002; 39: 13-17.
17. Abinaya M, Vaseeharan B, Divya M, Vijayakumar S, Govindarajan M and Alharbi NS: Structural characterization of Bacillus licheniformis Dahb1 exopolysaccharide antimicrobial potential and larvicidal activity on malaria and Zika virus mosquito vectors. Environ Sci Pollut Res Int 2018; 25: 18604–18619. doi: 10.1007/s11356-018-2002-6
18. Farida B, Sonia H, Hakima MK, Fatma B and Fatma H: Histological changes in the larvae of the domestic mosquito *Culex pipiens* treated with the entomopathogenic fungus *Beauveria bassiana*. Sci Res Essays 2018; 13: 1–10. doi: 10.5897/SRE2017.6544
19. Ragavendran C, Mariappan T and Natarajan D: Larvicidal, histopathological efficacy of *Penicillium daleae* against larvae of *Culex quinquefasciatus* and *Aedes aegypti* plus biotoxicity on *Artemia nauplii* a non target aquatic organism. Front Pharmacol 2017; 8: 773. doi: 10.3389/fphar.2017. 00773

20. Seye F, Faye O, Ndiaye M, Njie E and Afoutou JM: Pathogenicity of the fungus Aspergillus clavatus isolated from the locust *Oedaleus senegalensis* against larvae of the mosquitoes *Aedes aegypti* Anopheles gambiae and *Culexquin quefasciatus*. Journal of Insect Science 2009; 9(57): 7.
21. Silva RO, Silva HH and G. Luz C: Effect of Metarhizium anisopliae isolates originating from soil samples of the Central Brazilian Cerrado against *Aedes aegypti* larvae under laboratory conditions. Revista de Patologia Tropical 2004; 33: 207-216.
22. Liu J, Zhang M, Fu W, Hu J and Dai G: Efficacy of bioactive compounds from *Curcuma longa* L. against mosquito larvae. J Appl Entomol 2018; 142: 792–799.
23. Jia Liu, Diana Fernandez, Yanjin Gao, Silvie Pierre, Yongdong Gao and Guanghui Dai: Enzymology, Histological and Ultrastructural Effectsof Ar-Turmerone on *Culex pipiens* pallens Larvae. Insects 2020; 11(6): 336; https://doi.org/10.3390/insects11060336
24. Daniella G, Christelle D, Andric G, Cédric R, Thierry G, Frédéric F, Jean-Philippe D, Anubis VR and Florence F: Levels of insecticide resistance to deltamethrin, malathion, and temephos and associated mechanisms in Aedes aegypti mosquitoes from the Guadeloupe and Saint Martin islands (French West Indies). Infect Dis Poverty 2017; 6, 38.
25. Hecker H: Structure and function of midgut epithelial cells in culicidae mosquitoes (Insecta, Diptera). Cell and Tissue Research 1977; 1843321-341.
26. Murad AM, Naronha EF, Miller RNG, Costa FT, Peraira CW, Mehta A, Caldas RA and France OL: Proteomic analysis of Metarhizium anisopliae secretion in the presence of the insect pest Callasobruchus maculate. Microbiology 2008; 154: 3766-3774.
27. Rey D, Pautou MP and Meyran JC: Histopathological effects of tannic acid on the midgut epithelium of some aquatic dipteral larvae. Journal of Invertebrate Pathology 1999; 73: 173-181.
28. Mehmadi-Al RM and Al-Khalaf AA: Larvicidal and histological effects of Melia azedarach extract on Culex quinquefasciatus Say larvae Diptera: Culicidae. Journal of King Saud University Science 2010; 22: 77–85.
29. Sutter G and Raun ES: Histopathology of European corn borer larvae, treated with Bacillus thuringiensis. J Invertbr Path 1967; 9: 90–103.
30. Ndione RD, Faye O, Ndiaye M, Dieye A and Afoutou JM: Toxic effects of neem products *Azadirachta indica* A. Juss on *Aedes aegypti* Linnaeus 1762 larvae. African Journal of Biotechnology 2007; 6(24): 2846-2854.
31. Zhuang Z, Paul J, Linser and William R, Harvey: Antibody to H+ V-atpase subunit E colocalizes with portasomes in alkaline larval midgut of a freshwater mosquito (*Aedes aegypti* (L). The Journal of Experimental Biology 1999; 202: 2449–2460.
32. Cioffi M: The morphology and fine structure of the larval midgut of a moth Manduca sextain relation to active ion transport. Tissue Cell 1979; 11: 467-479.
33. Clark TM, Koch A and Moffett DF: The anterior and posterior 'Stomach'regions of larval *Aedes aegypti* midgut: regional specialization of ion transport and stimulation by 5-hydroxytryptamine. J Exp Biol 1999; 202: 247-252.
34. Rey D, Long A, Pautou MP and Meyran JC: Comparative histopathology of some Diptera and Crustacea of aquatic alpine ecosystems after treatment with Bacillus thuringiensis var israelensis. Entomol Exp Appl 1988; 88: 255-263.
35. Ferreira C, Ribeiro AF and Terra WR: Fine structure of the larval midgut of the fly Rhynchosciara and its physiological implications. J. Insect Physiol 1981; 27559-570.
36. Lehane MJ: Peritrophic matrix structure and function. Annu. rev. Entomol 1997; 42: 525-50.
37. Anjana Intodia, Arti Prasad and Bharati Veerwal: Histopathology of Beauveria bassiana (balsamo) vuillemin, an entomopathogenic fungus, infection in the midgut of termite, *Odontotermes obesus* (r.) Worker. International Journal of Recent Scientific Research 2019; 08: 34326-34330.
38. Billingsley PF: Vector-parasite interactions for vaccine development. Int J Parasitol 1994; 2453-58.
39. Mohamed KA, Sikorowski P and Bell JV: Histopathology of Nomuraea rileyi in larvae of Heliothis zea and *in-vitro* enzymatic activity. J Invertebr Pathol 1978; 3: 1345-52.
40. Intodia Anjana, Prasad Arti and Veerwal Bharati: Histophathological protocol to find out the mode of action of fungus *Beauveria bassiana* (balsamo) vuillemin preparation on cuticle of termite worker of *Odontotermes obesus* (r.). International Journal of Scientific Research and Reviews. 2019; 8(2): 2498-2509.
41. Roberts DW and Humber RA: Entomogenous Fungi. In: Biology of Conidial Fungi (Cole GT and Kendrick Beds.). Academic Press New York 1981; 201-236.
42. Pendland JC, Heath M and Boucias DG: Function of a galactose –binding lectin from Spodoptera exigua larval hemolymph: opsonization of blastospores from entomogeneous hyphomycetes. J Insect Physiol 1988; 34533-40.

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