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Examining the larval morphology of *Aedes aegypti* to Carica papaya extracts

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Abstract
Controlling mosquito larvae is a crucial strategy in managing outbreaks of severe vector-borne diseases such as dengue, chikungunya, and yellow fever. Utilizing plant-derived constituents for larviciding presents an environmentally friendly and effective approach to reducing mosquito populations. A significant advantage is that these constituents can be easily and economically prepared by farmers and small-scale industries, either in their crude form or as partially purified extracts. The *Aedes aegypti* larvae were treated with seed aqueous and ethanolic extracts, ranging from the lowest dose of 1% to the highest dose of 5%, for morphological analysis. The seeds of *Carica papaya* demonstrate notable efficacy as a mosquito larvicidal agent, with increased mortality observed in correlation with concentration. Treatment with *Carica papaya* seed extracts induces morphological changes in both the external and internal structures of *Aedes larvae*. This includes the development of a weakened and shrunken body, a delicate larval skin, and cytological alterations in the mid-gut, abdomen, and siphon region.

Keywords: *Carica papaya*, *Aedes aegypti*, larvicidal, biocontrol, morphology

Introduction
The mosquito *Aedes aegypti* serves as the primary vector for arboviral diseases like dengue, chikungunya, yellow fever, and Zika fever. These viral infections are transmitted by infected female Aedes mosquitoes, and controlling the Aedes mosquito is crucial for preventing these diseases. *Aedes aegypti* undergoes four larval stages, a pupal stage, and an adult stage, all of which occur in aquatic environments, while the adults are aerial. Identification keys often focus on the adult and fourth larval stage (Bar and Andrew, 2013) [1]. These mosquitoes thrive in areas with dense human populations, where human activities contribute to the accumulation of stagnant water environments, utilized by mosquitoes for breeding (Malliga *et al*., 2018; Rueda, 2008; Malathi and Kurinchivanan, 2021) [7, 2, 4]. The most common method for controlling mosquito vectors is the use of chemical insecticides. While these chemicals are effective and require minimal quantities to control mosquitoes, there is growing concern about their impact on public health and the environment.

Due to these concerns, researchers are increasingly exploring the use of biodegradable pesticides derived from plants. The category of plant extracts encompasses a vast array of phyto compounds, many of which exert significant control over insect pests (Chellappandiyan *et al*., 2019) [22]. Researchers worldwide have demonstrated that plant extracts and their bioactive compounds play a crucial role in managing destructive agricultural pests and disease-spreading arthropods, exhibiting comparable toxicity to chemical pesticides (Nathan *et al*., 2008; Thanigaivel *et al*., 2012; Escaline *et al*., 2015) [23-25]. *Carica papaya* seed, identified as an excellent biopesticide, possesses antiviral, insecticidal, bactericidal properties, and acts as an insect growth inhibitor. It has also demonstrated toxic effects against Aedes mosquitoes (Malathi and Vasugi, 2015) [3]. The diverse composition of chemical constituents in papaya seeds affects various aspects of bioactivity.

The larvae have a thin, tubular, and membranous neck connecting the head and thorax. The abdomen of Aedes larvae is elongated, segmented, and dorsoventrally horizontal, appearing more translucent after molting. Chitin, a tough, semi-transparent substance, constitutes the primary component of the arthropod exoskeleton.

The insect's midgut plays a crucial role in the secretion of digestive enzymes and nutrient absorption (Christophers, 1960) [3]. The respiratory system is responsible for supplying adequate oxygen to all cells and removing carbon dioxide produced during cellular respiration. *Aedes aegypti* larval abdomen features a respiratory siphon, with the respiratory opening located at its tip. Initially soft, the siphon becomes tougher and darker in later instars.

Morphological studies are essential not only for insect control but also for understanding various functional aspects such as insect physiology. The presence of active ingredients in botanicals can cause morphometric variations in the epithelial layer, potentially leading to digestive, food absorptive, and respiratory disorders in many insects. This study aimed to analyse both the internal and external morphological variations in larvae treated with papaya seed aqueous and ethanol extracts, along with the control group.

Materials and Methods

Bioassay Procedure (Malathi P and Vasugi SR, 2015) [3]

For the larvicidal study, laboratory colonies of mosquito larvae were utilized. The eggs or seeds were obtained from the Centre for Medical Entomology Research in Madurai, Tamil Nadu, and India. The egg samples were acclimatized to laboratory conditions. Only 4th stage larvae were selected for the study. These larvae were treated with seed aqueous and ethanolic extracts, ranging from the lowest dose of 1% to the highest dose of 5%, for morphological analysis. A control set was also maintained without adding any extract. After 24 hours, the larvae were fixed in a fixative for further microscopic observation.

External Morphology (Saravanan *et al*., 2007; Hostettmann *et al*., 1995) [12, 27]

Specimens treated with Carica papaya seed extract and untreated specimens were mounted on clean slides using DPX (mounting agent) carefully to avoid air bubbles. Subsequently, the slides were subjected to microscopic observation, and photomicrographs were captured with the assistance of the OLYMPUS U-CTR 30-2 image analyzer at a resolution of 40x.

Internal Morphology (Ochei and Kolhatkar, 2000) [26]

After the exposure period, mosquito larvae from the experimental and control groups were fixed with a 10% formalin solution (pH 7.4) for 24 hours. Following fixation, the larvae were washed in physiological saline, cut into pieces of the desired size, and then fixed in Bouin-Hallande fixative for an additional 24 hours. After removing excess picric acid, the larvae underwent dehydration in a graded series of alcohol. Subsequently, the larvae were infiltrated with molten paraffin at 58-60 °C through three changes and finally embedded in paraffin. Thin sections, 3-5 microns thick, were obtained using a rotary microtome (Weswox, India) and stained with Hematoxylin and Eosin as a counterstain. The mounted slides were observed using an image analyzer to identify any histological changes.

Results and Discussion

The entire body sections of untreated and treated 4th stage larvae were examined to observe the effects of seed extract on the head, mid-gut, and siphon regions. After treating *Aedes aegypti* larvae with Carica papaya seed aqueous and ethanolic extracts at doses ranging from 1% to 5%, the whole larval midgut tissues and siphon were studied through whole mount observation. Microscopic examination of the control larvae revealed normal, healthy morphology with well-developed structures in the head, thorax, body wall, membrane in the abdomen region, and siphon region, along with multiple setae tufts (Fig. 1, 2a, b, and c).

In our investigation, it was observed that severe morphological damage occurred in larvae treated with seed aqueous extract. The treated groups exhibited a darkened color in the entire larvae. Microscopic observations of seed aqueous extract-treated larvae showed an uneven and jagged cuticle, significant separation of the cuticle from the external body part, severe damage to the head region, dislocated body wall, digestive tract disruption, and severe destruction of the siphon region at the 1% dose. The highest dose of 5% showed an overall shrinkage of the head, necrosis in the abdomen, and siphon region with degraded setae tufts (Fig. 1 a1, b1, c1, and a2, b2, c3).

Figure 2 displayed the external morphology of ethanol extract-treated larvae at 1% and 5% concentrations along with the control. The ethanol extract-treated larvae exhibited fully impaired chitin, overall shrinkage in the three regions, and severe damage to the head and thorax. Particularly in the abdomen region, the digestive tract could not be perceived. At 1% concentration, necrosis was observed in the abdomen and siphon, while at 5%, the body wall appeared darkened, diminished, and dislocated, with a contracted siphon (Fig. 2 a1, b1, c1, and a2, b2, c3).

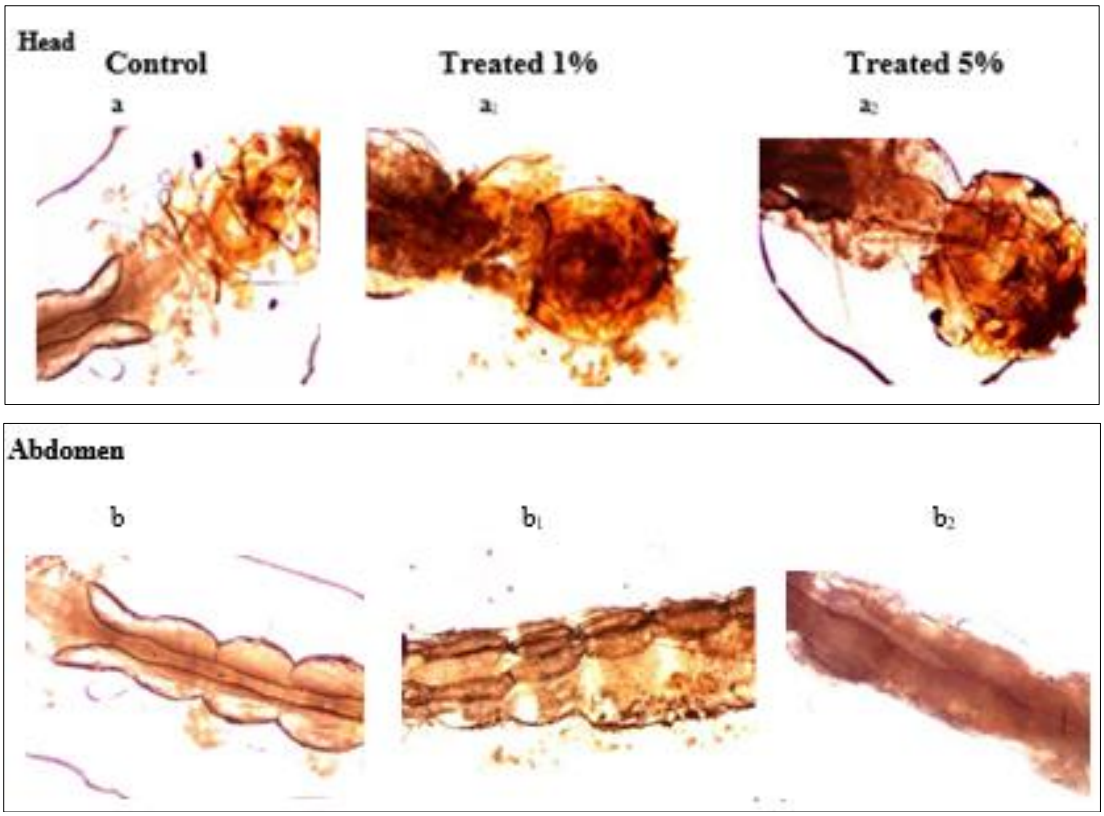
In Figure 3a, a1, and a2, a longitudinal section of *Aedes aegypti* 4th stage larvae treated with 5% aqueous extract is shown along with the control. The control larvae's central midgut regions consisted of well-spread cellular contents with a prominent nucleus. The midgut epithelium included an inner transparent peritrophic membrane (PM), basement membrane (BM), well-developed brush border (BB), and an outer midgut epithelial layer. Both inner and outer membranes were detached by the peritrophic space. Distinguished midgut epithelial layers and PM were observed in the posterior midgut regions of the control larvae. In contrast, the aqueous seed extract-treated larvae exhibited completely damaged residues of epithelial and PM in the mid-gut, with signs of intoxication continuing precisely in the middle of the gut. Most cells underwent lysis, with a rejection of cytoplasmic material towards the gut lumen, leading to total cell degeneration. Most epithelial cells degenerated and vacuolated (Fig. 3 b, b1, and b2).

In Figure 4 b, b1, and b2, a longitudinal section of *Aedes aegypti* 4th stage larvae treated with 5% ethanol is shown along with the control. The figure illustrated the detachment of epithelium from the basement membrane, with observed vacuolation in some places. Histological analysis revealed remarkable disorganization of the mid-gut epithelium in comparison to the control, with a large space between cells and the presence of tissue debris in the luminal space. Bursting of cells occurred, followed by a rejection of cytoplasmic material into the ectoperitrophic space. Muscle fibers appeared loose with typical striation and became shorter. Partial lysis of the posterior mid-gut began through local detachment, dilated BM, and destruction of PM.

Figure 5 showed the longitudinal section of *Aedes aegypti* 4th stage larvae treated with seed aqueous and ethanol extract, focusing on the gut region (40x). In the aqueous and ethanol-

treated larvae, the basement membrane became highly vacuolated and almost disintegrated. The larvae exhibited significant damage in the midgut, characterized by hypertrophic cells, damaged, sloughed, and detached cells, and separation of cuticle layers. Figure 6 displayed the longitudinal section of *Aedes aegypti* 4th stage larvae treated with seed aqueous and ethanolic extract, focusing on the siphon region (40x). The control larvae had a clearly defined siphon tube in the siphon region, whereas the treated larvae showed blockage in the siphon tube, losing their normal structure entirely.

Numerous studies have explored resources for eradicating mosquitoes primarily at the larval stage, which has proven more effective than controlling adult mosquitoes. Reports indicate that various phytochemicals act as toxicants, killing different life stages of the insect, while others interfere with growth and metamorphosis (Redwan *et al*., 2002) [20]. Rawani *et al*. (2009) [17] observed the activity of Carica papaya seed extract on different life forms of *Culex quinquefasciatus* and Anopheles stephensi. Mortality increased with concentration, confirming the positive correlation between concentration and larval mortality, as reported by Shadia *et al*., (2008) [18]. Okolie (2006) [21] studied the leaves and seeds of papaya crude extract, used to repel Aedes mosquitoes. Interestingly, during the screening of half-starved larvae, external pathological signs such as a weak and shrunken body, delicate larval skin, interruption of feeding, and the discharge of liquid from the hind end of the larvae were observed, indicating a cessation of midgut functioning due to damage in its cellular architecture. Our results align with the work of other researchers, such as Snodgrass and Erickson, (2003) [14] and Toppozada *et al*., (1968) [15], where they observed external and cytological symptoms when *Spodoptera litura* larvae were orally fed with insecticides. The progressive external harmfulness symptoms showed a positive synchronization with the external toxicity and a compulsive effect on the midgut epithelia. Furthermore, the plant acts as a stomach poison, is toxic, and acts as an antifeedant (Ross, 2001) [11]. Phytocompounds could be involved in producing larvicidal action against mosquito species. The present study suggests that there may be a block in the siphon region affecting the larvae's respiration, in line with previous studies (Dien *et al*., 2011; Youssif and Shaalan, 2011) [8, 16]. Flavonoid sulfate present in the extract could obstruct mosquito larvae through changes in the spiracular valves of the siphon and anal papillae. The deteriorating impact of the compounds on mosquito larvae results in a complete failure of adult emergence, possibly attributed to interference in the development of the chitinous cuticle, as elucidated by Saxena and Kaushik (1988) [13]. The deceased larvae in both extracts exhibited a thinning of chitin, indicating the presence of an inherent dechitinizing property within the extract. This study's findings are consistent with the results of a prior investigation conducted by Saravanan *et al*., (2007) [12]. Additionally, saponin-like phytocompounds extracted in both organic solvent and water interact with the chitin membrane of the larvae, ultimately disturbing the membrane, which is deemed the most plausible cause for larval death (Hostettmann and Marston, 2005) [9]. The observed biological action in the current study could be attributed to various substances, including phenols, terpenoids, and alkaloids present in plants (Park *et al*., 2000) [19].



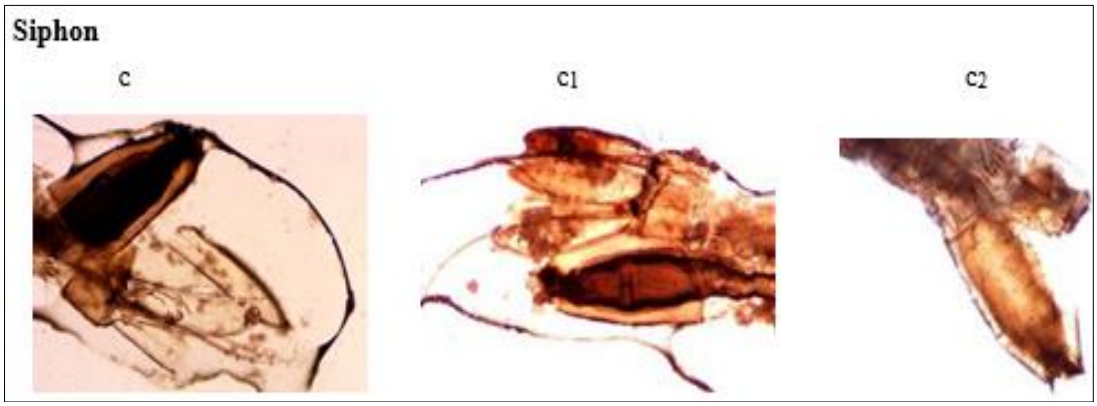


Fig 1: Histopathological alterations in *Aedes aegypti* larvae on exposure to Carica papaya aqueous seed extracts

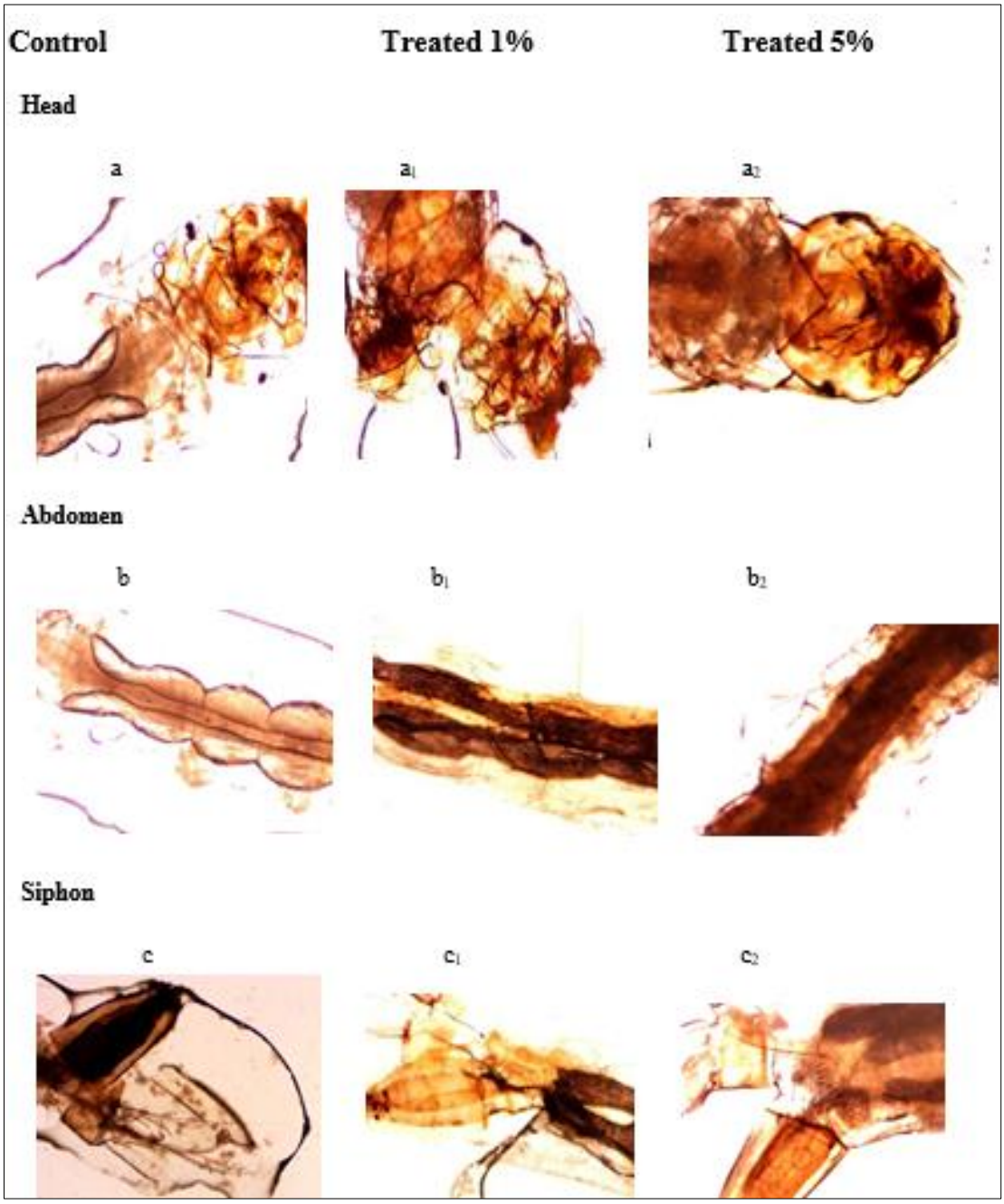


Fig 2: Histopathological alterations in *Aedes aegypti* larvae on exposure to Carica papaya ethanol seed extracts

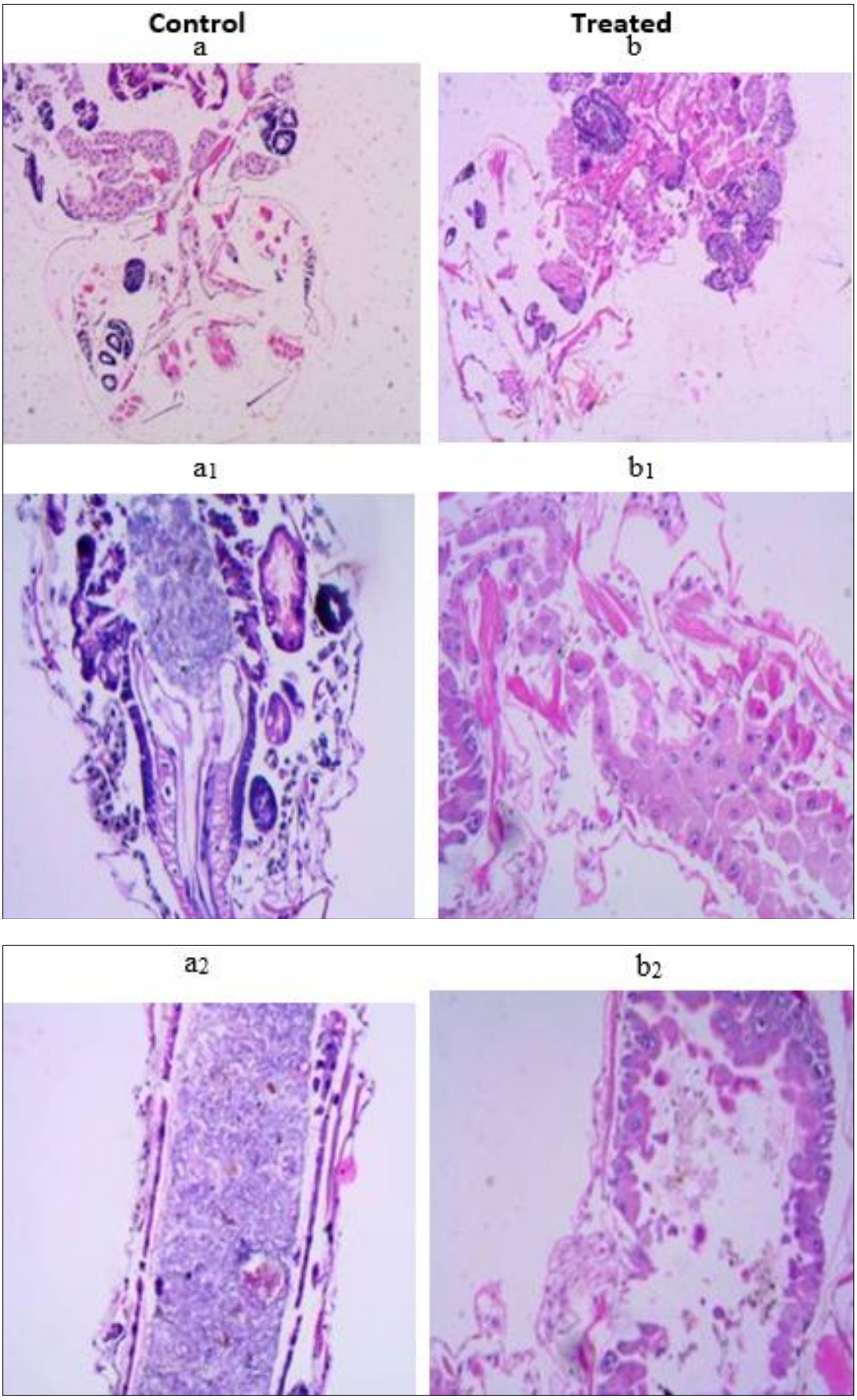


Fig 3: Longitudinal section of *Aedes aegypti* treated with 5% aqueous seed extract of Carica papaya a, a1 and a2-well defined head, digestive tract, body wall, Peritrophic Membrane, Brush Border, Basement Membrane and epithelial cells with nucleus.b, b1 and b2-rupture of membranes, degenerated epithelial cells, liberation of cytoplasmic material in to alimentary canal and masses of cellular material in the lumen vacuolation in some places.

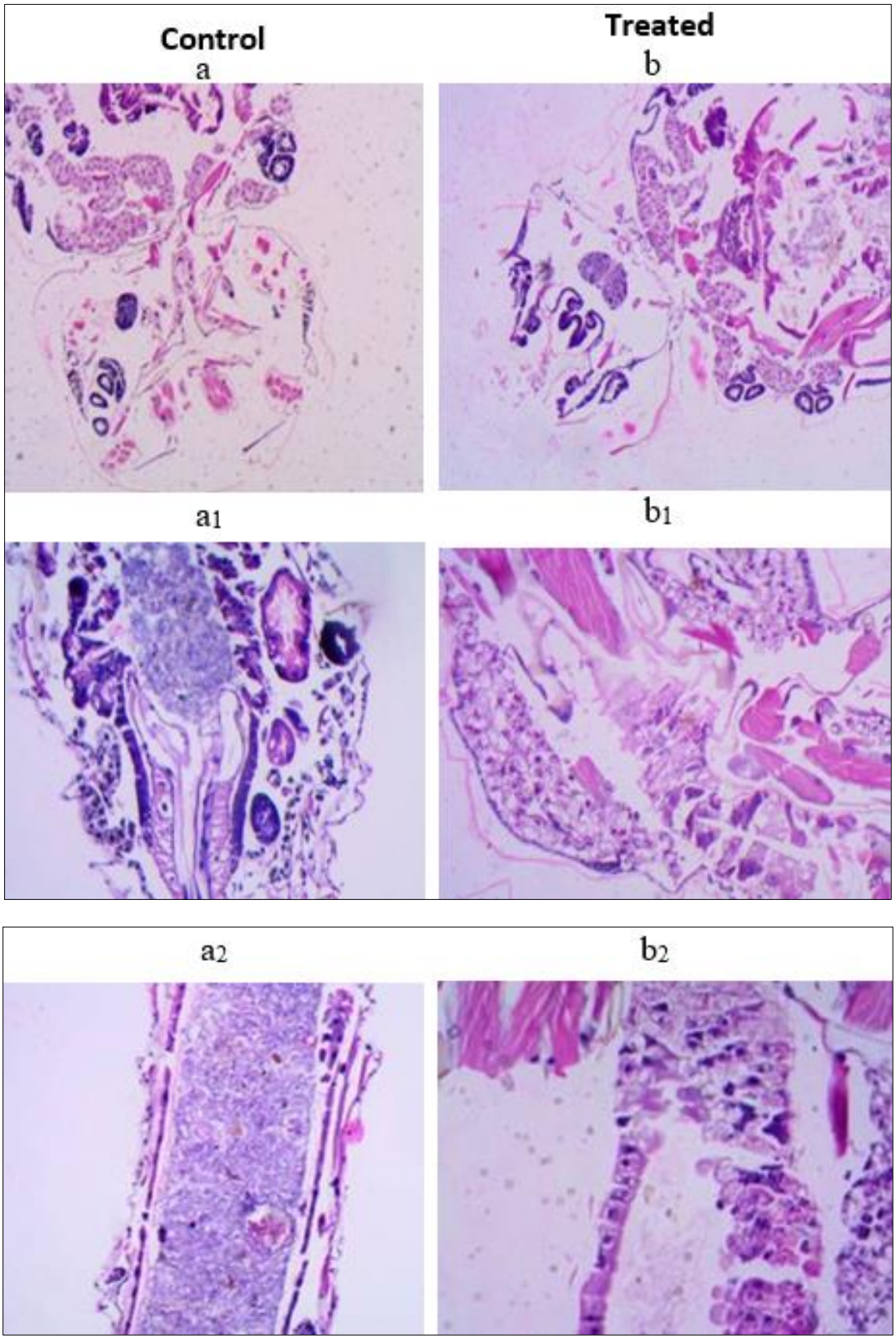
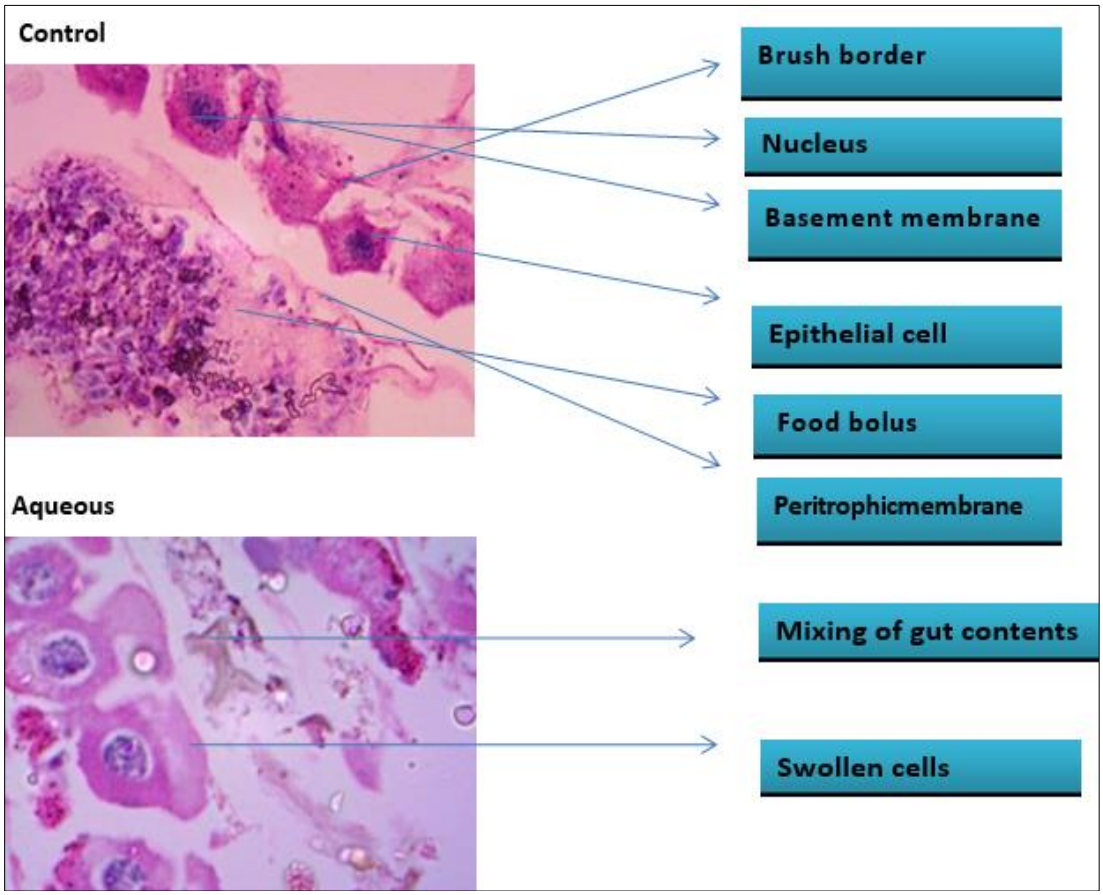


Fig 4: Longitudinal section of *Aedes aegypti* treated with 5% ethanol seed extract of Carica papaya a,a1 and a2 –well defined head, digestive tract, body wall, Peritrophic Membrane, Food bolus, Brush Border, Basement Membrane and epithelial cells with nucleus. b-damage of chitin,b1–masses of cellular material appeared in the lumen. Muscle become shorter and b2-rupture of membranes, degenerated epithelial cells, mixing of gut contents and vacuolation appears



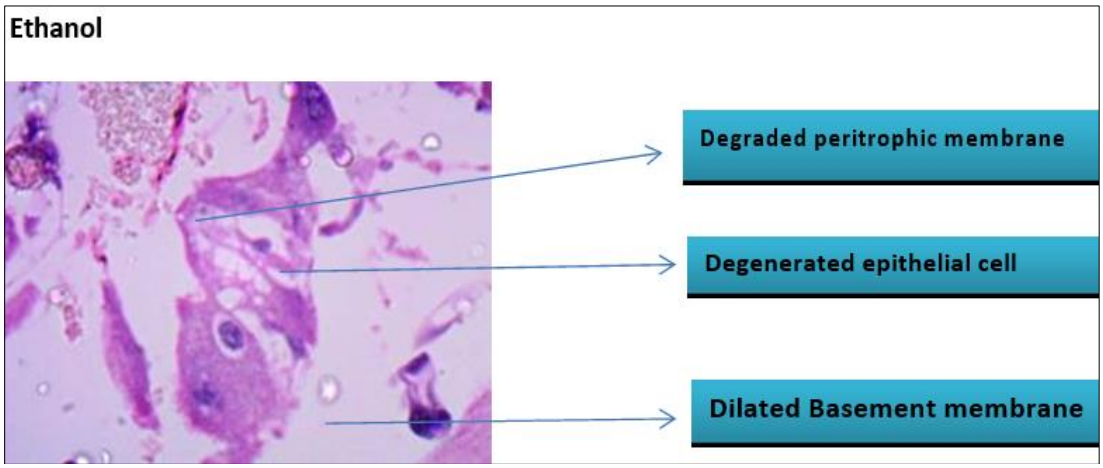
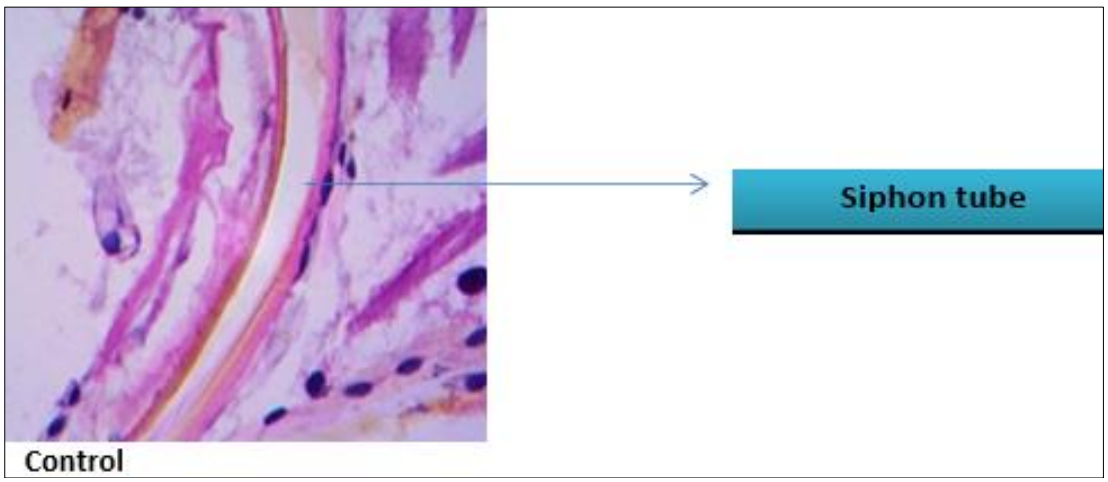


Fig 5: Longitudinal section of *Aedes aegypti* larvae gut region on exposure to Carica papaya seed extracts



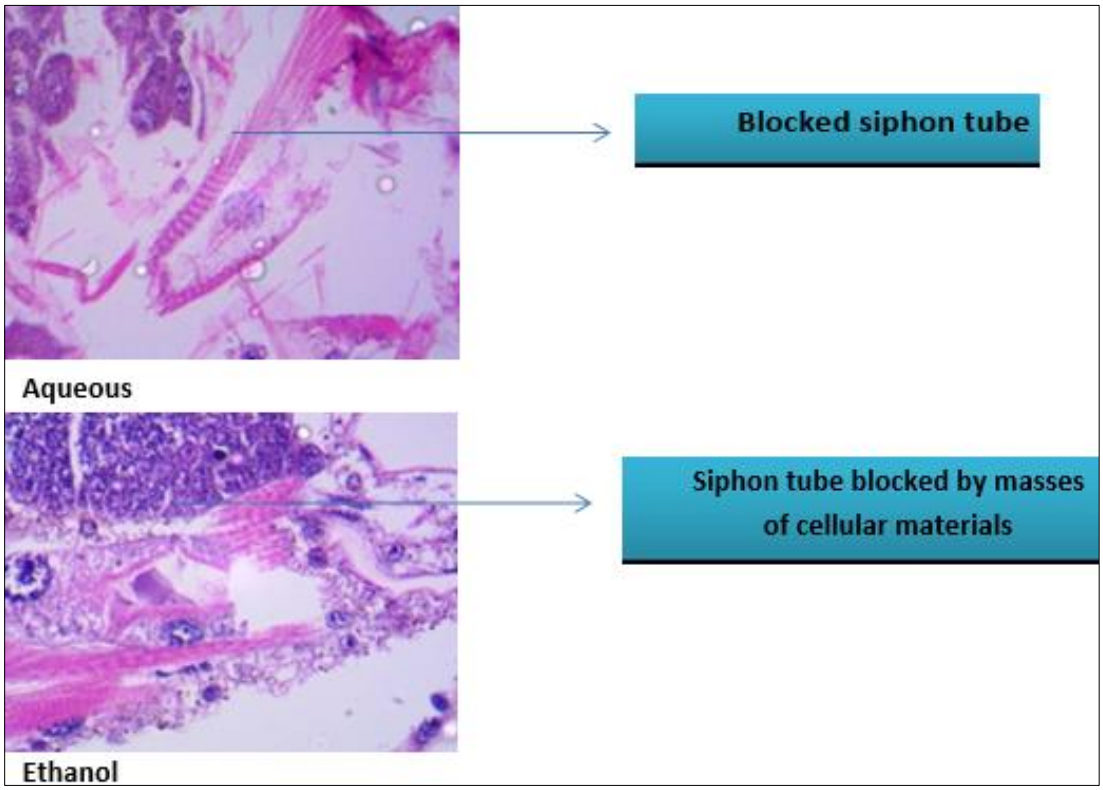


Fig 6: Longitudinal section of *Aedes aegypti* larvae siphon region on exposure to Carica papaya seed extracts

Conclusion

Carica papaya seeds are readily available and have demonstrated significant toxicity against mosquito larvae. Mosquito larvae can be effectively managed with seed extract at a low dosage, posing no harm to non-target organisms. Given that the extracts are easy to prepare, cost-effective, and safe for controlling mosquito larvae, they can be directly utilized as larvicidal and mosquitocidal agents in small-volume aquatic environments or breeding sites near human habitats. The results of the current study highlight the advantageous effects of Phytocompounds and their pro-oxidant impact at the cellular level.

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