# MORPHOLOGY AND HISTOLOGY OF THE DEFENSIVE GLANDS OF STICK INSECT, *Abrosoma johorensis*

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## ABSTRACT

This study was conducted to identify the morphology and histology of defensive glands of stick insect, *Abrosoma johorensis* (Phasmida: Aschiphasmatidae). Samples were collected from Mount of Ledang, Johor. Observation of gross morphology *in-situ* and *ex-situ* on the defensive gland of *A. johorensis* have been done using stereo microscope Carl Zeiss V12) connected to Canon EOS 1000D with LAS EZ software. Cell of *A. johorensis*'s defensive gland were stained using a special staining procedure that is Periodic Acid Schiff's reagent and Alcian Blue method. Histological slides were viewed using light photomicroscope Carl Zeiss Axio Scope A1 with iSolutionLite v1.0 software. The defensive gland morphology showed that male individual of *A. johorensis* have a smaller size of gland compared to female individual. Histological study showed that the defensive gland structures consist of secretory epithelium and surrounded by layer of muscles known as longitudinal muscles and circular muscles. Observation on ultrastructure of the defensive gland was able to discover several components and organelle lies within the secretory epithelium region that might take part in the production of chemical secretion such as epithelial cells, pigment granules, ductules, vesicles, mitochondria, tracheols and secretory apparatus.

**Keywords**: Stick Insects, Histology, Morphology, Ultrastructure, Defensive Gland

## ABSTRAK

Kajian ini telah dijalankan untuk mengenal pasti morfologi dan histologi kelenjar rembesan pertahanan serangga ranting, *Abrosoma johorensis* (Phasmida: Aschiphasmatidae). Sampel bagi kajian ini diperoleh dari Gunung Ledang, Johor. Pemerhatian morfologi kasar secara in situ dan ex situ bagi kelenjar rembesan pertahanan *A. johorensis* dilakukan dengan menggunakan mikroskop stereo Carl Zeis V12 yang disambungkan dengan Canon EOS 1000D dan menggunakan perisian LAS EZ. Sel bagi kelenjar rembesan pertahanan *A. johorensis* diwarnakan dengan menggunakan teknik pewarnaan spesifik iaitu Reagen Asid Periodik Schiff dan Biru Alsian. Slaid histologi diperhatikan dengan menggunakan fotomikroskop cahaya Carl Zeiss Axio Scope A1 dengan perisian iSolutionLite.v1.0. Morfologi kelenjar rembesan pertahanan menunjukkan bahawa individu jantan *A. johorensis* mempunyai saiz yang lebih kecil berbanding individu betina. Kajian histologi menunjukkan bahawa struktur histologi bagi kelenjar rembesan pertahanan terdiri daripada epitelium perembes dan dikelilingi oleh lapisan otot yang dikenali sebagai otot memanjang dan

membulat. Pemerhatian terhadap ultrastruktur kelenjar rembesan pertahanan berupaya mengenal pasti beberapa komponen dan organel yang berada pada bahagian epitelium perembes yang mungkin terlibat dalam penghasilan rembesan pertahanan kimia seperti sel epitelial, pigmen granul, duktul, vesikel, mitokondria, trakeol dan kelenjar perembes.

**Kata kunci**: Serangga Ranting, Histologi, Morfologi, Ultrastuktur, Kelenjar Pertahanan

## INTRODUCTION

Chemical strategy has been employed by insects as one of their defense mechanism. Insect chemical weapons might present in the form of gases, poisons, burning and foul-smelling liquids. Chemical defense among insects included both passive and active strategy. Passive form does not require any behavioural mechanism meanwhile for the other type, specific behavioural mechanism activation is needed and displayed by the insects (Unkiewicz-Winiarczyk & Gromysz-Kalkowska 2012).

Like other insects that have the ability to produce chemical compounds for the avoidance of predators or insectivorous animals included bird and mammals as the defense mechanism, Phasmid appears to be no exception (Dossey et al. 2007; Stolz et al. 2015). There are relatively over 3000 stick insect or walking stick (Phasmid; Order Phasmatodea) species exist around the whole world (Dossey et al. 2012). Phasmid is a terrestrial phytophagous insect and can be found in nearly all temperate and tropical ecosystem (Jin et al. 2013). Phasmid have two types of defense mechanisms that have been used and are best known is their primary defense mechanism which is mimetic or use of camouflage resembling the twig or leaves however, there is few species able to produce chemical spray as a secondary defense mechanism against predators (Dossey et al. 2012; Stolz et al. 2015). For some species such as *Carausius morosus*, its body colour change depending on the condition of environment because their body pigmentation can be affected by their visual stimulation of its compound eyes (Jin et al. 2013). While in *Anisomorpha buprestoides*, one of the available species that are able to produce and emit chemical secretion Anisomorphal that is strong enough to damage human eye (Clark 1976). This American stick insect (Pseudophasmatidae) can shoot its venom towards the predators and while the predator begins cleaning, the stick insect has enough time to escape and prevent them from being captured ((Unkiewicz-Winiarczyk & Gromysz-Kalkowska 2012). For *Peruphasma schultei*, the defensive secretion of this species consist of peruphasmal and glucose (Stolz et al. 2015)

Defensive glands (exocrine glands) can be found on a pair of tubercles that are located at anterior prothorax of a phasmid species or also known as prothoracic gland (Strong 1975; Stolz et al. 2015). The glands are soft and yellow in colour and for those stick insect species that as large as *Extatosoma tiaratum*, the length of the gland can reach nearly 7 mm to 8 mm (Clark 1976). Each gland has a pair of tubes connected with tubercles on the bottom (Strong 1975). The whole gland develops in the muscles layer (Strong 1975).

*Abrosoma johorensis* is one of the very few species of phasmid that manage to produce and secrete chemical secretion from their defensive gland. *Abrosoma johorensis* are highly abundance species in Gunung Ledang in altitude 1000-1200 m due to the highly density presence of host plant *Clidemia hirta* and *Melastoma malabthricum* (Rabihah et al. 2016). Very few researches have been conducted on histological studies of phasmid species that have the defensive gland and chemical spray mechanism and none have been conducted

on *A. johorensis* regarding on their morphological, histological and ultrastructures of defensive glands that may be useful in understanding the production and elaboration of the liquid chemical defensive secretion of the gland. Thus, this study outcome was to identify morphology, histology and ultrastructures of the defensive gland of *A. johorensis*.

## MATERIALS AND METHODS

### Sample Collection

Adults of *Abrosoma johorensis* were captured alive using hand during night sampling on Mount Ophir or known as Gunung Ledang, Johor (2.374112, 102.607679). Total of six (three males and three females) fresh adult samples were used in this study. *Abrosoma johorensis* were dorsally dissected from abdomen into thorax to expose the defensive gland and the glands were removed for further analysis on morphological, histological and ultrastructure studies.

### Gross Morphology Observation

Samples were weighed and measured before dissected to ensure the size of all samples were in the same range. Dissection was done in phosphate-buffered saline (PBS) to avoid the defensive glands from dry and images of the glands *in-situ* and *ex-situ* were captured using a stereo microscope (Carl Zeiss V12) connected to Canon EOS 1000D and with LAS EZ software.

Further observations of the gross morphology of the defensive gland been performed using scanning electron microscopy Table-Top (Table Top SEM Hitachi TM-1000). This method was used for observing the structure of the external morphology of the defensive gland and also tubercles that secrete the gland's secretions.

### Histological Study

The defensive glands were immersed in 10% formalin fixative overnight. The glands were dehydrated through a series of alcohol 70%, 80%, 90% and 100% for an hour each. Tissues were left in sub-xylene for an hour. The glands tissues were infiltrated with paraffin wax 3% at 58°C and embedded. Blocks of wax that contain tissues were sectioned 3 - 5 µm using Leica RM2245 microtome. Tissues sectioned were placed on the clean slides before left dried overnight in an oven. Dried sectioned tissues were then stained according to Periodic Acid Schiff's reagent PAS and Alcian Blue staining method. This staining method used to describe the histology and structure of cells of the defensive gland of *A. johorensis* and suitable for cell structures of mucoid gland because it can distinguish between natural mucin and acid dye mucin structure of defensive gland with a specific color. Microphotography of the stained tissues on slides were then observed and taken using photomicroscope Carl Zeiss Axio Scope A1 with iSolutionLite v1.0 software.

### Ultrastructural Study

Transmission Electron Microscopy (TEM) was used to observe the histological structures of cross sections of the defensive gland. Observations by TEM (Philip TEMCM12), requires a sample size that has a thickness between 3mm$^3$ volume up to 5 mm$^3$. Tissues were fixed into a solution of 2% Glutaraldehyde for 12 to 24 hours and then stored at 4°C. Then, samples were purified in solution of 2.0 M Phosphate buffer (PBS) for 10 minutes and repeated three times. Samples were then immersed in a solution of 2% Osmium tetroxide for two hours at 4°C for post-fixation. Next, the samples undergone a second process of purification with a

solution of PBS and repeated three times for 10 minutes each. Dehydration process was carried out on the sample to eliminate water from the sample. Samples were placed in a series of alcohol 30%, 50%, 70%, 80%, 90% and followed by 100% and repeated for three times. Infiltration of samples were done later with different ratio of resin and acetone mixture (Table 1) before it was embedded in resin. The sample cell was placed into the capsule and then the capsule was labelled and filled up with resin. The cell was filled with resin through the polymerisation process at 60° C in the oven for 24 hours

## RESULTS

The gross morphological study on defensive gland of *Abrosoma johorensis* have found that the gland is located on the right and left side of prothorax segment at the back of the head. The gland is brownish yellow in colour and both male and female have a similar physical appearance of the gland but slightly different in their sizes. Females have a slightly larger size of gland compared to male (Figure 1) and (Figure 2). Table 2 showed the average length of defensive gland of female and male individual for both left and right side.

The secretion area known as small openings or tubercles and believed to be function as the site where the secretions of the defensive glands will be secreted from the glands when *A. johorensis* stimulated or triggered by any external stimuli. The tubercles are present in both left and right anterior prothorax segment at the cuticles back of the head of *A. johorensis* (Figure 3). There is also a pair of smaller tubes that connect beneath the tubercles to the defensive gland (Figure 4).

Gross morphological observations of the defensive gland and tubercles of *A. johorensis* using SEM Table Top TM 1000 shows that the glands are surrounded by a layer of muscle [Figure 5 a), b) and c)]. Observations on the tubercles showed the presence of a small openings with a slit-like aperture that secrete fluid defensive secretion from the gland in Figure 5 d).

Based on the histological photograph (Figure 6), the histological structures of the defensive glands of *A. johorensis* consisting of three principal layers: an inner cuticle, secretory epithelium layer and several muscle layers.

Observations of the secretory epithelium region of the gland that surrounded by muscle layers with TEM showed the inner cuticular intima of the secretion reservoir layer and beneath it is a layer of epithelial cells along the cuticular layer (Figure 7 a). Based on Figure 7 a) and b), there are several other components that also can be found within the epithelial cells such as pigment granules, ductules and mitochondria.

Meanwhile, beneath the epithelial cells layer is a layer of secretory cells in Figure 7 b). There is a feature known as secretory apparatus which is the largest secretory cells that lies at the center within the secretory epithelium region Figure 7 a) There are small vesicles that located scattered at random in the cytoplasm of Figure 7 b). While in Figure 7 d), showed the pigment granules that is penetrated by cuticular ductules and surrounded by epithelial cells.

## DISCUSSIONS

In this study, the male and female of *Abrosoma johorensis* are able to secrete the defensive liquid secretion. This was in accordance by the study of Strong (1975) that both female and male Phasmid individual are able to discharge the defensive secretion from their defensive gland however female usually ejects more liquid than the male. From this study, *A. johorensis* male and female individual have slightly different in size of the gland. This might be due to the size of the body. The size of the defensive gland is directly proportional to the body size. Size of the gland will contribute to the amount of secretion produce in the gland. The bigger size of the gland, the more spacious for the chemical defensive secretion to be produced inside. Thus, this might be the reason why female individual secretes more compared to male. Besides, Stolz et al. (2015) stated that the defensive gland's size varies between species, for *Peruphasma schultei*, the defensive gland similar to other species but rather larger in size and extended from prothorax into the mesothorax region. For *A. johorensis*, the size of defensive gland is slightly smaller compared to those bigger species like *P. schultei* since *Abrosoma* genus mostly consist of only smaller size of stick insect and the defensive gland located only in the anterior prothorax region. Thus, the size of the gland might vary according to sex and species although similar in the morphological appearance.

The secretion discharged from the gland is through a pair of tubercles located on both side of the prothorax, near to the base of the front leg of Phasmid since a small droplet of colourless secretion remains attached to their tips of tubercles after a discharge (Strong 1975). Identically for *A. johorensis*, from the observation there was no visible spraying of the chemical secretions and merely one drop of volatile milking secretion attach on the thoracic tergum. The tubercle is a protrusion of the exoskeleton lined with epidermis (Strong 1975). Similar with findings on *A. johorensis*, the tubercles of these species is having a slit-like structure at the opening area. Is also could be seen that the tubercles opening is lined with muscle layers beneath it. This muscle layers connected to a minute duct that have similar description with what has been reported by Stolz et al. (2015) that this defensive secretion secreted through a smaller ejaculatory duct with an opening in the anterior prothorax. Each tubercle's center form a cavity that receives the secretions from the glands (Strong 1975). This can be seen by the puffy shaped of the surface area of the tubercle and also the shape of the gland.

The defensive gland is surrounded by layers of muscle called inner circular muscle and outer longitudinal muscle (Happ et al. 1966). The muscle layers might be involved in contraction movement for elaboration of the secretion within the secretory epithelium region or also involved in emitting the secretion from the gland to the outside of the body when *A. johorensis* was triggered by external stimuli.

The inner cuticle lining the lumen of the defensive glands is continuous with the cuticle of the exoskeleton, thus *A. johorensis* defensive glands can be regarded as invaginations of the integument which similar with findings on *Extatosoma tiaratum* that consists of both exo- and endocuticle (Strong 1975). Tracheol is another component found with a cuticular structure consist of taenidium and is believed involved in control access of oxygen to the cells such as muscles cells (Ghiradella 1977). For this study tracheal structure scattered randomly both in secretory and epithelium region of *A. johorensis*. Giglio et al. (2011) mentioned in their research on exocrine defensive gland of carabid beetle that the presence of tracheal associated with mitochondria showed of aerobic metabolism in the cell.

*Abrosoma johorensis* possess a similar structure with *E. tiaratum* reported by Strong (1975) on the complex layer of secretory epithelium located external to the cuticle layer that consist variety of cytoplasmic elements. The secretory epithelium layers that coating much-infolded surface of the secretion reservoir under the muscle layers is responsible for elaboration of the defensive toxicant (Happ et al. 1966). Secretory epithelium cell layers also function in defensive fluid production, storage and secretion of the fluids for defense purposes (Happ et al. 1966; Stolz et al. 2015).

Vesicles that scattered randomly in the cytoplasm may have contained non-osmiophilic lipid similar with findings on *Anisomorpha buprestoides* (Happ et al. 1966). Cytoplasmic process of *A. johorensis* defensive gland might be similar to those in *A. buprestoides* since the elucidation of the specification of structures and components of cytoplasmic elements involved in the process were corresponding to each other. Pigment granules is penetrated by cuticular ductules and then surrounded by epithelial cells. Epithelial cells follow the ductules as the ductules passing towards the secretory apparatus. The cytoplasm that surround the ductules may contain pigment granule and also small mitochondria and they are might bonded by desmosomes to the plasma membrane of the secretory cell. Deposit of the osmiophilic material that lie within the ductule are believed to be the secretory product or the defensive secretion. The epicuticular tip of the ductule projects into the central cavity that lies within the secretory apparatus where the secretion will be deposited. These structures were first described by Happ et al. (1966). However, the full cycle of defensive secretion production within one defensive gland of *A. johorensis* is quite complex and it is actually exceeding the area of this study.

## CONCLUSION

This study managed to identify almost similar results with the existing findings of previous studies on the same interest of stick insect. Therefore, the histological and ultrastructure components discovered in this study may composed similar properties in synthesizing the chemical secretion for their defense purposes. However, more detailed characterization of the components and the biosynthesis pathways involved in the production of the defensive chemical secretion will be necessary for future research studies as this species is endemic to Singapore and Peninsular Malaysia and it is found on specific food plant.

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## REFERENCES

Clark, J. 1976. The dissection of a stick insect. *Journal of Biological Education* 10(1): 31-34.

Dossey, A. T., Walse, S. S., Conle, O. V. & Edison, A. S. 2007. Parectadial, a monoterpenoid from the defensive spray of *Parectatosoma mocquerysi*. *Journal of Natural Products* 70(8):1335–1338.

Dossey, A.T., Whitaker, J.M., Dancel, M.C.A., Vander Meer, R.K., Bernier, U.R., Gottardo, M. & Roush, W.R. 2012. Defensive spiroketals from *Asceles glaber* (Phasmatodea): absolute configuration and effects on ants and mosquitoes. *Journal of Chemical Ecology* 38(9):1105–1115.

Ghiradella, H. 1977. Fine structure of the tracheoles of the lantern of a Photurid firefly. *Journal of Morphology* 153:187-204.

Giglio, A., Brandmayr, P., Talarico, F. & Brandmayr, T. Z. 2011. Current knowledge on exocrine glands in carabid beetles: Structure, function and chemical compounds. *ZooKeys* 100:193-201.

Happ, G.M., Strandberg, J.D. & Happ, C.M. 1966. The terpene-producing glands of a phasmid insect cell morphology and histochemistry. *Journal of Morphology* 119(2):143–159.

Jin, G.L., Hee, D.K., Chang, S.K., Ae, G.S., Sang, H.L., Young, K.P. & Young, B.L. 2013. Study on the hatching characteristics and diet of the stick insect, *Baculum elongatum* (Phasmida: Phasmatidae) for artificial mass rearing. *International Journal of Industrial Entomology* 26(1):61-66.

Rabihah, I., Marwan, K. & Azman, S. 2016. Stick insect of Taman Negara Johor, Gunung Ledang according to the altitudes. *Serangga* 21 (2): 57-70.

Stolz, K., von Bredow, C.-R., von Bredow, Y. M., Lakes-Harlan, R., Trenczek, T. E. & Strauß, J. 2015. Neurons of self-defence: neuronal innervation of the exocrine defence glands in stick insects. *Frontiers in Zoology* 12(1):1.

Strong, L. 1975. Defence glands in the giant spiny phasmid *Extatosoma tiaratum*. *Journal of Entomology Series A, General Entomology* 50(1): 65-72.

Unkiewicz-Winiarczyk, A. & Gromysz-Kalkowska, K. 2012. Ethological defence mechanisms in insects. III. Chemical defence/Etologiczne mechanizmy obronne owadów. III. Obrona chemiczna. *Annales UMCS,*. https://www.degruyter.com/view/j/umcsbio.2012.67.issue-2/v10067-012-0021-/v10067-012-0021-0.xml. [24 January 2017].

## APPENDICES

Table 1 Ratio of resin and acetone mixture

| Acetone | Resin | Period |
|---|---|---|
| 1 | 1 | 1 hour |
| 1 | 3 | 2 hours |
| 0 | 1 | 24 hours |
| 0 | 1 | 2 hours |

Table 2 Average length for female and male of *Abrosoma johorensis*

| Position | Length (mm) | |
|---|---|---|
| | Female | Male |
| Left | 2.59 ± 0.39 | 1.67 ± 0.29 |
| Right | 2.66 ± 0.36 | 1.72 ± 0.11 |

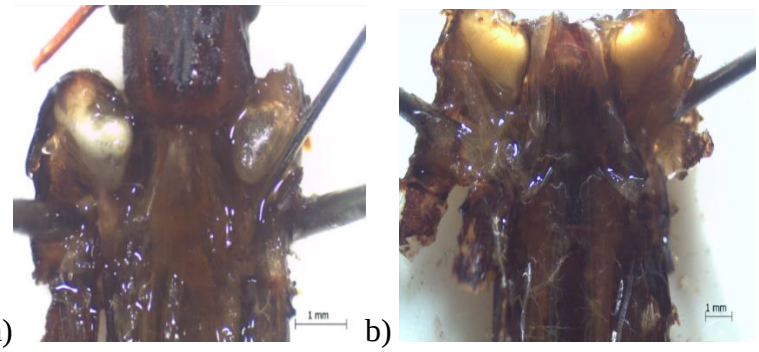



Figure 1 Gross morphology observation in-situ of the defensive gland of an individual *Abrosoma johorensis* a) male b) female.

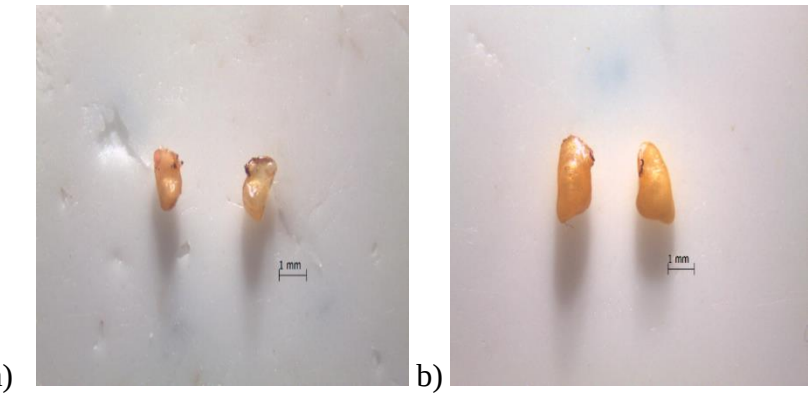
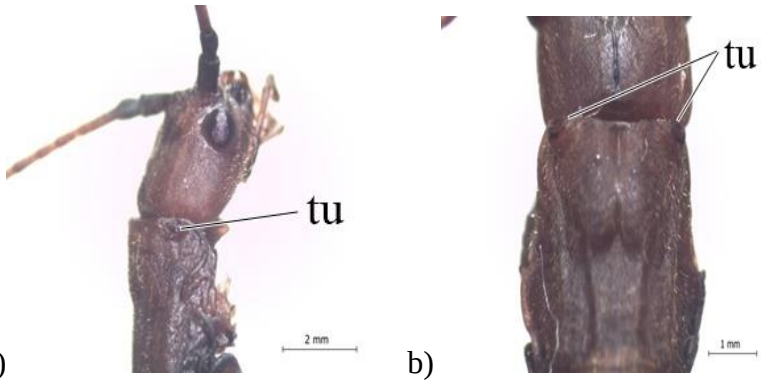



Figure 2 Gross morphology observation ex-situ of the defensive gland of an individual *Abrosoma johorensis* a) male b) female.




a) b)

Figure 3 Tubercles; tu, the site area that secrete defensive secretions of *Abrosoma johorensis.*

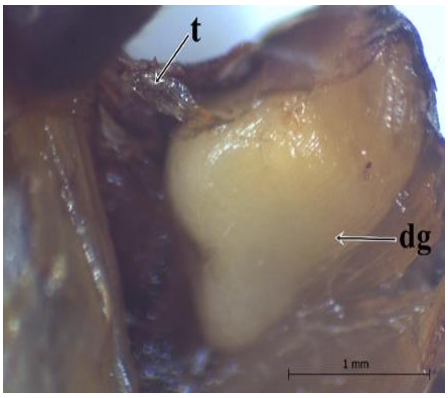



Figure 4 Tubes; t that connects the defensive gland; dg with tubercles of *Abrosoma johorensis.*

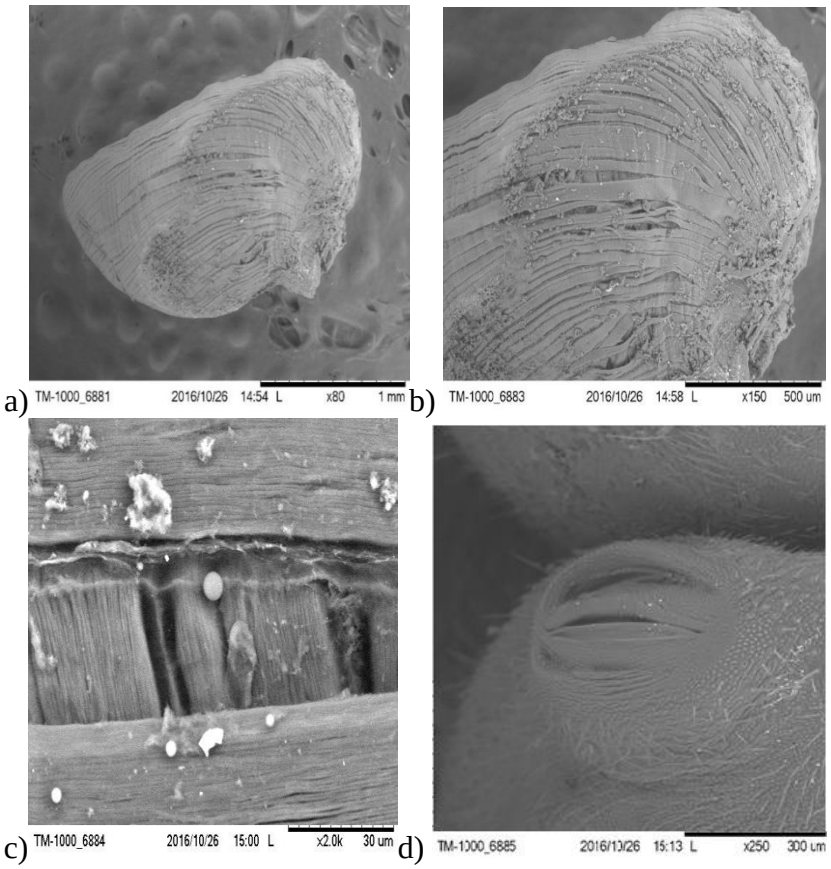



Figure 5 Gross morphology structure *ex-situ* of the defensive gland a), b) and c) and (d) tubercles of *Abrosoma johorensis*

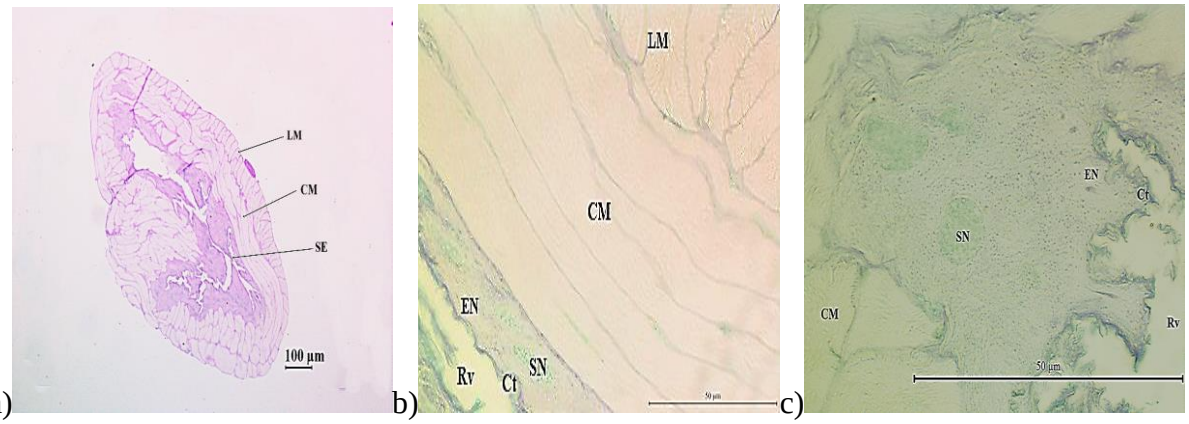



Figure 6 Cross-section through the defensive gland of *Abrosoma johorensis* shows histological cells structures such as secretory epithelium; SE, inner circular muscle; CM, outer longitudinal muscle; LM, several nuclei of secretory cells; SN, several nuclei of epithelial cells; EN, reservoir; Rv and cuticle; Ct (a) 10x, (b) 100x and (c) 100x.

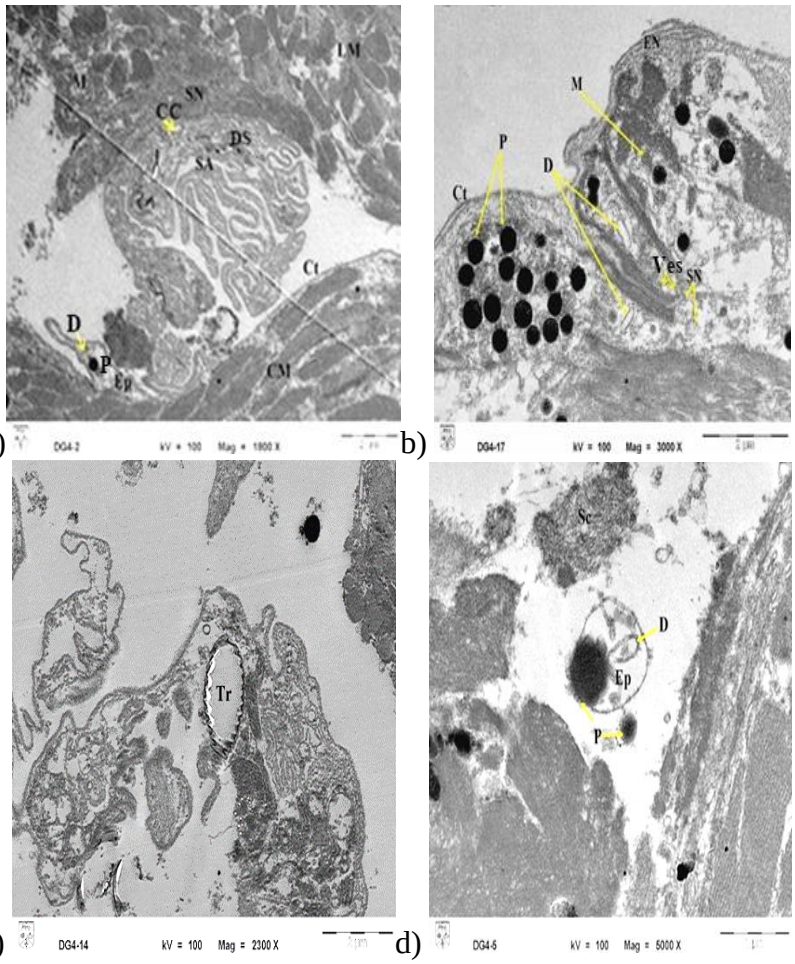



Figure 7 Observation of the secretory epithelium by using electron micrograph. (a), (b), (c) and (d) Longitudinal muscle; LM, circular muscle; CM, several nuclei of secretory cells; SN, several nuclei of epithelial cells; EN, pigment granules; P, cuticle; Ct, mitochondria; M, ductule; D, pigment granule; P, vesicles; Ves, Tracheol; Tr, epithelial cell; Ep, secretory cell; Sc, secretion in ductules; DS and central cavity; CC.