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Ultrastructural and Chemical Comparative Characteristic Studies on the Free Coelomocytes, Coelomic Epithelia and Fluids Between the Sea Urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*"

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ABSTRACT

Remarkably, day after day, new echinoderm species were added to Phylum Echinodermata that include starfish, sea cucumbers, feather stars, and sea urchins. In this work, the sea urchins especially "***Tripneustes gratilla***" and "***Echinometra mathaei***" are the animals of choice due to their popularity, medical and economic importance. The coelomic epithelium and the free coelomocytes of both sea urchins were ultrastructurally studied in addition to the chemical analyses of their coelomic fluids, followed by protein docking. The coelomic epithelium (CE) of "*Tripneustes gratilla*" exhibits 4 main cells which are 1) basal cells, 2) short cells, 3) elongated-wide cells, 4) elongated-narrow cells, all of which are polygonal coelomocytes. Additionally, the CE of the sea urchin "*Echinometra mathaei"* is composed of 1) small cells, 2) red spherules 3) leucocytes, and 4) colourless spherules, which are irregular shape coelomocytes. The free coelomocytes of both sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" appeared in the amoeboid state (resembling the human phagocytic cells) with three distinctive forms: the leucocytes, the red spherules (type "1" and type "2"), and the colorless spherules. The nuclear egress was noticeable from the nuclei of the coelomic epithelial cells of both the adult sea urchins, "*Tripneustes gratilla*" and "*Echinometra mathaei*". The CE of *Tripneustes gratilla* showed circular mitochondria with semi-circular cristae, while the CE of *Echinometra mathaei* showed elongated mitochondria. Chemically, the coelomic fluid extracted from both the adult sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" exhibited glycogen synthase kinase3-*β* protein. Ultrastructurally, the coelomic epithelium from both adult sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" showed 4 different units of coelomocytes, in which the coelomocytes of "*Tripneustes gratilla*" are elongated and polygonal; while the coelomocytes of *Echinometra mathaei* are morphologically irregular. In addition, the free coelomocytes from both adult sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" are irregular in shape resembling the human phagocytes, *i.e.* the shape meets the function. Chemically, the coelomic fluids extracted from both adult sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" exhibit glycogen synthase kinase3-*β* protein that could be used as an effective or potent agent for wound healing or skin regeneration.

INTRODUCTION

Remarkably, echinoderms always amaze us with new species discoveries, which reflects the importance of this phylum. On the **14th** of **July 2023, McLaughlin *et al.*** announced the discovery of a new feather star species *Promachocrinus kerguelensis*. The amazing criteria of this new feather star species are their discovery on the coast of

Antarctica; it ranges in colour from purplish to dark reddish, and can live at anywhere, with depths ranging from 65 feet to about 6,500 feet beneath the ocean's surface. Additionally, **McLaughlin *et al.* (2023)** found another eight unique species during their mission, including four never before named by scientists. Echinoderms include starfish, sea cucumbers, feather stars, and sea urchins. In this work, the sea urchins, especially "*Tripneustes gratilla*" and "*Echinometra mathaei*" are the animals of choice due to their popularity, medical and economic importance. Many authors reported that, at least 21 commercial species of sea urchins are listed in the market, particularly in Asia. The total production of the sea urchins reaches 73,000 ton/ year, which economically represents a total market share ranging from 200 million $ to 300 million $, noting that Japan represents the main market for sea urchins **(Shimabukuro, 1991; Micael *et al.*, 2009; Sun & Chiang, 2015; Castilla-Gavilán *et al.*, 2018; Tourón *et al*., 2023)**.

Natural bioactive materials were extracted and separated from many species of echinoderms of which the most popular species in these experimental trials was the starfish. Moreover, such extracted bioactive materials proved to form a pool of regenerative therapeutic molecules. There is no doubt that the free-living coelomocytes play an extremely important role in the regeneration processes of various echinoderms. Collectively, the coelomocytes are considered a primitive system of innate immunity compared to that of the vertebrate immune system, with homologs phylogenetic relationship, as reported in the studies of **Smith (1991)**, **Smith and Davidson (1994)** and **Kudryavtsev and Polevshchikov (2004)**.

As far as we know, the selected species for this study; namely, the sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" are poorly studied in Egypt. Moreover, **Die *et al.* (2016)** studied tissue regeneration using starfish body extract, and the results showed wide biological implications throughout the animal kingdom. Most mammals, including humans, heal wound tissues with scar repair, whereas most invertebrates, such as sea stars, Asteroidea, and planarians can regenerate almost any part of their bodies. Among echinodermatous invertebrates, the starfish – with typical developmental characteristics – possess a striking repair capability. Unlike most vertebrates, the regenerative capacity is generally limited to the healing of wounds based on the aforementioned trial of **Die *et al.* (2016)** about the usage of tissue extract from starfish for regeneration/wound healing aligned with the fact that the human blood (cardiovascular system) delivers nutrients and oxygen to all cells in the body **(Vaz *et al.*, 2016)**. The idea of studying coelomic fluid (haemocoel) sparks in our minds, and in turn, encourages us to study its characteristics and how to use it optimally for human benefits. In addition, in order to be on the right track, molecular docking as one of the most frequently used methods in structure-based drug design was listed in our consideration and methodology due to its ability to predict the binding-conformation of small molecule ligands to the appropriate target binding site. Characterization of the binding behaviuor

plays an important role in the rational design of drugs, as well as in elucidating fundamental biochemical processes **(Kitchen *et al.*, 2004; Mostashari-Rad *et al.*, 2019, Abdel-Ghaffar & Youssef, 2022; Abdel-Ghaffar *et al.*, 2023)**.

The main purpose of this work was to describe and identify the basic ultrastructure of the obtained free coelomocytes and the coelomic epithelia of both sea urchins "***Tripneustes gratilla***" and "***Echinometra mathaei***". In addition, this investigation addressed the chemical composition of the coelomic fluids of the sea urchins "***Tripneustes gratilla***" and "***Echinometra mathaei***" using GC/MS analysis followed by protein docking.

MATERIALS AND METHODS

1- Materials:

a- Chemicals:

In this study, all the used chemicals, solvents, and reagents were of analytical and pure grade. All chemicals, solvents, and reagents utilized in the TEM and GC/MS methods were of analytical and pure quality.

25% glutaraldehyde (100ml), osmic acid (EMS, 2g), acetone (HPLC), ethyl alcohol (HPLC), hexane, diethyl ether, Araldite kit (EMS), sodium hydroxide, paraformaldehyde, formalin, sodium citrate, methanol, ethyl acetate, glass knives (EMS), carbon-coated mesh (200, EMS), copper 200 mesh (EMS), Eppendorf, 15cm Falcon tubes & 50cm Falcon tubes, 7.5% $MgCl_2$, and toluidine blue were used in the present study. In addition to laboratory supplies were: yellow tips, blue tips, gloves, 50ml wide plastic containers (tightly-sealed), 20L container, large ice backs, freon B-100, 9cm Petri dish glass, and 18cm filter papers. 58*58*35 cm tanks with cover (each tank contains a filter and oxygen pump for preserving the specimen in seawater during extraction), and sea salt (to adjust water salinity).

b- Animals: "*Tripenustes gratilla*" & "*Echinometra mathaei*"

Both sea urchin species were collected from Hurghada, the Red Sea, Egypt. This work started in November 2021. **Prof. Dr. Ahmed Metwaly Hellal** (Zoology Department, Faculty of Science, Al-Azhar University "Boys-Branch") identified both specimens of the collected sea urchins. Both sea urchins of this study are known as the collector and the burrowing urchins according to **Linnaeus (1758)** and **Kroh (2010)**; the latter classification is based on World Echinoidea Database identified by **Blainville (1825)**.

1- ***Tripenustes gratilla*** **(The collector sea urchin).** The classification of this sea urchin is listed below.

Kingdom:	Animalia
Phylum:	Echinodermata
Class:	Echinoidea
Order:	Camarodonata

Family:	Toxopneustidae
Genus:	*Tripenustes*
Species:	***T. gratilla***
Binomial name	***Tripenustes gratilla***

2- ***Echinometra mathaei*** **(the burrowing sea urchin).** The classification of this sea urchin is listed below.

Kingdom:	Animalia
Phylum:	Echinodermata
Class:	Echinoidea
Order:	Camarodonata

Family:	Echinometridae
Genus:	*Echinometra*
Species:	***E. mathaei***
Binomial name	***Echinometra mathaei***

Both species of sea urchins were gathered to extract their own coelomic fluid as described below in the "Methods".

2- Methods

A. **Samples collection, and morphology of the sea urchins:** ***Tripenustes gratilla*** and ***Echinometra mathaei*****:**

- ***Tripneustes gratilla*** and ***Echinometra mathaei*** specimens were collected from Hurghada, the Red Sea, Egypt. The animals were collected throughout 18 months, covering seven seasons starting from November 2021, February/ May/ August/ November- 2022; January/ and April – 2023, *i. e.* seven intervals. Six specimens were hunted from each species of the two types of sea urchins. Thus, 6*7 intervals= 42 specimens from each species (*Tripneustes gratilla*" and "*Echinometra mathaei*). After collection throughout each interval, the coelomic fluids were drained using a sterilized syringe; these animals of choice were carefully handled, and a local anesthetic was first sprayed before piercing the skin with the syringe. In addition, the selected specimens for dissection were anesthetized using 7.5% $MgCl_2$ for 15min. During the whole experiment duration, 7 specimens from each species were dissected to get the coelomic epithelium from each; and the rest of the species were returned to their habitat. The animals were translocated in equipped tanks (58*58*35 cm) with filters and oxygen pumps to the labs of the Zoology Department – Faculty of Science – Ain Shams University.

B-1) The Ultrastructure preparation of the sea urchins *Tripenustes gratilla* and ***Echinometra mathaei* specimens of coelomic epithelia and coelomocytes for TEM examination.**

- Seven specimens from each species of the selected sea urchins "***Tripenustes gratilla*** and ***Echinometra mathaei*"** were dissected and their coelomic epithelia (CE) were processed for ultrastructure examination using a transmission electron microscope (TEM) according to **Williams and Carter (2009)**. The duration was adjusted in each step according to **Abdel-Ghaffar (2023)**.

-The sea urchins' "***Tripenustes gratilla*** and ***Echinometra mathaei*"** coelomic fluids (CF) were prepared by applying the same procedure described by **Baveja *et al.* (2018, 2019)**, in which the coelomocytes were separated as pellets after centrifugation [lengthy details were published by **Abdel-Ghaffar and Youssef (2022)**]. After centrifugation, the pellets were prepared for TEM examination. Finally, the stained ultrathin grids were examined and photographed using a JEOL 1200 EX II Electron Microscope, E. M. Unit at the Faculty of Science, Ain Shams University. The supernatant representing the acellular part was prepared for GC/MS application and protein docking.

B-2) Chemical compositions of *n*-hexane fraction obtained from the sea urchins *Tripenustes gratilla* and *Echinometra mathaei* coelomic fluids using gas chromatography accompanied by mass spectrometry (GC/MS).

GC/MS analysis was handled using Shimadzu GCMS-QP 2010 (Shimadzu Corporation, Koyoto, Japan) implemented by Rtx-5MS (30m × 0.25mm i.d. × 0.25µm film thickness) capillary column (Restek, PA, USA) and stuck to a Shimadzu mass spectrometer. The resulting compounds were detected using records from the Wiley Library Database and the National Institute of Standards and Technology (NIST), according to **Youssef *et al.* (2014)**, **Ayoub *et al.* (2015)**, **Mamadalieva *et al.* (2019)**, **Youssef *et al.* (2021)** and **Abdel-Ghaffar and Youssef (2022)**. The analyses run were fulfilled in GC/MS unit, Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University.

C- *In silico* molecular docking studies

In this study and others, we are concerned about the possible medical usage of the extracted coelomic fluids from the sea urchins *Tripenustes gratilla* and *Echinometra mathaei* and their possible medicinal effects especially on diabetics and burned-diabetic patients, and the efficacy of using the extracted coelomic fluid on their wounds. Molecular docking analysis was done on the major chemical constituents detected in the *n*-hexane fraction obtained from both sea urchins coelomic fluids using GC/MS and detected enzyme implicated in the process of wound healing identified as glycogen synthase kinase3-β protein (PDB ID 5K5N; 2.20 Å). The aforementioned protein was loaded from the protein data bank, and docking experiments were done according to

Discovery Studio 4.5 (Accelrys Inc., San Diego, CA, USA) utilizing the C-Docker protocols, as previously reported by **Labib *et al*. (2017)**, **Talaat *et al*. (2018)**, **Thabet *et al*. (2018)** and **Altyar *et al*. (2020),** where binding energies (Δ G) were calculated from the following equation:

$\Delta G_{binding} = E_{complex} - (E_{protein} + E_{ligand})$ Where;

$\Delta G_{binding}$: The ligand-protein interaction binding energy,

$E_{complex}$: The potential energy for the complex of protein bound with the ligand,

$E_{protein:}$ The potential energy of protein alone and

E_{ligand}: The potential energy for the ligand alone.

RESULTS

A- Samples collection and morphology of the sea urchins "*Tripenustes gratilla*" and "*Echinometra mathaei*"

Adults of both sea urchins "*Tripenustes gratilla*" and "*Echinometra mathaei*" were collected from Hurghada, the Red Sea, Egypt (global positioning system (GPS-GP80) is 27°17'7.67"N and 33°46'22.02"E). The specimens were gathered and delivered to the research laboratory of the Zoology Department, Faculty of Science, Ain Shams University using acrylic containers fitted with oxygen pumps and filters. Bottles of seawater were translocated from the original habitat at Hurghada to be delivered to the laboratory of Ain Shams University (Fig. 1A), mimicking its original habitat. The coelomic fluids were drained using a syringe, as shown in Fig. 1B.

The sea urchin "*Tripenustes gratilla*" is also known as the collector sea urchin; the body is discoid and almost pentagonal in shape, as shown in Figs. (1C, D). The sea urchin "*Echinometra mathaei*" is also called the burrowing urchin. The body shape is globose with unequally-projected spines (Fig. 1E).

B-1) Ultrastructure results of the coelomic epithelia and coelomocytes of both sea urchins: *Tripenustes gratilla* and *Echinometra mathaei*

-Tripenustes gratilla

In this work, the coelomic epithelial tissue of *Tripenustes gratilla* sea urchin (Figs. 2–13) was examined carefully. The coelomic epithelial tissue is formed of highly elongated cells that are arranged side by side and interrupted by short coelomocytes. The outermost layer is covered by thick glycocalyx (Figs. 2, 3, 4).

The longitudinal ultrathin sections of the coelomic epithelium (CE) were found to be composed of four main cells. In this study, the nomenclature of the building units of the coelomic epithelium was done based on the location of the cells (coelomocytes) and their ultrastructural characteristics. The cells are differentiated into short basal "No. 1", active protein-forming "No. 2", and elongated in length with variable widths that are named elongated-wide "No. 3" and elongated-narrow "No. 4" (Figs. 3, 4). The coelomocytes, named the basal cells, are found underneath the layer of the glycocalyx, as illustrated in Figs. (3, 5). This cell is slightly-pyramidal in shape with a wide base that is characterised by the presence of fenestrae (Figs. 3, 5). Its cytoplasm is rich in vesicles. The second type is named "the short coelomocytes", which show cytoplasm highly-loaded with ribosomes; these cells are known as active protein-forming cells.

The long cells are either elongated-narrow or elongated-wide cells (Figs. 2, 3, 4, 6). The two above-mentioned elongated cells are found side by side adhered to each other, and interrupted by the short active protein-forming coelomocytes. The cytoplasm of these elongated coelomocytes exhibits a prominent nucleus with nucleolus that appears as a rectangular patch (Fig. 9), and prominent mitochondria (Fig. 8). The mitochondria are circular in shape with semi-circular cristae (Fig. 8). The perinuclear space was distended due to the presence of numerous secretory granules, and nuclear envelope accordingly forms what is known as nuclear egress (Fig. 9). The terminal part of these cells is characterized by the presence of numerous apocrine secretory granules, which might discharge their contents to be delivered to the coelomic fluid. Finally, the cross ultrathin sections of the coelomic epithelial cells are polygonal in shape (Figs. 10–13).

Free Coelomocytes of "*Tripneustes gratilla*":

Remarkably, the free coelomocytes appear in amoeboid shape, as shown in Figs. (14A– J). Such amoeboid/irregular forms resemble human phagocytic cells. The free coelomocytes of the sea urchin "*Tripneustes gratilla*" show three distinctive forms:

- **The leucocytes**, as shown in Figs. (14A– C and E– G). The leucocyte exhibited a prominent nucleus, well-recognized mitochondria and smooth endoplasmic reticula.
- **The red spherules**: were easily identified due to their red pigment; echinochrome A. The nucleus had an almost regular outline, but the nuclear pores were not commonly recognized. The cytoplasmic organelles away from the spheres were usually hard to see. Scattered vesicular and tubular smooth ER were common in addition to glycogen. The red spherules are furtherly subdivided into:

  - **Type "1"**: in which the spheres are filled with electron-dense particles, as described in previous publications. This type is found more darkly-stained than the second type (Fig. 14I).

- **Type "2":** faintly-stained, in which it exhibits two types of spheres/vesicles, mostly with numerous almost empty vacuoles beside the other spheres that exhibit electron-dense particles, as shown in Figs. (14F, H, J).

- **The colorless spherules**: in this type, the spherules are almost colorless and highly intact, as shown in Figs. (14A, C, D, J)

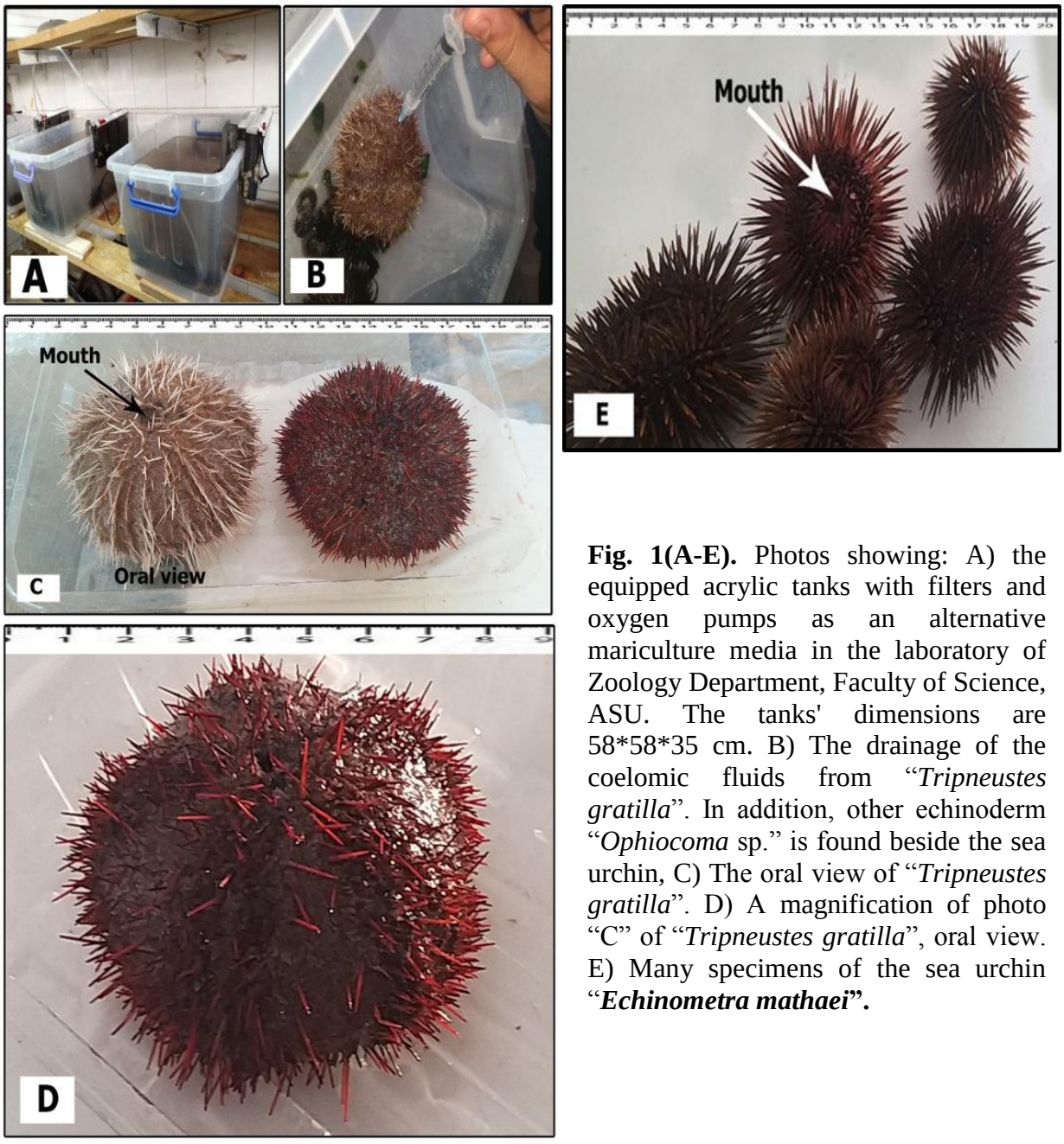


Fig. 1(A-E). Photos showing: A) the equipped acrylic tanks with filters and oxygen pumps as an alternative mariculture media in the laboratory of Zoology Department, Faculty of Science, ASU. The tanks' dimensions are 58*58*35 cm. B) The drainage of the coelomic fluids from "*Tripneustes gratilla*". In addition, other echinoderm "*Ophiocoma* sp." is found beside the sea urchin, C) The oral view of "*Tripneustes gratilla*". D) A magnification of photo "C" of "*Tripneustes gratilla*", oral view. E) Many specimens of the sea urchin "***Echinometra mathaei*".**

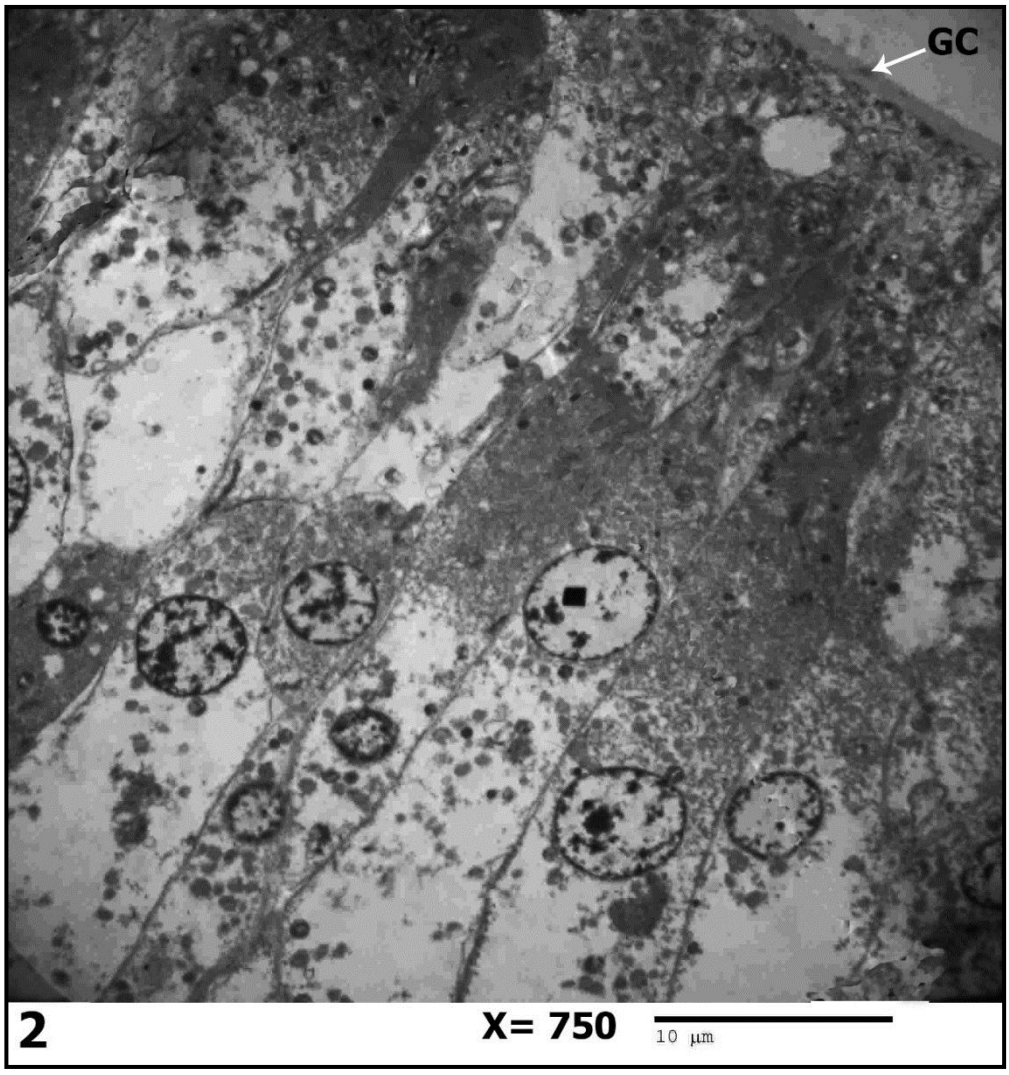


Fig. 2. An electron photomicrograph displaying a longitudinal ultrathin section of the coelomic epithelium of the sea urchin "*Tripneustes gratilla*". A prominent glycocalyx "GC" is found at the apical part of the photomicrograph. The cell lining starts with short cells and then long cells which are either elongated-narrow or elongated-wide. In between the elongated cells, short cells are found, in which their cytoplasm is highly-loaded by ribosomes and known as active protein-forming cells. Each type of the aforementioned cells exhibits a prominent nucleus.

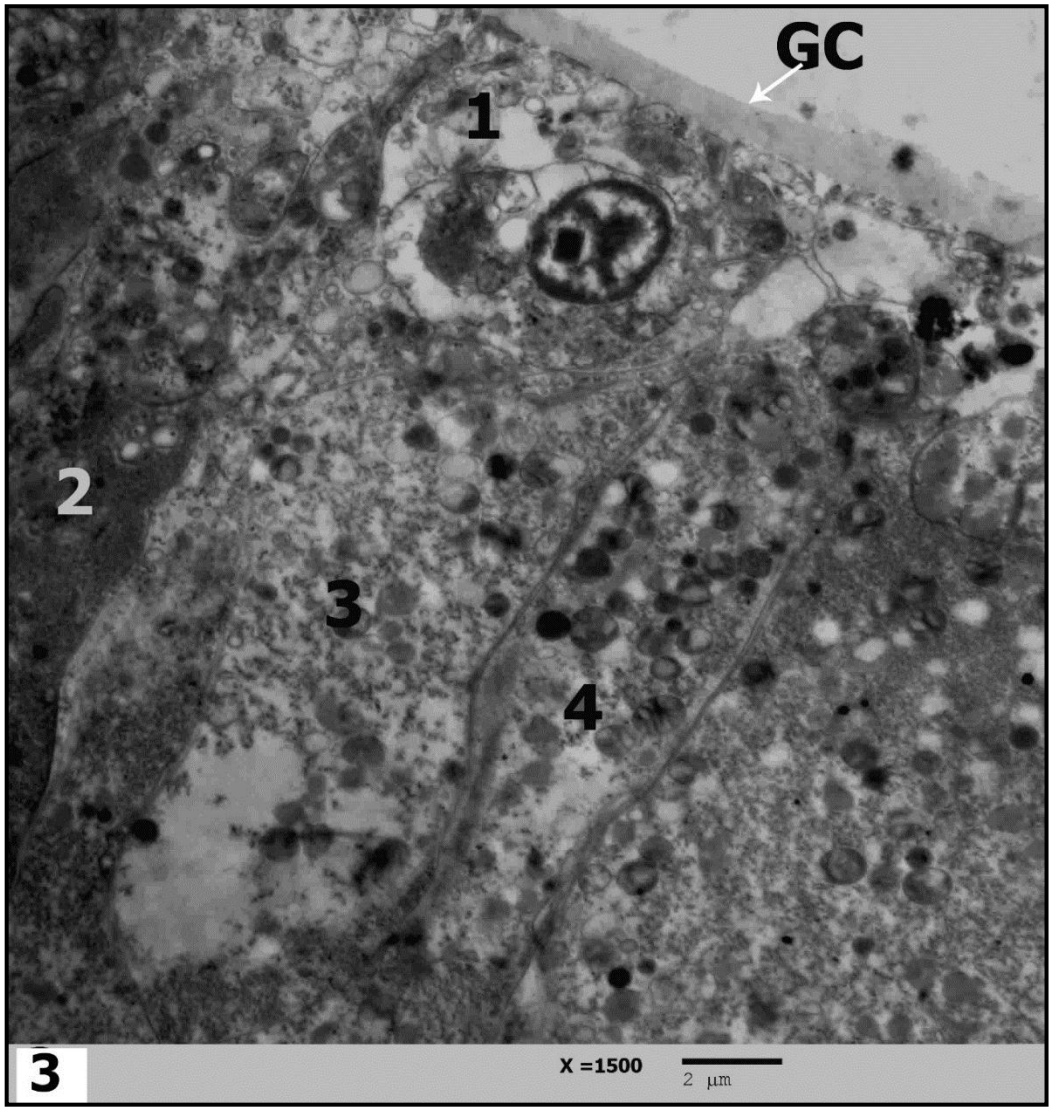


Fig. 3. An electron photomicrograph showing a magnified portion of Fig. (2) that represents an ultrathin section of the coelomic epithelium of the sea urchin "*Tripneustes gratilla*". Four types of cells are noticed: no.1: The basal cells underneath the layer of the glycocalyx "GC"; no. 2: Short cell; no. 3 & 4 represent the elongated-wide cell and elongated-narrow cell, respectively.

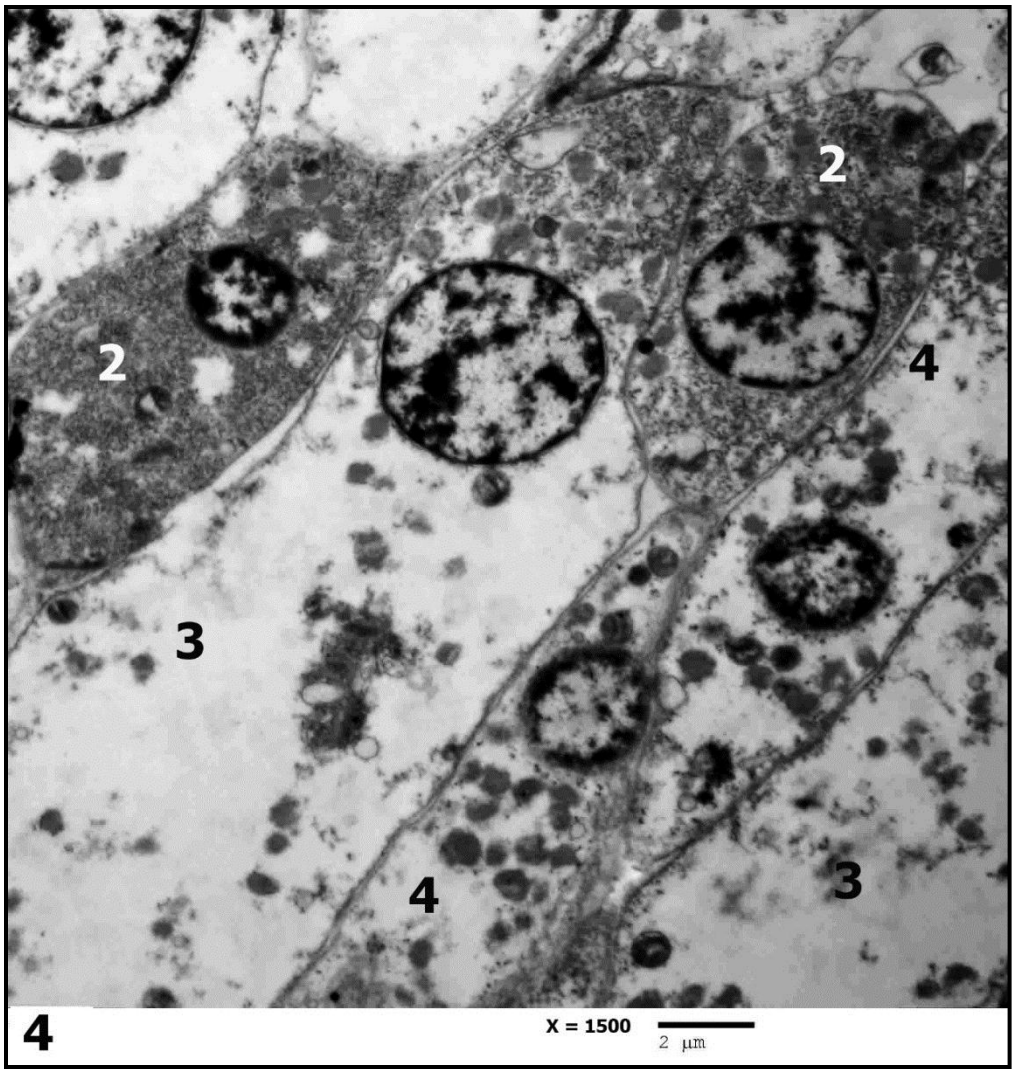


Fig. 4. An electron photomicrograph exhibiting a magnified portion from Fig. (2) that represents an ultrathin section of the coelomic epithelium of the sea urchin "*Tripneustes gratilla*". This magnified portion focuses on the short cells (no. 2), the elongated-wide cells (no. 3), and the elongated-narrow cells (no. 4).

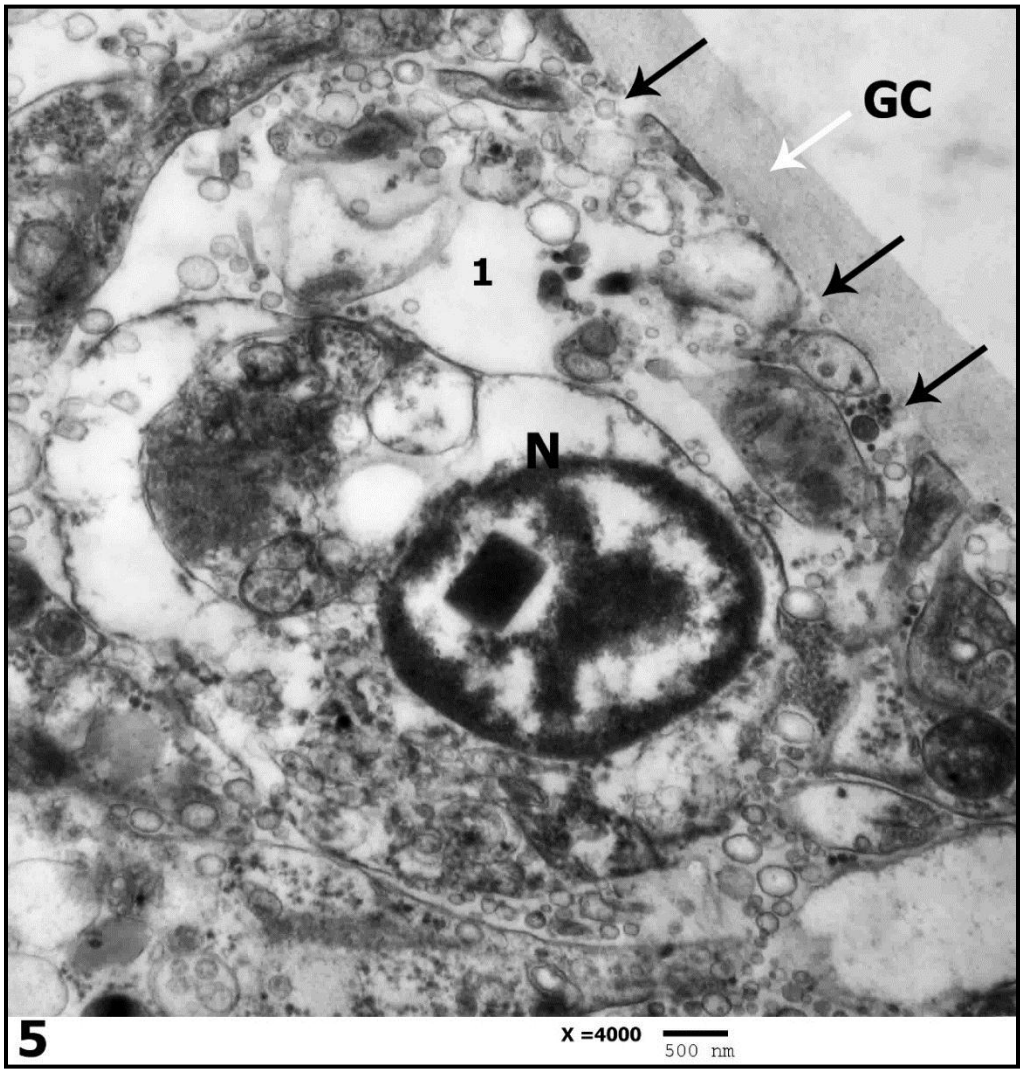


Fig. 5. An electron photomicrograph showing a magnified portion from Fig. (2) that represents an ultrathin section of the coelomic epithelium of the sea urchin "*Tripneustes gratilla*". This magnified portion focuses on a basal cell underneath the thick layer of the glycocalyx (GC). Notice the fenestrae on the plasma membrane of the basal cell (black arrows). The cytoplasm is rich in vesicles.

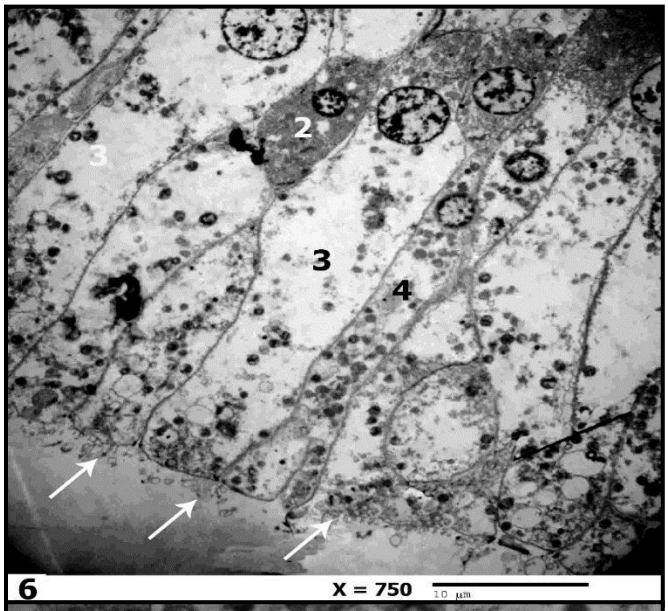


Fig. 6. An electron photomicrograph showing an ultrathin section of the coelomic epithelium of the sea urchin "*Tripneustes gratilla*" terminal portion of the elongated-wide (no. 3), and the elongated-narrow cells (no. 4). The terminal portion is characterized by the apocrine secretory granules (arrows).

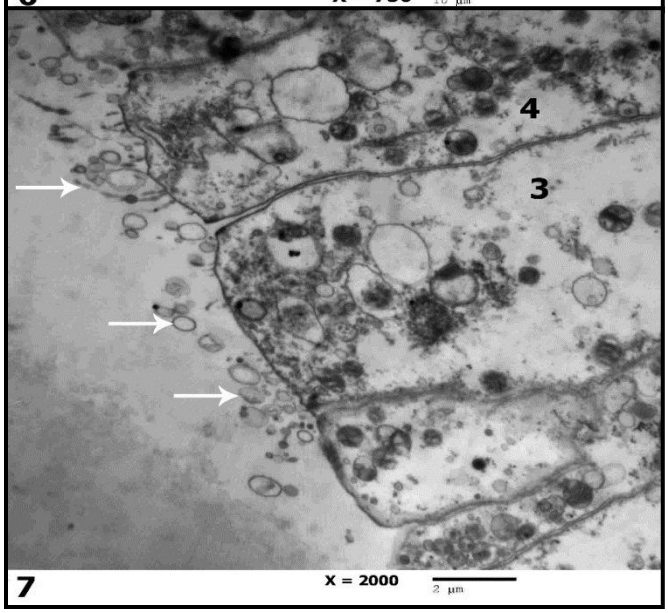


Fig. 7. An electron photomicrograph displaying a magnified ultrathin section of the coelomic epithelium of the sea urchin "*Tripneustes gratilla*" terminal portion of Fig. (6). The neighboring cells which are known as elongated-wide (no. 3), and the elongated-narrow cells (no. 4) discharge their apocrine secretory granules and vesicle towards the coelomic fluid (arrows).

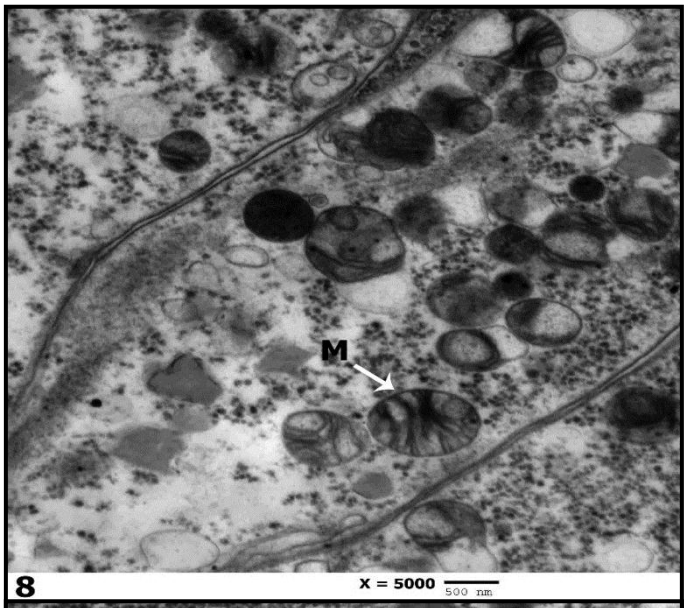


Fig. 8. An electron photomicrograph of an ultrathin section of an elongated cell from the coelomic epithelium of the sea urchin "*Tripneustes gratilla*" focusing on its cytoplasm. The cytoplasm exhibits prominent mitochondria (M), ribosomes, lysosomes, and secretory vesicles. The circular mitochondria are characterized by semi-circular cristae.

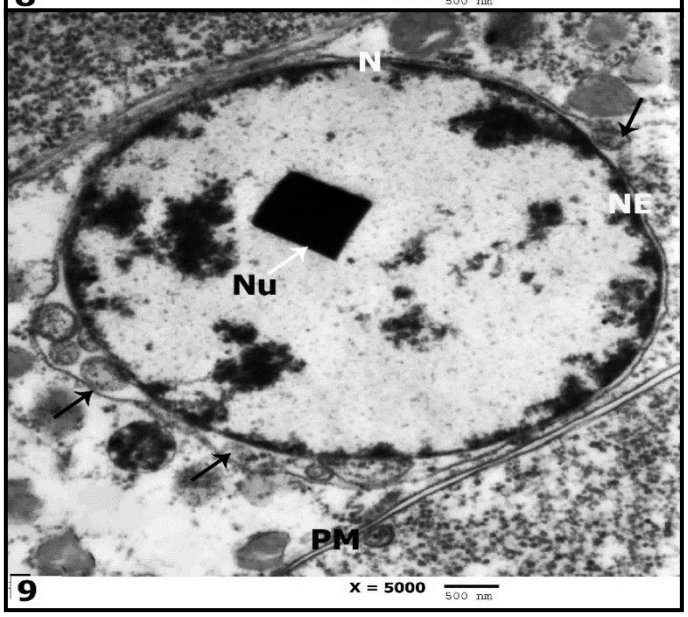
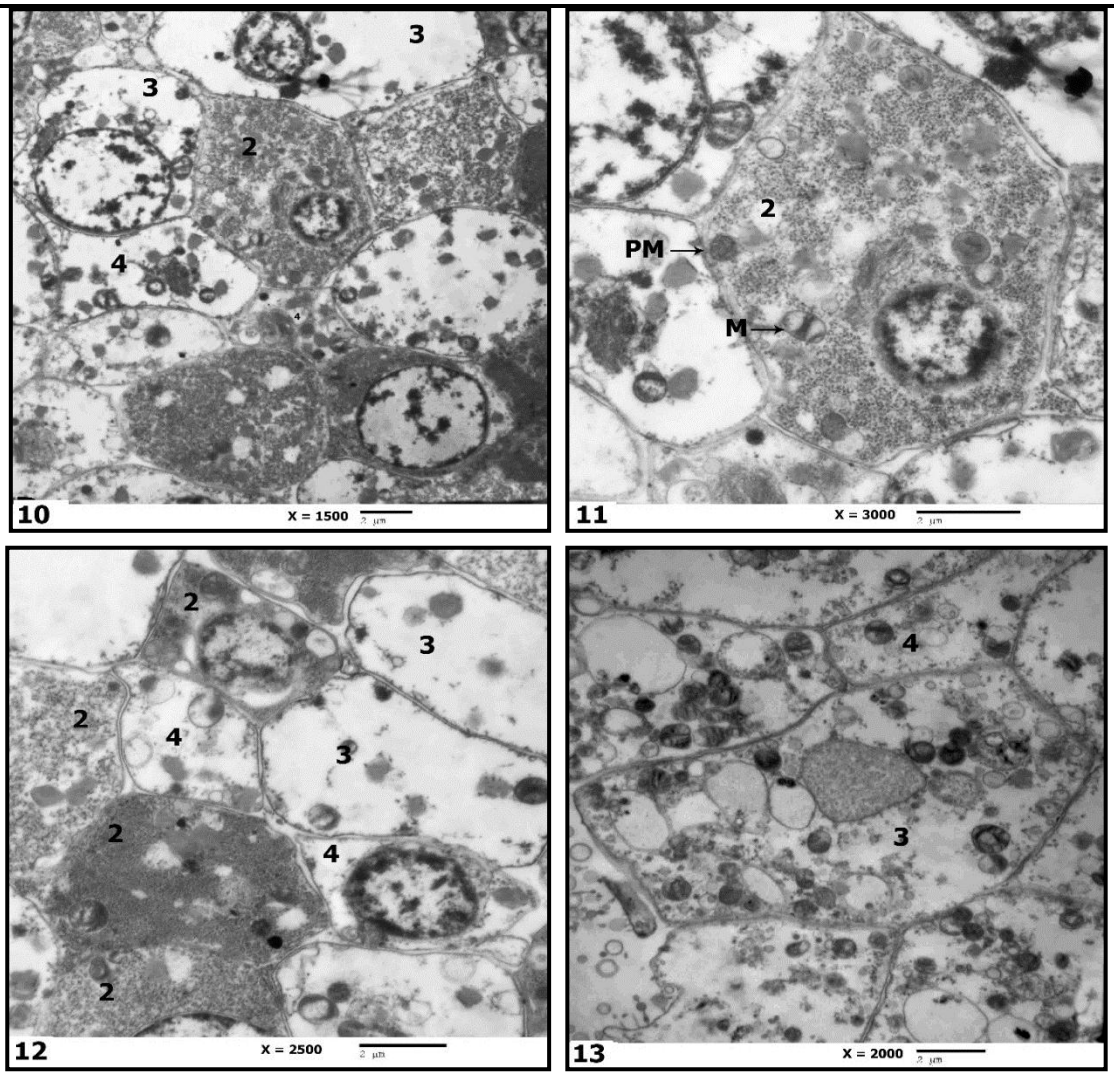
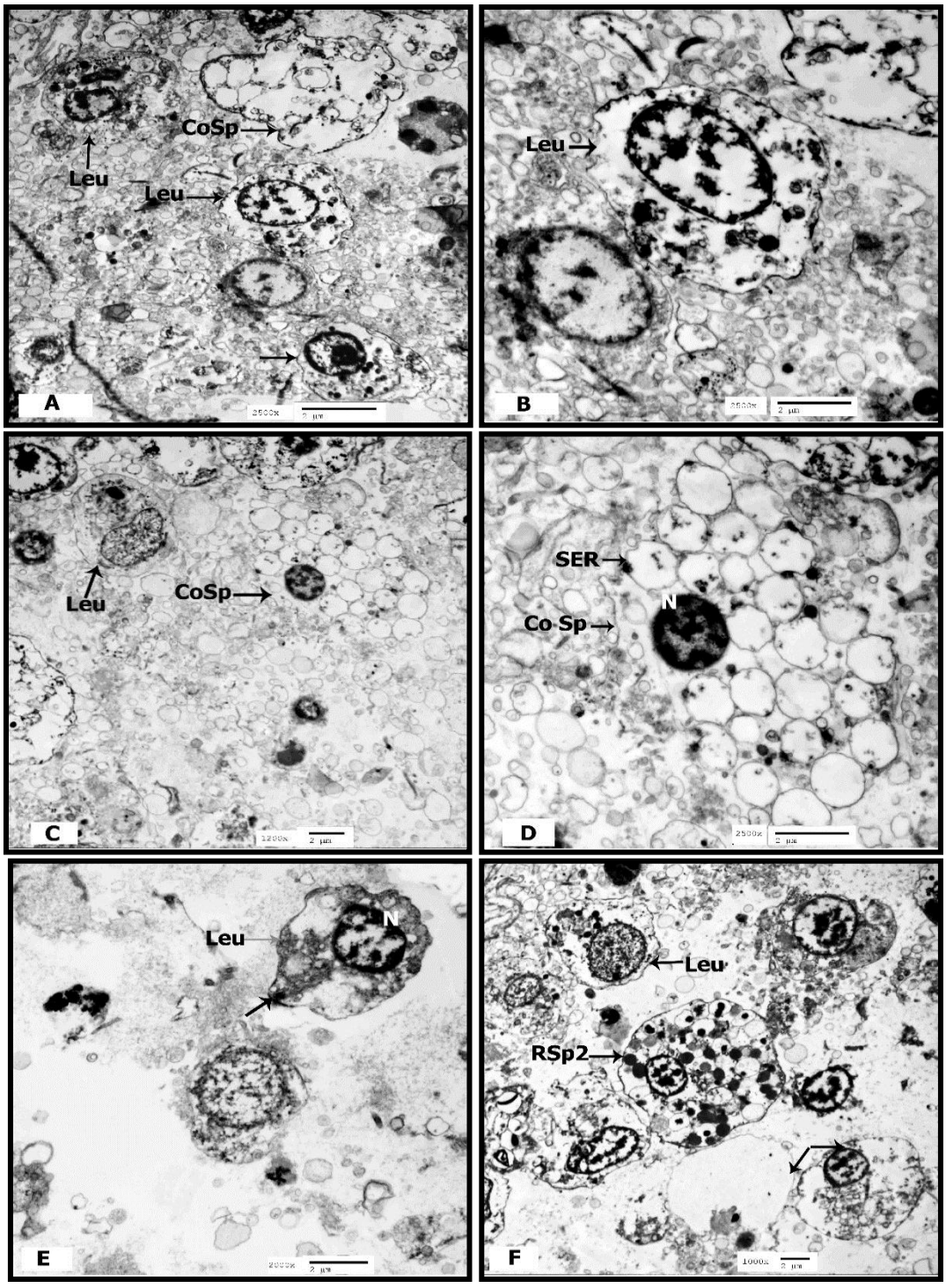


Fig. 9. An electron photomicrograph of an ultrathin section of an elongated cell from the coelomic epithelium of the sea urchin "*Tripneustes gratilla*" focusing on its active nucleus (N). The perinuclear space is distended due to the presence of numerous secretory granules (also known as perinuclear vesicles, "arrows") and forms what is known as nuclear egress. The nucleolus (Nu) forms a rectangle patch of heterochromatin that characterises this type of cell in addition to the basal cells. PM: Plasma membrane. NE: Nuclear envelope.



Figs. 10- 13. Electron photomicrographs showing cross ultrathin sections of the coelomic epithelium of the sea urchin "*Tripneustes gratilla*". Fig. (10) focuses on the short cells (no. 2), the elongated-wide cells (no. 3), and the elongated-narrow cells (no. 4). These sections show that the building units of the coelomic epithelium of the sea urchin "*Tripneustes gratilla*", which are polygonal in shape. Fig. (11) magnifies the short cell and shows cytoplasm rich with ribosomes that indicate a high affinity for protein production. Figs. (12, 13) illustrate mainly the difference in size between the elongated-wide cells (no. 3), and the elongated-narrow cells (no. 4).


A
CoSp
Leu
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B
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C
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CoSp
D
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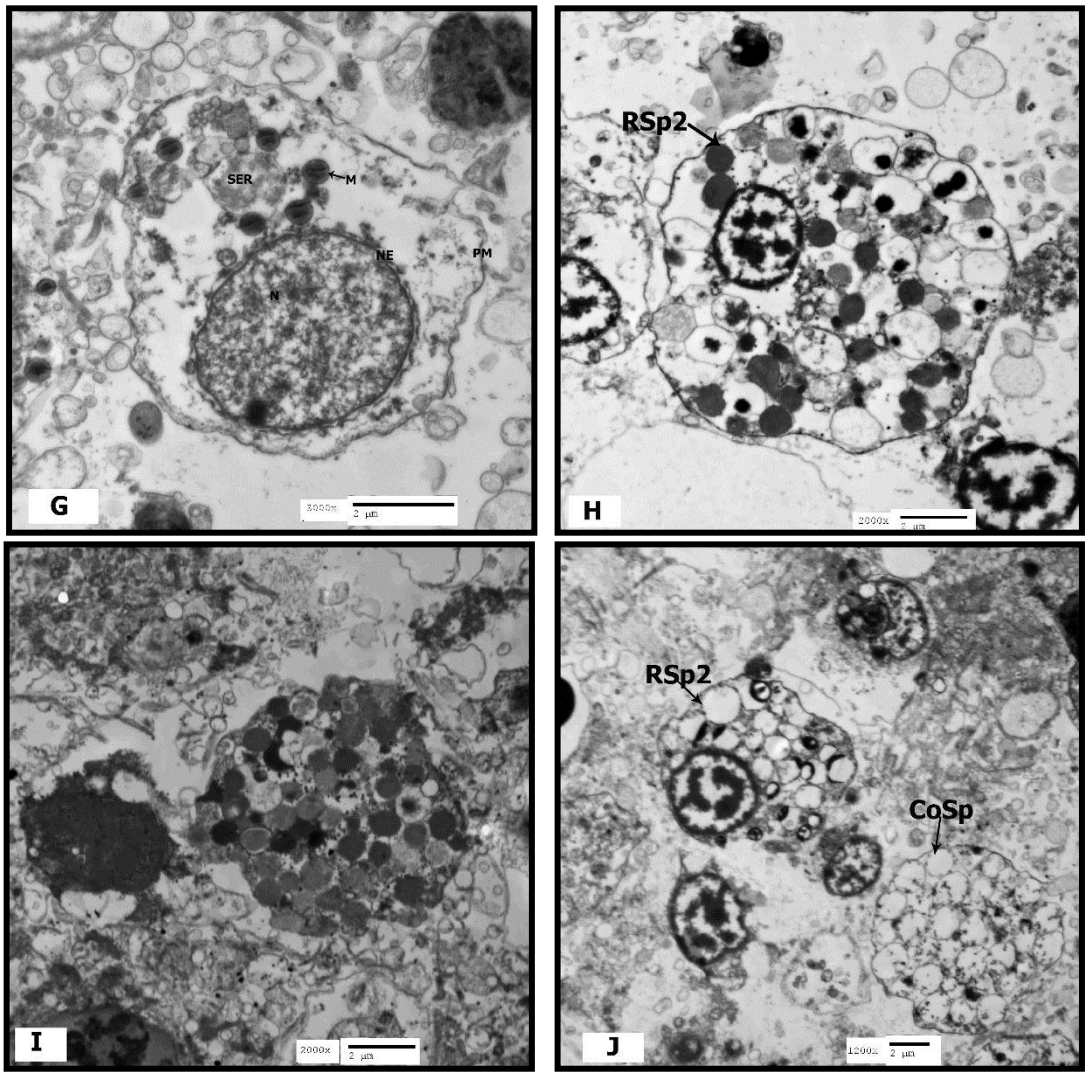


Fig. 14(A–J). Electron photomicrographs of the free coelomocytes of the sea urchin "*Tripenustes gratilla*". Three types of free coelomocytes are shown, and are differentiated into: leucocytes (Leu, E; shows protrusion "black arrow"; by which it tries to scavenge the surrounding); red spherules that are subdivided into type 1 (RSp), and type 2 (RSp2), and finally; the colourless spherules (CoSp). All types are shown in the amoeboid/irregular shapes.

2- ***Echinometra mathaei*:**

Both the coelomic epithelium and the free coelomocytes of the sea urchin "*Echinometra mathaei*" showed amoeboid/irregular shapes (Figs. 16–27). The coelomic epithelium showed four main types of coelomocytes, as shown in Figs. (15, 16), with 1) small cells; 2) the red spherule type 1 and 2, which are also known as secretory granular coelomocytes; 3) the leucocyte: is characterized by the presence of elongated mitochondria (M), and 4) the colorless spherules, which are also known as secretory mucus cells.

The small cells represent the majority of the coelomic epithelia and the free coelomocytes (Figs. 17– 25 & 27). The small cells are numerous and tightly-packed (Figs. 17–21). The cytoplasm of these SCs is structureless, while the intermediate junctions among those cells are filled with transporting vesicles. The cells are irregular in shape or show an amoeboid state. The translucent cytoplasm is only observed in the winter season; while in the summer, the examined coelomic epithelia and the free coelomocytes showed opaque cytoplasm due to the high quantity of glycogen, ribosomes in the SER or cytoplasm (Figs. 15, 16, 22–24, and 27). The nuclei of the tightly-packed small cells show nuclear blebbing or the characteristic phenomenon of the sea urchins known as nuclear egress (Figures 18 & 19). Numerous vesicles are shown nearest to the nucleus (Figs. 19–21). The intermediate junctions among those cells are filled with transporting vesicles (Figs. 17–21). In Figs. (18, 19), the cytoplasm embodies glycogen. The cells are irregular in shape (amoeboid state) and size. In Fig. (25), a leucocyte with an expanded nucleus with numerous granules is shown.

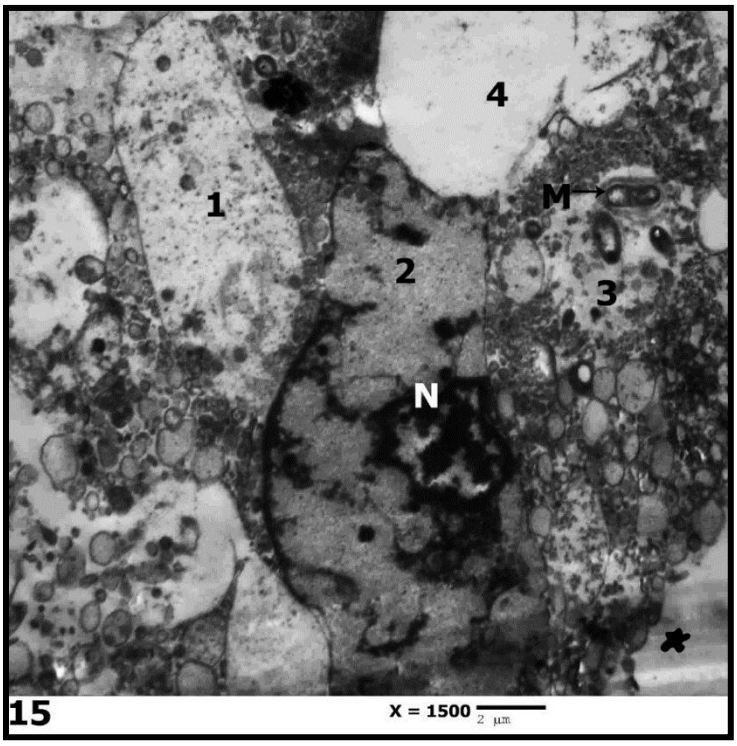


Fig. 15. An electron photomicrograph showing a longitudinal ultrathin section of the coelomic epithelium "CE" of the sea urchin "*Echinometra mathaei*". Four main types of CE are recorded:1) small cell; 2) the red spherule type 1; 3) the leucocyte which is characterized by the presence of elongated mitochondria (M) and 4) the colorless spherule.

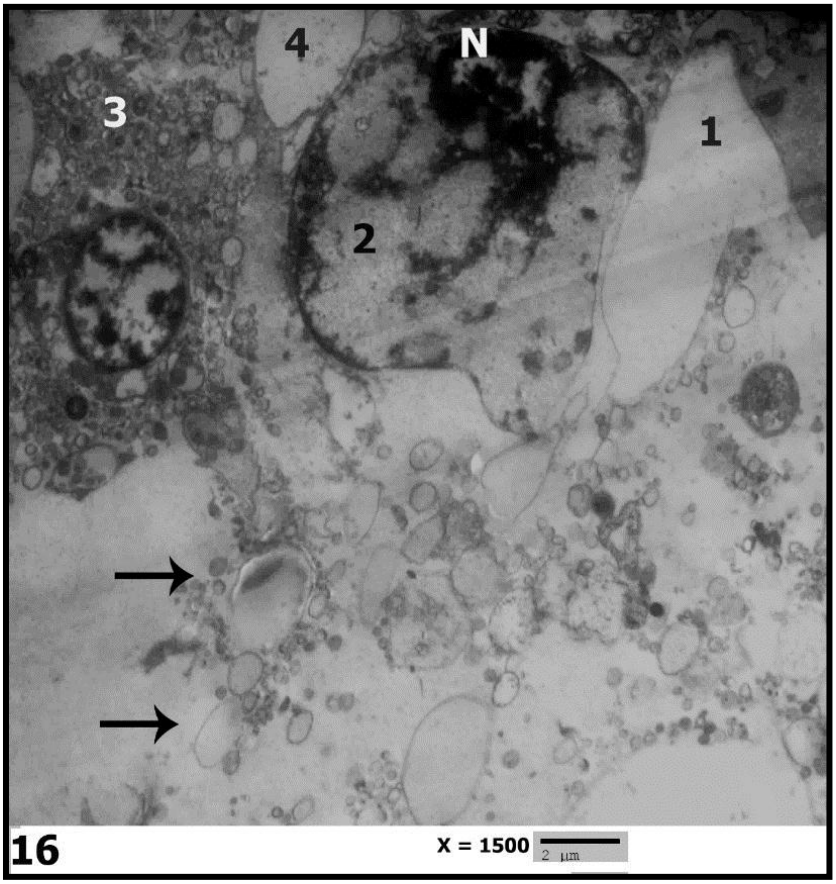


Fig. 16. An electron photomicrograph displaying a cross ultrathin section of the coelomic epithelium of the sea urchin "*Echinometra mathaei*". Four main types of CE are shown:1) small cell; 2) the red spherule type 1; 3) the leucocyte, and 4) the colorless spherule. The cells are amoeboid or irregular in shape.

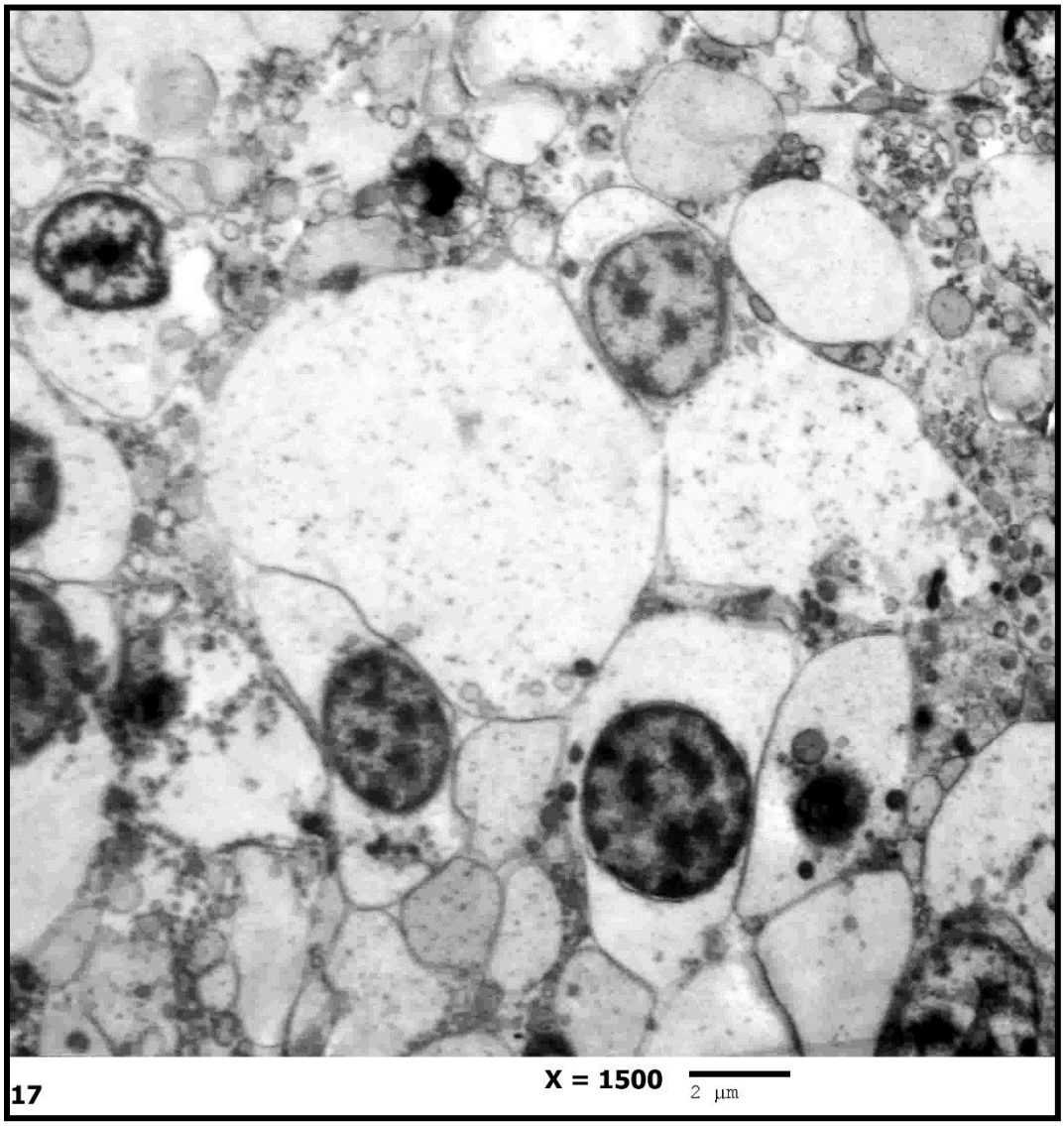
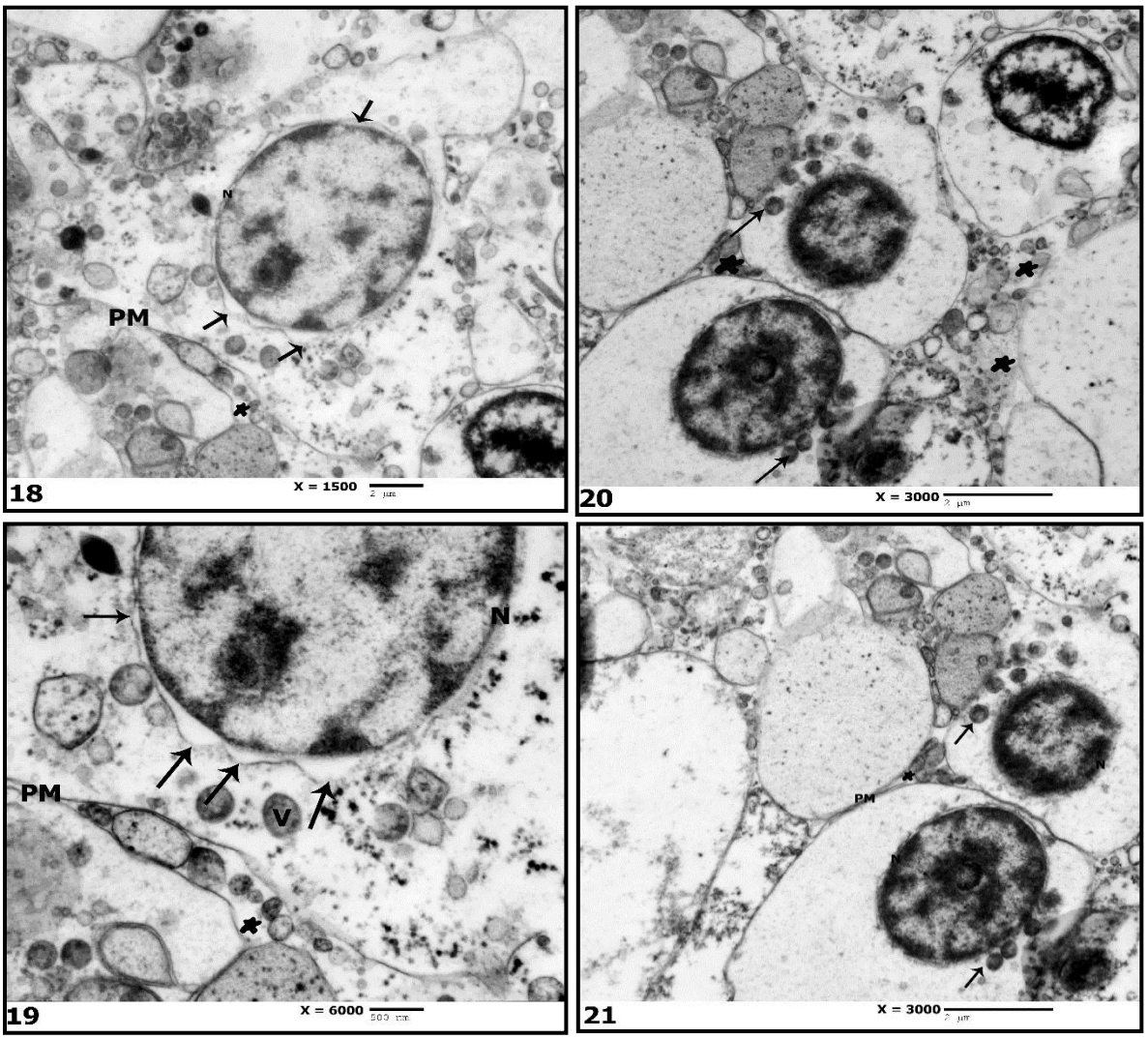
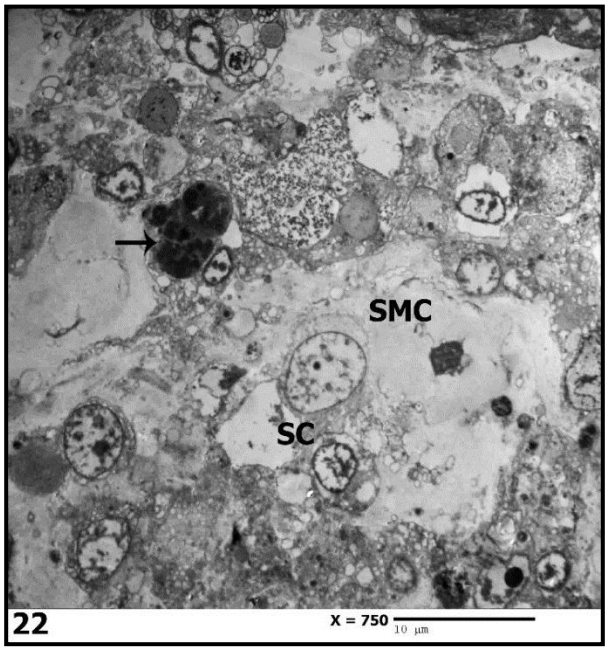
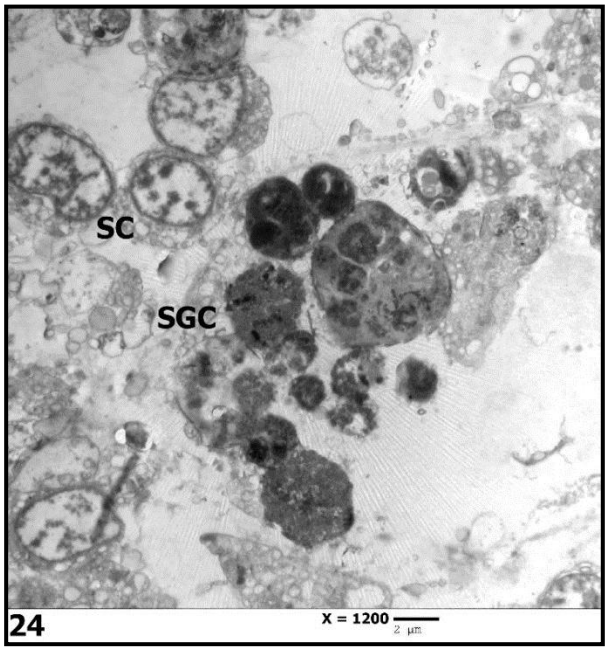


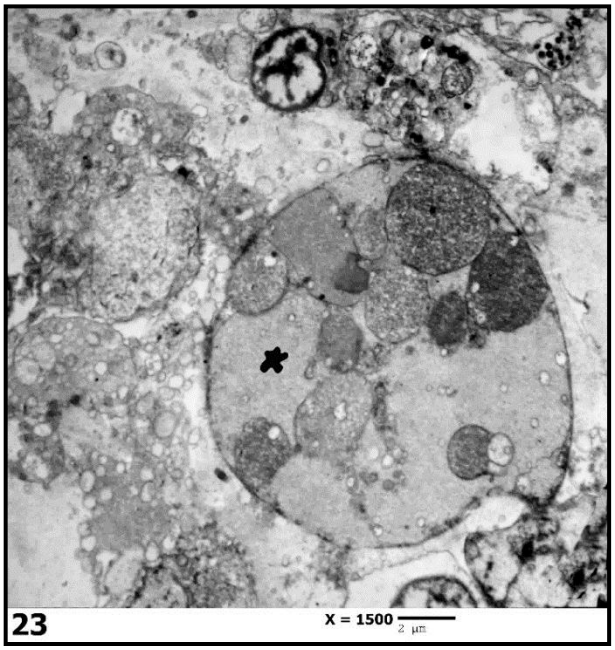
Fig. 17. An electron photomicrograph displaying a cross ultrathin section of the coelomic epithelium of the sea urchin "*Echinometra mathaei*". Numerous tightly-packed small cells "SCs" are shown. The cytoplasm of these SCs is almost structureless, while the spaces among those cells are filled with transporting vesicles.



Figs. 18–21. Electron photomicrographs showing cross ultrathin sections of the coelomic epithelia of the sea urchin "*Echinometra mathaei*". This translucent cytoplasm is only observed in the winter season. The nuclei of tightly-packed small cells show nuclear blebbing which is the characteristic phenomenon of the adult sea urchins known as nuclear egress (arrows in Figs. 18, 19). Numerous vesicles are shown near the nucleus (V in Fig. 19, and arrows in Figs. 20, 21). The cytoplasm of these SCs is structureless, while the extracellular spaces among those cells are filled with transporting vesicles (*). In Figs. (18, 19), the cytoplasm contains glycogen. The cells are irregular in shape and variable in size.







Figs. 22–24: Electron photomicrographs show cross ultrathin sections of the coelomic epithelia of the sea urchin "*Echinometra mathaei*". This darkened cytoplasm is only observed in the summer season. The Figs. show mainly small cells (SC), and secretory granular cells (SGC type 2) in which the dilated SER is filled with or heavily loaded by glycogen particles/ ribosomes (* in Fig. 23).

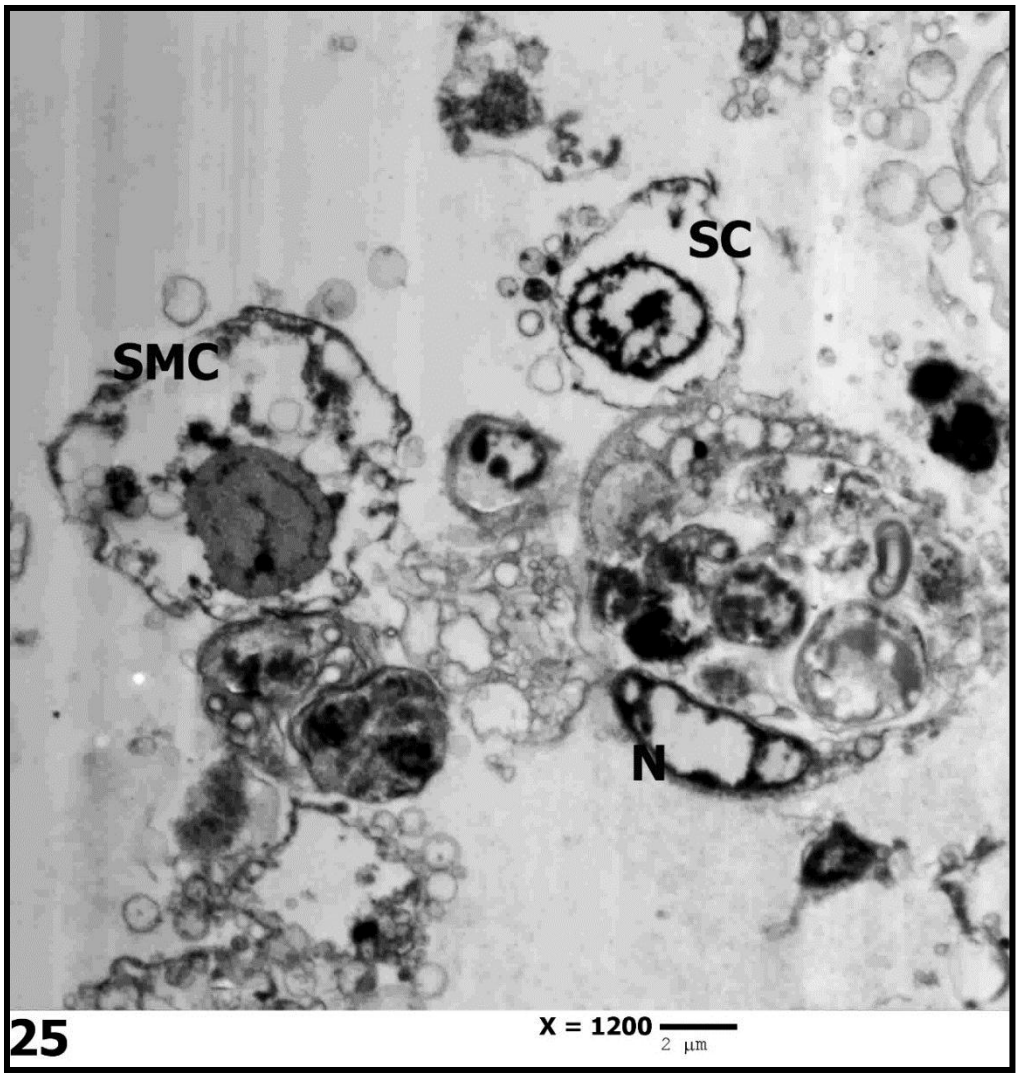


Fig. 25. An electron photomicrograph an ultrathin section of free coelomocytes of the sea urchin "*Echinometra mathaei*". The free coelomocytes are known as small cells (Sc), secretory mucous cells (SMC), and a leucocyte with an elongated nucleus (N). The cytoplasm contains numerous granules. The leucocyte scavenges a neighboring secretory granular cell.

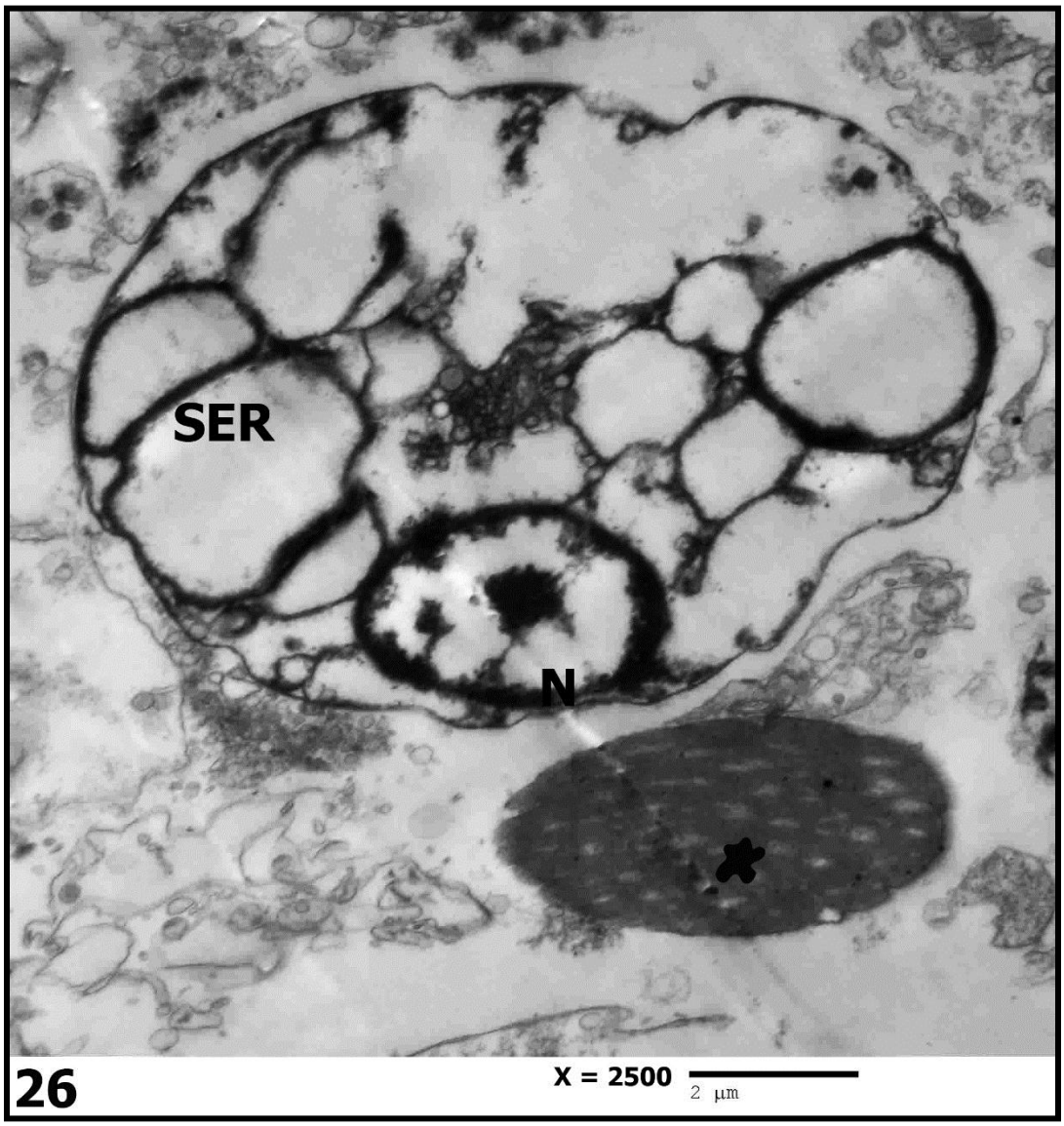


Fig. 26. An electron micrograph of an ultrathin section of the free secretory mucous coelomocyte (SMC) of the sea urchin "*Echinometra mathaei*". The SMC contains numerous dilated smooth endoplasmic reticula (SER), and a prominent nucleus (N). Notice the neighbouring vesicle (*), which might represent the red echinochrome A pigment.

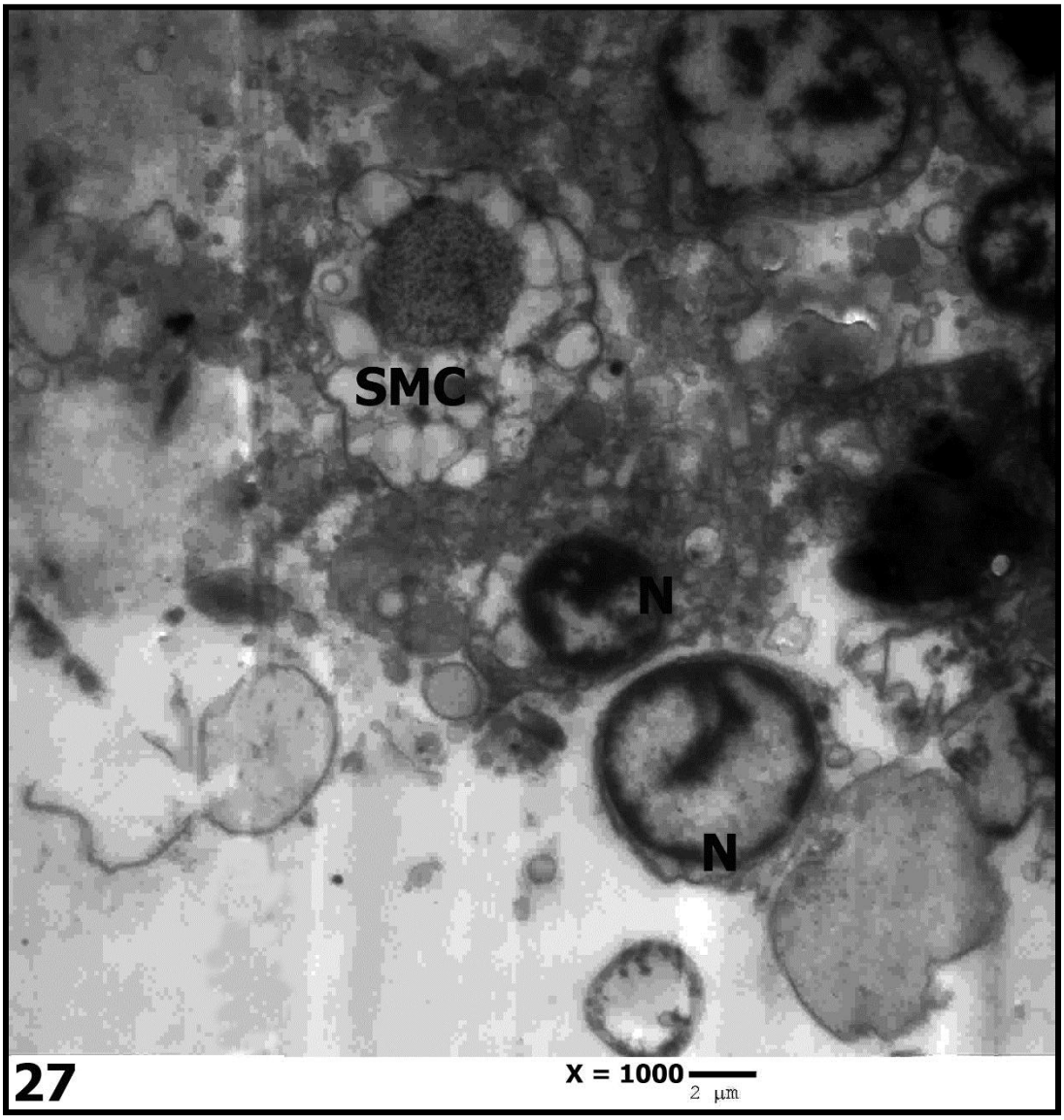


Fig. 27. An electron micrograph of an ultrathin section of free coelomocytes of the sea urchin "*Echinometra mathaei*". The Fig. shows a secretory mucous cell (SMC) that is engulfed by a leucocyte.

C-1) Chemical compositions of *n*-hexane fraction obtained from the sea urchin "*Tripenustes gratilla*" coelomic fluid using GC/MS

Chemical characterisation of the *n*-hexane fraction obtained from sea urchin "*Tripenustes gratilla*" coelomic fluid using GC/MS revelaed the presence of five main compounds; namely, 4,6-dimethyldodecane, 2,6,11-trimethyldodecane, *n*-heptadecane, 2-methylheptadecane and 8-hexylpentadecan. They conistute the major identified compounds in the coelomic fluid *n*-hexane fraction, belonging mainly to fatty acid derivatives (Table 1). A scheme showing the predominant chemical conistiuents in the *n*-hexane fraction is illustrated in Fig. (28).

Table 1. Chemical compositions of *n*-hexane fraction obtained from *Tripenustes gratilla* coelomic fluid using GC/MS supplied with Rtx-5MS column

No.	Compounds[	R_I		% Composition	References
		Measured	Reported		
1.	4,6-Dimethyldodecane	1276	1285	1.69	MS, RI
2.	2,6,11-Trimethyldodecane	1322	1320	7.23	MS, RI
3.	*n*-Heptadecane	1704	1711	5.97	MS, RI
4.	2-Methylheptadecane	1744	1746	6.08	MS, RI
5.	8-Hexylpentadecan	2172	2045	4.66	MS, RI

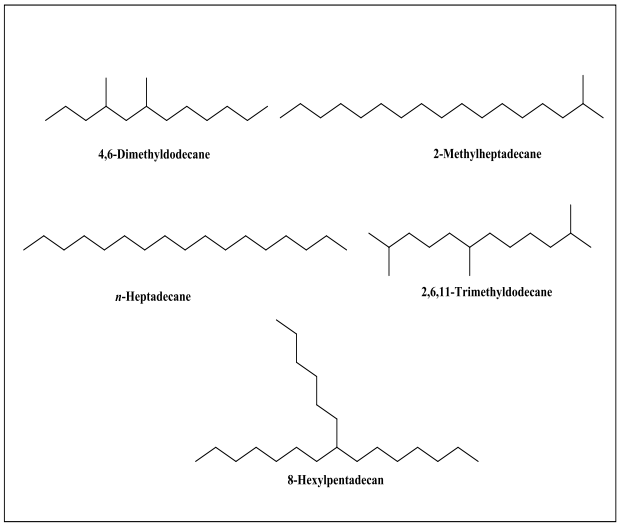


Fig. 28. Scheme showing the chemical compositions of *n*-hexane fraction obtained from "*Tripenustes gratilla*" sea urchin coelomic fluid using GC/MS supplied with Rtx-5MS column

In silico molecular docking studies

Results illustrated in Table (2) show that all the tested compounds exhibited the notable inhibitory potential of 8-hexylpentadecan and represent the best fitting within glycogen synthase kinase3-β protein enzyme active site, followed by 2-methylheptadecane, n-heptadecane and 4,6-dimethyldodecane, displaying free binding energies (ΔG) of –39.31, –38.81, –38.56 and –28.49 kcal/mol, respectively. Fig. (29A) indicate that 8-hexylpentadecan forms one π-alkyl bond with Tyr140, whereas *n*-heptadecane exhibited one alkyl bond with Ile62 (Fig. 29B). Meanwhile, 4,6-dimethyldodecane forms alkyl and π-alkyl bonds with Tyr134, Ala83, Cys199, Leu188, Leu132 (Fig. 29C). Besides, all tested constituents exerted Van der Waals's interaction, with the residues of the amino acid.

Table 2. Free binding energies (kcal/mol) of major compounds in the *n*-hexane fraction obtained from *Tripenustes gratilla* sea urchin coelomic fluid using *in silico* studies

Compound	Glycogen synthase kinase3-*β* protein	Number of formed Hydrogen bonds	Number of other formed bonds
4,6-Dimethyldodecane	–28.49	-	5 ; Tyr134, Ala83, Cys199, Leu188, Leu132
2,6,11-Trimethyldodecane	–28.47	-	3; Val70, Leu132
n-Heptadecane	–38.56	-	1 ; Ile62
2-Methylheptadecane	–38.81	-	3; Arg141, Tyr140
8-Hexylpentadecan	–39.31	-	1; Tyr140

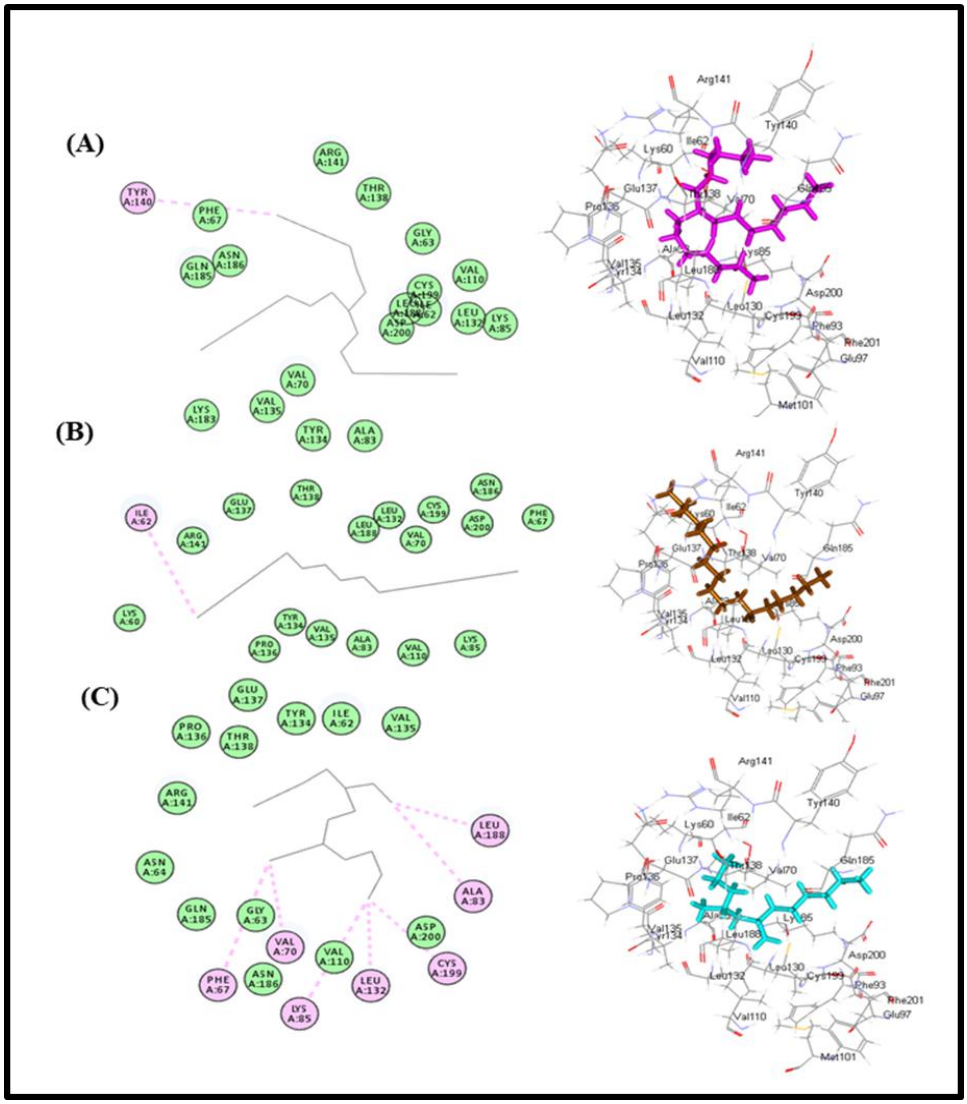


Fig. 29. 2D and 3D binding modes of 8-hexylpentadecan (A), *n*-heptadecane (B) and 4,6-dimethyldodecane (C) within the active sites of glycogen synthase kinase3-*β* protein.

C-2) Chemical compositions of *n*-hexane fraction obtained from the sea urchin "*Echinometra mathaei*" coelomic fluid using GC/MS

Chemical charcterization of the *n*-hexane fraction obtained from the sea urchin "*Echinometra mathaei*" coelomic fluid using GC/MS revealed the presence of five major compounds namely, 5,8-diethyldodecane, nonanoic acid, methyl ester, 2-methylheptadecane, *n*-octadecane and 5-*n*-butylhexadecane. They constitute the major identified compounds in the coelomic fluid *n*-hexane fraction belonging mainly to fatty acid derivatives (Table 3). A scheme showing the predominant chemical constituents in the *n*-hexane fraction is illustrated in Fig. (30).

Table 3. The chemical compositions of *n*-hexane fraction obtained from ***Echinometra mathaei*** coelomic fluid using GC/MS supplied with Rtx-5MS column.

No.	Compounds	R_I		% Composition	References
		Measured	Reported		
1.	5,8-Diethyldodecane	1491	1483	11.91	MS, RI
2.	Nonanoic acid, methyl ester,	1536	1536	9.96	MS, RI
3.	2-Methylheptadecane	1744	1746	13.67	MS, RI
4.	*n*-Octadecane	1786	1800	18.30	MS, RI
5.	5-*n*-Butylhexadecane	1917	1896	8.88	MS, RI

***In silico* molecular docking studies**

Results illustrated in Table (4) show that all the examined compounds exhibited notable inhibitory potential of 5-*n*-butylhexadecane, representing the best fitting within glycogen synthase kinase3-β protein enzyme active site, followed by nonanoic acid, methyl ester and 2-methylheptadecane displaying free binding energies (ΔG) of –41.98, –40.28 and –38.81 kcal/mol, respectively. 5-*n*-Butylhexadecane forms three π-alkyl bonds with Ile62, Arg141, Tyr140 at glycogen synthase kinase3-β protein binding site (Fig. 31A). However, nonanoic acid, methyl ester forms one H-bond with Tyr134 in addition to three C-H bonds with Pro136, Glu137, Arg141 (Fig. 31B). Regarding 2-methylheptadecane, it exerts three π-alkyl bonds with Arg141, Tyr140 with the active pocket of the enzyme (Fig. 31C). Besides, all tested constituents showed Van der Waals's interaction with the residues of the amino acid.

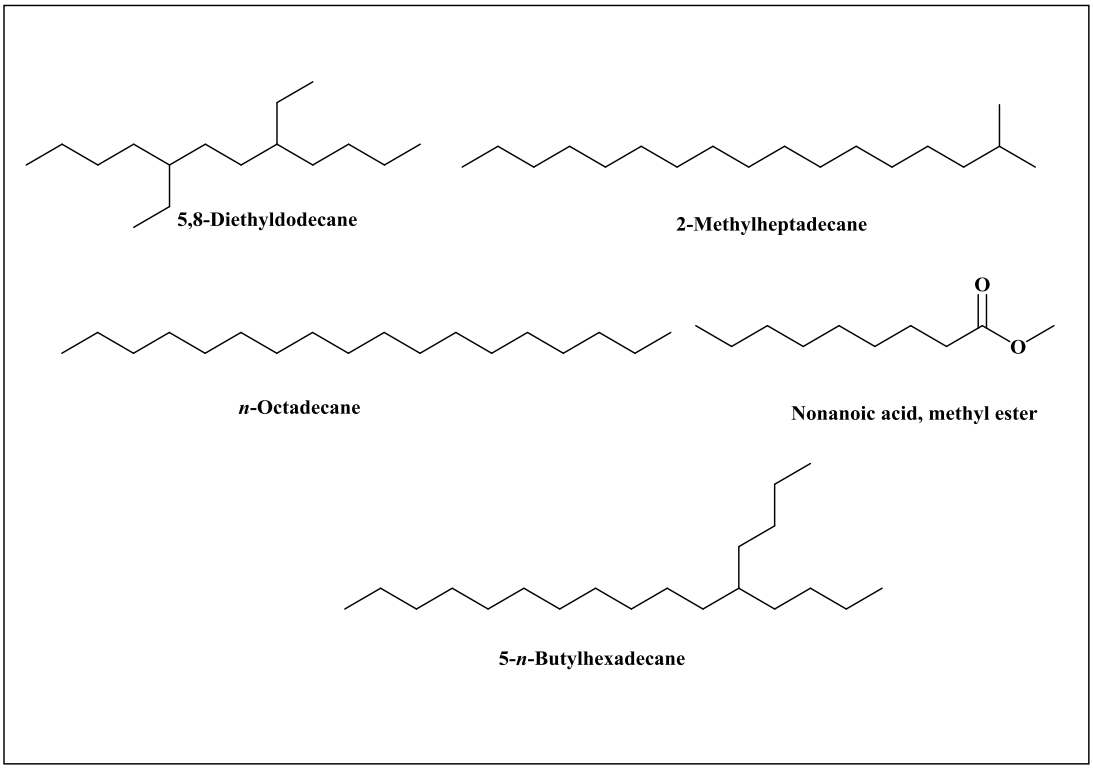


Fig. 30. Scheme showing the chemical compositions of *n*-hexane fraction obtained from ***Echinometra mathaei*** sea urchin coelomic fluid using GC/MS supplied with Rtx-5MS column.

Table 4. Free binding energies (kcal/mol) of major compounds in the *n*-hexane fraction obtained from ***Echinometra mathaei*** sea urchin coelomic fluid using *in silico* studies.

Compound	Glycogen synthase kinase3-*β* protein	Number of formed Hydrogen bonds	Number of other formed bonds
5,8-Diethyldodecane	–29.06	-	7; Phe67, Val70, Lys85, Leu132, Cys199, Ala83, Leu188
Nonanoic acid, methyl ester,	–40.28,	1; Tyr134	3; Pro136, Glu137, Arg141
2-Methylheptadecane	–38.81	-	3; Arg141, Tyr140
n-Octadecane	–29.88	-	2; Phe67, Val70
5-*n*-Butylhexadecane	–41.98	-	3; Ile62, Arg141, Tyr140

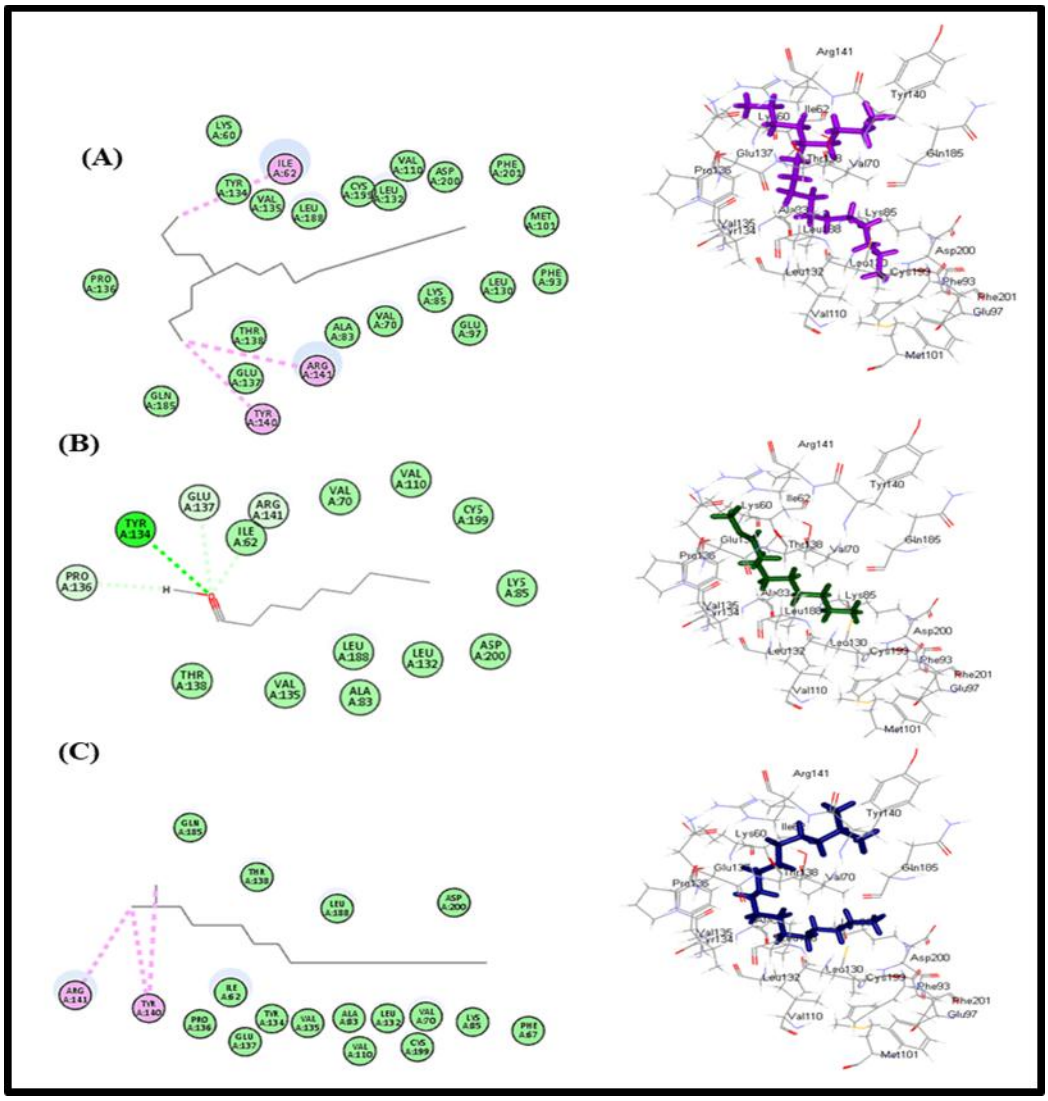


Fig. 31. 2D and 3D binding modes of 5-*n*-butylhexadecane (A), nonanoic acid, methyl ester (B) and 2-methylheptadecane (C) within the active sites of glycogen synthase kinase3-*β* protein.

DISCUSSION

The discoveries and importance of echinoderms exponentially grow day after day till it becomes crystal clear that this phylum principally exists to improve human health and sustain society's needs at the commercial, medicinal, and economical levels. The sea urchins are marine benthic invertebrates belonging to class Echinoidea which contains over 700 species worldwide **(Reich *et al.*, 2015; Luparello *et al.*, 2020)**. Both echinoderms and vertebrates emerged from the deuterostomes group and shared together some immunological genes **(Li *et al.*, 2015; Coates *et al.*, 2018)**. The sea urchins

"*Tripneustes gratilla*" and "*Echinometra mathaei*" are characterised by having globose and discoid bodies. Additionally, "*Tripneustes gratilla*" and "*Echinometra mathaei*" are the most popular echinoderms with voluminous haemocoel (coelomic fluid). By this study, we have reached the end of our list that contains the most popular examples of echinoderms. Moreover, this work and the previously-published articles (Kindly refer to **Abdel-Ghaffar and Youssef, 2022; Abdel-Ghaffar *et al.*, 2022, 2023**) give us plenty of choices to avoid overconsumption of certain species when applied as promising medicine for human benefits. In addition, these publications serve the benefits of human health, without affecting the species biodiversity due to overconsumption by relying on certain species, which might lead to become distinct or classified as endangered species if applied.

As far as we know, we own the privilege of studying the free coelomocytes and the coelomic epithelium "CE" ultrastructurally of both sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" in Egypt. By examining the coelomic epithelium of both sea urchins, we found that, both "*Tripneustes gratilla*" and "*Echinometra mathaei*" exhibit 4 main cells which are: 1) the basal cells; 2) short cells; 3) the elongated-wide cell, and 4) the elongated-narrow cell – as shown in Figs. (2–13) – and 1) small cells; 2) the red spherule; 3) the leucocyte, and 4) the colorless spherule – as shown in Figs. (15–24) –, respectively. Furthermore, the ultrastructure cross sections of the CE of both sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" revealed that the former animal cells are polygonal in shape, while the latter animal cells are amoeboid or irregular in shape. Although both the sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" differ in the main structural unit of the coelomic epithelium ultrastructurally, they exhibit almost the same ultrastructure of their free coelomocytes. Remarkably, the free coelomocytes of both sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" appeared in the amoeboid state, as shown in Figs. (14A–J) and Figs. (25–27), such as amoeboid/irregular forms resembling human phagocytic cells. The free coelomocytes of the sea urchin "*Tripneustes gratilla*" showed three distinctive forms: the leucocytes; the red spherules (type "1" and type "2"), and the colorless spherules. Although the obtained shape of the examined coelomic epithelia of the sea urchin "*Tripneustes gratilla"* showed a polygonal shape of the plasma membrane, the free coelomocytes extracted from the coelomic fluid showed an irregular/amoeboid shape opposing the definitive regular shape of the examined coelomic epithelia (Figs. 2–14). We attribute this hypothetically to the fact that cells are located in and moving in a fluid medium (haemocoel), in which they need to move faster and freely, like the amoeba, *i.e.* the shape meets the function. The free coelomocytes of the sea urchin "*Echinometra mathaei*" are named as small cells, leucocytes, secretory mucous cells (SMC), and secretory granular cells (SGC). Both coelomocytes that were identified as red spherule and colourless spherule are the same coelomocytes known as secretory granular and secretory mucous cells, respectively; the former coelomocytes are named according to **Chein *et al.* (1970)**, and the latter two cells

are named according to **Xing *et al.* (2008)**, **Gorshkov *et al.* (2009)**, **Sharlaimova and Petukhova (2016)** and **Sharlaimova *et al.* (2021)**; both coelomocytes nomenclature could be used interchangeably. **Xing *et al.* (2008)** illustrated that the small cells are called lymphocytes, and progenitor cells are named here as the young coelomocytes. At the same time, he called the mature coelomocytes "amoebocytes". It appears with different shapes and variable materials inside its own cytoplasm, depending on the heterogeneous materials of variable sizes they engulfed *via* amoeboid phagocytosis. Meanwhile, according to **Gorshkov *et al.* (2009)**, the mature secretory coelomocytes differentiate into secretory granular and mucous cells, representing two types of cells. In this work, we did not agree with the two opinions, we supposed that these small cells (progenitor cells) bearing clotting granular/mucus materials formed at the site of the animal injury **(Xing *et al.*, 2008)** harbor such secretory granules, whether granular or mucous secretion, to the site of injury and then transformed to mature or large coelomocytes variable in their size according to the type of the secretory material it bears and its quantity.

Ultrastructurally, the nuclear egress or the nuclear envelope budding is shown in the coelomic epithelial cells of both the sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" (Figs. 9, 18–21). **LaMassa *et al.* (2017)** published a research article on the phenomenon of nuclear egress in the sea urchin gastrula "*Strongylocentrotus purpuratus*", but in the present study, we documented such phenomena in the coelomic epithelial cells of both adult sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*". The authors stated that nuclear egress allows large macromolecules to reach the cytoplasm by budding from the nuclear membrane. The CE of *Tripneustes gratilla* showed circular mitochondria with semi-circular cristae, while the CE of *Echinometra mathaei* showed elongated mitochondria. Interestingly, the morphology of the mitochondrial cristae in both "*Tripneustes gratilla*" and "*Echinometra mathaei*" are unique and unusual to our knowledge, and this might be due to the scarce ultrastructure studies on echinoderms especially, and invertebrates generally. A recent study by **Crabtree *et al.* (2023)** mentioned the changes in the morphology of the mitochondrial cristae, but actually, it does not meet our obvious results since it represents the results of pathological alterations of mammalian animals.

It is worth mentioning that, the sea urchins have survived in regard to their enhanced immunity (**Chiaramonte *et al.*, 2019; Moreno-García *et al.*,2022)**. Besides, sea urchins have been used as a tratment for cardiovascular diseases and cancer **(Rahman *et al.*, 2015)**. The overconsumption of sea urchins refers to their wide spectrum range of treatment as antibiotic, antiviral, antiprotozoal, and antifungal **(Luparello *et al.*, 2020)**. The chemical constituents of these valuable compounds differ largely among the variable species of sea urchins **(Stabili *et al.*, 2018)**. Recently, **Sibiya *et al.* (2021)** innumerated the medicinal characteristics of sea urchins in cancer treatment and their efficacy as

antioxidant, anticoagulant, and antibacterial; however, other properties, as our concern in wound healing and other important pharmacological properties, are still disregarded.

One of the disregarded properties of the sea urchins is the medical importance of the coelomic fluid. Recently, numerous research papers proved that different echinoderms exhibit GSK-3β protein, which is essential for wound healing **(Abdel-Ghaffar & Youssef, 2022; Abdel-Ghaffar *et al.*, 2022, 2023)**. Accordingly, other publications stated that the coelomic fluids enhanced the immunity of the animal *via* providing proteins with haemolytic and haemagglutinating properties **(Soboleva *et al.*, 2018)**. Haemagglutinin strengthened and improved the animal immunity enabling the animal to face the environmental changing factors **(Oren *et al*., 2019)**. Also, according to **Brusca *et al.*, (2016); and Pinsino & Matranga (2014),** the coelomic fluids exhibit activating factors enabling coelomocytes formation. Accordingly, the coelomic fluids improved the animal immunity. Furthermore, the enhanced immune system conjugates with other cellular and molecular mechanisms, for example in case of thermic shock and metallothioneins, and in case of serious harm, they showed efficacious potentiality for apoptosis and autophagy if regeneration is needed **(Chiarelli *et al.*, 2016)**. Collectively, sea urchins generally develop complex molecular elements to use in diverse biological processes; hence, as benthic organisms, they are also considered as a rich source of bioactive compounds, with the potential for the discovery and development of new pharmacological compounds **(Luparello *et al.*, 2020; Strahsburger *et al.*, 2020)**.

Chemically, the proteins resulting from the GC/MS data from the extracted coelomic fluid of both adult sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" in this work led to the identification of five main compounds in both sea urchins, which are: 4,6-dimethyldodecane, 2,6,11-trimethyldodecane, *n*-heptadecane, 2-methylheptadecane and 8-hexylpentadecan (Table 1) in addition to 5,8-diethyldodecane, nonanoic acid, methyl ester, 2-methylheptadecane, *n*-octadecane and 5-*n*-butylhexadecane (Table 3), respectively. The metabolites identified in the adult sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" coelomic fluid (results are presented in Tables 2, 4) revealed that 8-hexylpentadecan and 5-*n*-butylhexadecane showed the best fitting within the active site of glycogen synthase kinase3-*β* protein, respectively, in the coelomic fluid extracts from both sea urchins; these protein factors were obtained from the protein docking analyses. Additionally, it might be considered as a promising constituent for many medicinal applications including wound healing **(Abdel-Ghaffar & Youssef, 2022; Abdel-Ghaffar *et al.*, 2022, 2023)**, especially for diabetic patients. Based on this finding, the coelomic fluids of the sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" could be used as an effective potent agent for wound healing or skin regeneration **(Harish *et al*., 2008; Naika *et al*., 2015; Aksoy *et al.*, 2021, Abdel-Ghaffar & Youssef, 2022; Abdel-Ghaffar *et al.*, 2022, 2023)** due to its characteristic glycogen synthase kinase3-*β* protein, which is identified by the protein docking (Table 2, 3 & Fig. 7A, B) of

this work. In their study, **Soleimani *et al.*** (2021) worked on the coelomic fluid of a certain type of sea urchin and listed it as a new perspective of medicinal antioxidants.

CONCLUSION

Ultrastructurally, the coelomic epithelium from both adult sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" showed 4 different units of coelomocytes, in which the coelomocytes of "*Tripneustes gratilla*" are elongated and polygonal, while the coelomocytes of *Echinometra mathaei* are morphologically irregular. Additionally, the free coelomocytes from both adult sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" are irregular in shape, resembling the human phagocytes, *i.e.* the shape meets the function. Chemically, the coelomic fluids extracted from both adult sea urchins "*Tripneustes gratilla*" and "*Echinometra mathaei*" exhibit glycogen synthase kinase3-β protein that could be used as an effective or potent agent for wound healing or skin regeneration.

Abbreviations:

H&E: Haematoxylin and Eosin. **CE**: Coelomic epithelium. **SMC**: Secretory mucous cells. **SGC**: Secretory granular cells. **SC:** Small Coelomocytes/Cells. **GSK3-** β: Glycogen synthase kinase3-β protein.

Author contribution statement:

Abdel-Ghaffar WH: Conceived, designed, and performed the experiments; Abdel-Ghaffar WH, and Youssef FS: analyzed and interpreted the data; contributed materials, and wrote the paper equally in the area of interest.

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