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Sea Cucumber "*Holothuria (Thymiosycia) arenicola*" induced-autotomy for sustainable development in Egypt: Histological, Ultrastructure, and Chemical studies.

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ABSTRACT

The importance of Echinoderms in Egypt grows day after day, as well as, the usage of the available untapped resources, in particular our interest in mariculture. Sea cucumber fishing was prohibited in Egypt and listed as endangered species, due to overfishing. Although the above decision was right at its time, it is not acceptable to us, due to the economic, nutritional, and pharmaceutical importance of the sea cucumber. So; this work was designed to study the animal well microscopically, apply the artificial induction of autotomy on the animal for the numerical increase and reproduction asexually, and chemically investigate the medical importance of the coelomic fluid of the sea cucumber "*Holothuria arenicola*". The body wall and the coelomic epithelium were studied microscopically. For the animal numerical increase, the autotomy was induced in 3 groups by manual ligation of the body from ¼, ⅓, and ½ of the whole body length of the adults; respectively, in addition to the control group. Side by side with the abovementioned studies, the acellular chemical content of the coelomic fluid was analyzed using GC/MS, and the resulting constituents were furtherly-analyzed by protein docking to investigate the ability of the coelomic fluid to accelerate wound healing. Histologically; the body wall of the sea cucumber's "*Holothuria arenicola*" is composed of five layers. Ultrastructurally; the coelomic epithelium small (progenitor) cells (coelomocytes) seem to be hypothetically differentiated into two main types: the neighboring/surrounding cells (that might supply the neighboring dividing cells with the membranes or any cytoplasmic organelles, hypothetically *via* exocytosis or endocytic recycling trafficking events or by plasma membrane remodeling), and defense cells that resemble the human neutrophils. Experimentally, the artificially-induced autotomy of the half-body-length ligated group is the only successful group that divides completely. Chemically, we found the presence of bioactive GSK3-β protein in the sea cucumber-extracted coelomic fluid which makes them a valuable resource for wound healing treatment especially in diabetics. Finally, the sea cucumber "*Holothuria arenicola*" is considered a gift for Egypt, due to its proven significant potential for economic and medical applications; both criteria of significance are concomitant with the sustainable developmental goals and Vision of Egypt 2030.

INTRODUCTION

Sea cucumbers are marine invertebrates with long, cylindrical bodies that resemble cucumbers. Just like sea urchins, starfish, and sand dollars, they are classified as Echinodermata. Sea cucumbers are known for their economic and health benefits, making them valuable organisms for researchers and businesses. Known to have medicinal properties, these organisms have been harvested and studied for centuries. One of the most known species in Egypt is *Holothuria arenicola*, the animal of interest in this study. *Holothuria arenicola*, also known as the donkey dung sea cucumber (**Semper, 1868**), contains bioactive substances with potential medicinal benefits, making it one of the most economically significant species eaten owing to its high nutritional value.

Holothuria arenicola, specifically, has gained attention due to its high growth rate, tolerance to different environmental conditions, and high nutritional value. Traditional Asian medicine makes use of this species' extracts as an anti-inflammatory, anti-coagulant, and wound-healing agent, and its meat is considered a delicacy in many Asian nations. **(Purcell *et al.*, 2016).** As a result of rising consumer demand, aquaculture initiatives have been established to cultivate *Holothuria arenicola*, providing a sustainable food source and boosting local economies in coastal regions. **(Yahel *et al.*, 2005)**.

Sea cucumbers are known for their bioactive compounds and high nutritional value. Glycosaminoglycans like chondroitin sulfate, fucans, triterpene glycosides, sphingoid bases, and peptides are among the bioactive compounds found in these foods. These compounds have been linked to a variety of pharmacological activities, including anti-cancer, anti-tumor, anti-inflammatory, anti-viral, immunomodulatory, and anti-oxidant effects (**Kim and Wijesekara, 2010; and Kang *et al*., 2011**). In addition, sea cucumber extracts have been shown to have beneficial effects on various disorders such as arthritis, gastric ulcers, and wound healing **(Yang *et al.*, 2011; and AOAC International, 2005).**

The mariculture of sea cucumbers occurs by two methods either sexually or asexually *via* autotomy. Beginning in May, gametogenesis allowed for sexual reproduction, with the months of June and July being the peak of spawning for both sexes (**Omar *et al.*, 2013; and Venâncio *et al.*, 2022). Mladenov (1996), and Dolmatov (2014)** published detailed reviews of asexual reproduction.

Dry sea cucumbers are the most common kind of sale. Popular in Asia because of their nutritional and therapeutic value, they are known all over the world by the name "bêche de-mer." Prices per kilogram of dry weight fall between €43 and €348. Inexplicably, the price of high-quality species like *Holothuria (Metriatyla) scabra* is more than €1593/kg ($1800/lb) **(Purcell *et al.*, 2018).** Overfishing of natural supplies is a serious problem in many regions of the globe due to the rising demand for this seafood commodity, necessitating strong protection initiatives **(Toral-Granda *et al.*, 2008)**. As a result of overexploitation, natural populations of more than 70 species are on the decrease

(Meloni and Esposito, 2018; and Purcell *et al.*, 2018), which has increased demand for this product in several areas, including the Mediterranean and the Northeastern Atlantic.

By examining the sea cucumber "*Holothuria arenicola*" from a variety of angles, this effort hopes to highlight the creature's critical significance. First: histologically, examining the body wall. Then, ultrastructurally; studying the coelomic epithelium and coelomocytes. Expermintaly, investigating the ability of the selected species of the sea cucumber "*Holothuria arenicola*" to increase numerically *via* mariculture using artificial-induced-autotomy as a type of mariculture application for sustainable development in Egypt. Finally, chemically; extracting the coelomic fluid and prepared the acellular part for GC/MS analyses. The chemical constituents obtained by the latter technique; were furtherly-examined using protein docking to investigate the ability of the coelomic fluid to accelerate wound healing, especially for diabetics.

MATERIALS AND METHODS

1- Materials:

A-Chemicals: Analytical and pure-grade chemicals, solvents, and reagents were employed throughout this investigation.

B-Animals: Twenty-four individuals of adult sea cucumbers ranging in length from 15 cm to 27 cm were hunted from Ras Sedr, Red Sea, Egypt. The collected specimens were identified by **Prof. Dr. Ahmed Mitwaly Hellal** (Zoology Department, Faculty of Science, Al-Azhar University "Boys-Branch") and **Prof. Dr. Magdy T. Khalil (**Zoology Department, Faculty of Science, Ain Shams University**)** according to **Semper (1868)** as ***"Holothuria (Thymiosycia) arenicola*".** Also, a confirmatory method based on the ossicles was used to identify the animal in addition to its morphology using ossicles as shown in Methods 2A-1.

Kingdom	Animalia
Phylum	Echinodermata
Class	Holothuroidea
Order	Aspidochirotida

Family	Holothuriidae
Name	*Holothuria arenicola*
	H. arenicola
sea cucumber	

2-Methodology:

A- Samples collection, morphology, histology, ultrastructure (TEM) of the coelomic epithelium and coelomocytes, and the practical experimental design of artificial-autotomy-induction in the sea cucumber *Holothuria arenicola*:

Adults of the sea cucumbers were collected from the subtidal region of Ras Sedr in the Gulf of Suez, Red Sea, Egypt (global positioning system (GPS-GP80) is **30°05'17.1"N** and **31°20'13.6"E**). We try to collect any specimens from Hurghada, Red Sea, Egypt, for two seasons but this did not successfully accomplish.

1- Specimens were identified by taking a small part of the body wall and identifying the ossicles or spicules according to the procedure of **Wirawati *et al*. (2007),** and confirmed by **Prof. Ahmed Mitwaly Hellal** and **Prof. Magdy T. Khalil;** as previously mentioned. One cm^2 of the body wall was rinsed and placed in a test tube with domestic bleaching as Clorox, genital heat for 5 min, remove the supernatant and pour the bottom of the test-tube into a watch glass in which the spicules exist. Then, the 70% ethyl alcohol was added to the obtained spicules for more bleaching and preservation too. By using a dropper on a clean slide, the spicules were identified microscopically, and photographed.

2- Paraplast blocks were produced after processing the tissue samples of the body wall that were fixed in 10% formalin. Standard histological stains; including haematoxylin and eosin (H&E) and Mallory trichrome (MTC), were used to examine all levels of the animal's body wall.

- The obtained histological slides of the sea cucumber "*Holothuria arenicola*" body wall that stained with routine H&E or MTC; were examined and photographed using a Leica DM1000 LED Research microscope for the examination of semithin Toluidine blue stained slides in a transmitted light bright field. The microscope is supported by Leica FlexaCam (C1 12M 4K/FHD) camera and image Morphometry system (LASX software).

3- The coelomic epithelium was prepared for TEM in addition to the drained coelomocytes using a syringe as described in section "2B", in which the cellular part was examined using TEM.

4- Animal groups for asexual reproduction "artificial-induced-Autotomy": After hunting 24 adult specimens of the sea cucumber *Holothoria arenicola*; the acquired specimens were collected in the morning and sent to the laboratory of the Zoology Department, Faculty of Science, Ain Shams University within three hours using acrylic containers fitted with oxygen pumps and filters. Immediately after hunting, the specimens were distributed into four groups, six specimens per each group, and the length was measured then the autotomy was induced by using hard wool threads for ligation around the body in different zones:

- The control group: No ligation was initiated, as shown in Figure "**5A**".
- The second group: In which the ligation was initiated at ¼ of the total body length **(Conand *et al.*, 1997);** shown in Figure "**5B**".
- The third group: the ligation was initiated at ⅓ of the whole-body length **(Conand *et al.*, 1997);** shown in Figure "**5C**".
- The fourth group: in this group, the ligation was initiated at ½ of the whole body of the animal **(Dolmatov *et al.*, 2012);** shown in Figure "**5D**".

- Bottles of sea water in addition to the sea sediments were translocated from the original habitat at Ras Sedr to be delivered to the laboratory of Ain Shams University, mimicking its original habitat.

B. Coelomic fluid extraction:

The preparation of the sea cucumber "*Holothuria arenicola*" coelomic fluid (CF) was done by applying the same procedure described by **Baveja *et al.* (2018 and 2019)**; additional details were published by **Abdel-Ghaffar and Youssef** (**2022**). After centrifugation; the pellet was prepared for microscopical examination, and the supernatant that represents the acellular part was prepared for GC/MS application and protein docking.

1-The preparation of the separated and processed tissue specimen and cellular content of the coelomic fluids for microscopical examination:

- The isolated cellular part was processed for ultrastructure by applying the preparation procedures for transmission electron microscope (TEM) according to **Williams, and Carter (2009)**.

- Finally, the stained ultrathin grids of the coelomic epithelia and coelomocytes were examined and photographed using a JEOL 1200 EX II Electron Microscope, E. M. Unit, at the Faculty of Science, Ain Shams University.

2-Chemical compositions of *n*-hexane fraction obtained from sea cucumber "*Holothuria arenicola*" coelomic fluid using GC/MS

Analyses by GC/MS were carried out using a Shimadzu GCMS-QP 2010 (Shimadzu Corporation, Koyoto, Japan) equipped with a Restek Rtx-5MS (30 m 0.25 mm i. d. 0.25 m film thickness) capillary column (Restek, PA, USA). Compounds were identified after a multi-step process (please see **Abdel-Ghaffar and Youssef (2022)** for more information) based on retention indices relative to a series of *n*-alkanes (C8-C28) injected under identical conditions, and by comparing mass spectra of detected compounds to those recorded in the Wiley library database and the National Institute of Standards and Technology (NIST) **(Youssef *et al.*, 2014; Ayoub *et al*., 2015; Mamadalieva *et al.*, 2019; and Youssef *et al.*, 2021).** The GC/MS analysis was performed at the GC/MS facility at the Department of Pharmacognosy in the Faculty of Pharmacy at Ain Shams University.

C- *In silico* molecular docking studies

Glycogen synthase kinase3-β protein (PDB ID 5K5N; 2.20), which is thought to be among the enzymes implicated in the process of wound healing, was the target of the molecular docking analysis performed on the major chemical constituents detected in the *n*-hexane fraction obtained from sea cucumber *"Holothuria arenicola"* coelomic fluid using GC/MS. Docking experiments were performed with Discovery Studio 4.5 (Accelrys Inc., San Diego, CA, USA) using the C-Docker protocol, as previously reported **(Labib *et al.*, 2017; Thabet *et al.*, 2018; Talaat *et al.*, 2018; and Altyar *et al.*, 2020)**, with binding energies (G) determined using 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 morphology, histology, ultrastructure (TEM) of the coelomic epithelium and coelomocytes, and the practical experimental design of the induced- autotomy in the sea cucumber *Holothuria arenicola*:

1-**Samples morphology:**

- Morphologically, the colour of the live specimens ranged from cream to brown, with paired dark brown or black spots forming longitudinal series dorsally **(Figure 5A)**.

- The superficial layer covering the body wall exteriorly is characterized by the presence of ossicles or spicules. By microscopical examination; it was found that these ossicles formed from different shapes known as tables, buttons, and rods; which are unique features for each species specimen, and based on these forms; the species was precisely determined. The spicules are differentiated into: Tables (Figures **1A-C**) bearing square smooth discs with four large central holes (Figure **1D**). In addition, narrow disc rims are characterised by a ring of 4-8 smaller holes around their periphery (Figure **1E**). The buttons are small with 3-4 pairs of holes (rarely 4 or 5 pairs as shown in Figure **"1 J"** of comparatively large holes and regular in outline). Figures **"1 F-I"** depicted various rods used for structural support.

2-The histology of the body wall of the sea cucumber "*Holothuria arenicola*" (H&E- and MTC-stained sections): the microscopical examination of processed tissue specimens of the body wall from the individual animals of the sea cucumbers stained by H&E and MTC revealed: The body wall integument is formed of ridges and furrows (Figs. **2A, 3A, 3E-H**).

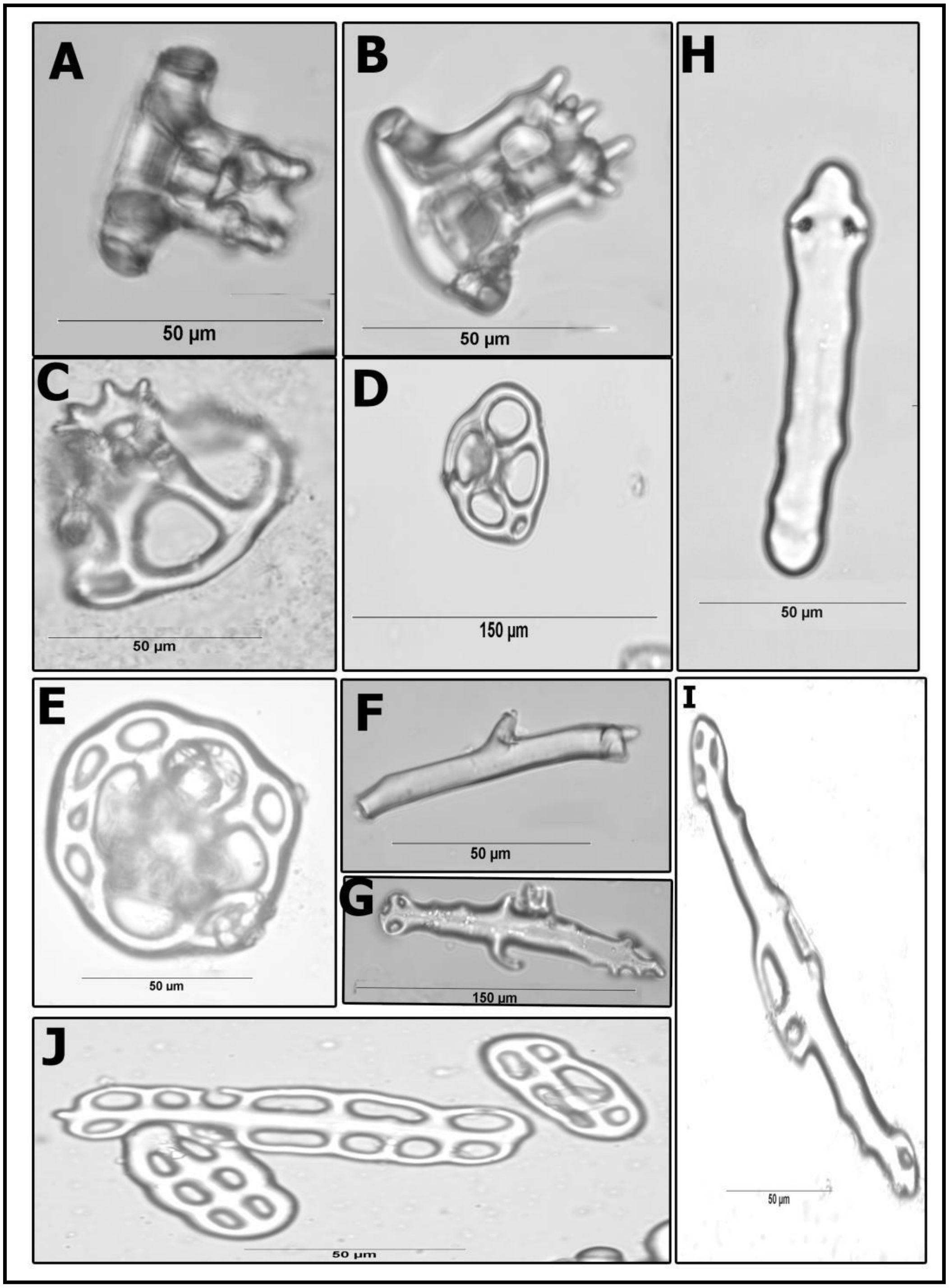


Fig. 1(A-J): Photomicrographs illustrate the unstained ossicles extracted from the sea cucumber "*Holothuria arenicola*". **A-C**) represent the tables. **D&E)** shows the discs that are smooth and square in the outline; usually with 4 large central holes and 4-8 regular, small peripheral holes. **F-I)** Supporting rods. **J)** Buttons. A-C, E-F, H-J) X 1000. D&G) X 200.

The integumentary tissue of *Holothuria arenicola* has the following layers:

a-**The Cuticle:** The cuticle, a thin acellular coating covering the body, stands in for the spicules. The latter were seen dispersed throughout their integument, staining brown with MTC. Additionally, reservoir sacks beneath the dermis and in between the circular and longitudinal muscles contain brown entities, which cluster and seem like brown granules without a discernable nucleus or cytoplasm when stained with MTC (Figs. **3Q & 3R**).

b-**The epidermis:** single layer of squamous cells (Figs. **2A, 3A& 3E-H**).

c-The dermis and superficial dermis: represent the subepidermal layer of the sea cucumber's integument, which is mostly made of connective tissue (Figs. **2A-B. 3A-B, & 3E-K**). Small fibroblasts, long oval bipolar cytoplasmic developments, basic substances, and collagen fibres grouped in regular or irregular nuclei make up the connective tissue of the deep dermis. Based on the relative abundance and distribution of its constituents, this tissue may be classified into two broad categories: One, tight dermis; two, slack dermis (deeper). After the tight dermis comes the slack dermis. After the layer of connective tissue, the layer of circular smooth muscles appeared.

d-The circular smooth muscle: delimits the dermis with several elastic fibres (Figs. **2B-C, 3B-D. & 3L-R**).

e-The longitudinal skeletal muscles: this layer contacts with the coelom and the coelomic fluid, and is shown as crypts (Figs. **2C-J, 3C-D**, and **3O-U**). The crypts are lined with mesothelial cells (peritoneum, Figs. **2D-E, 2J, 3Q, 3I,** & **3S-U**). The crypts are formed of several rachis (axis) rays around which the segments of the skeletal muscles are distributed in an organized manner for mechanical contraction and relaxation.

2-**TEM of the Coelomic Epithelium and Coelomocytes:**

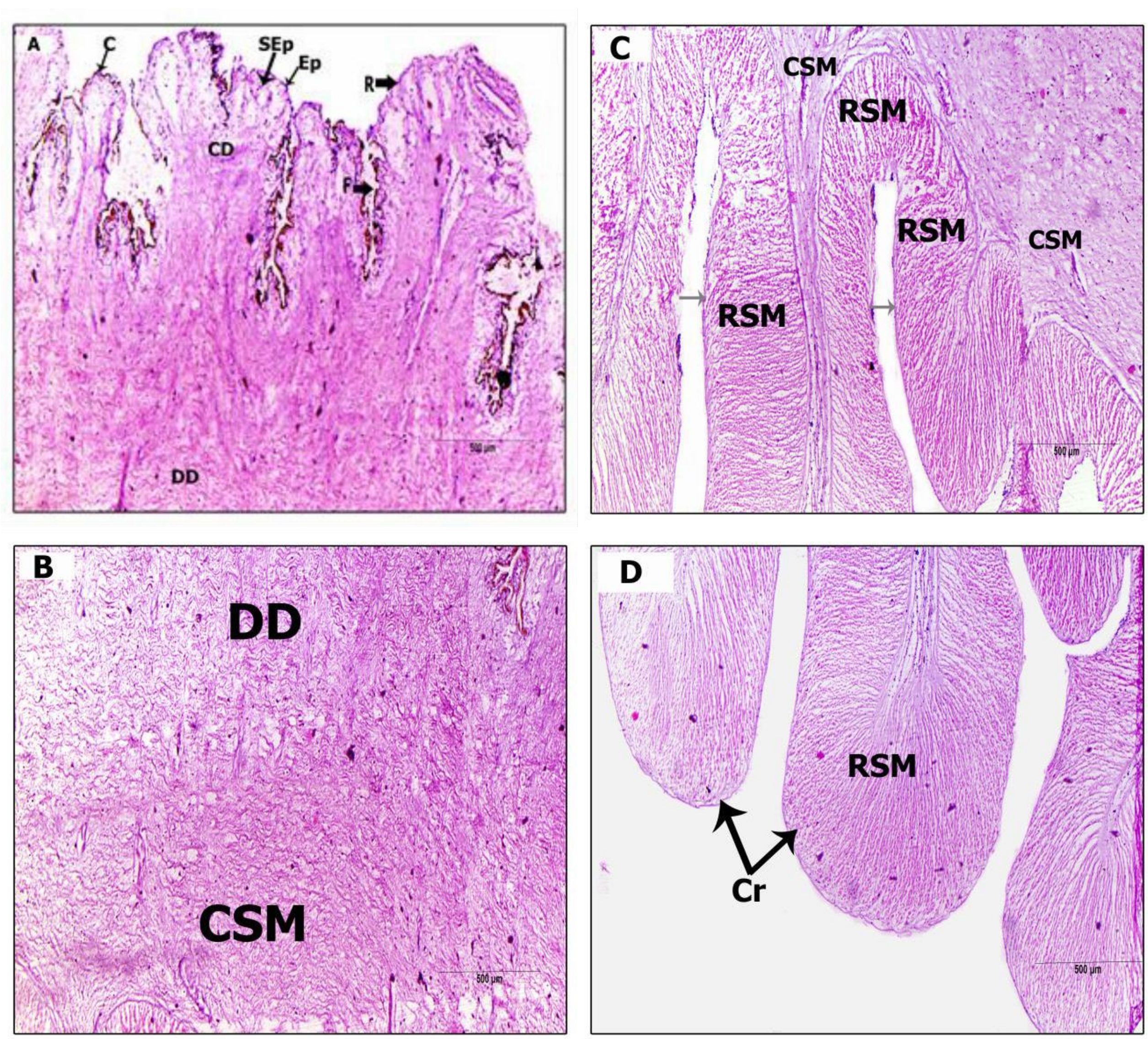
The examination of the coelomic epithelium of the sea cucumber using the TEM showed that it is mainly composed of two types of cells, numerous small cells (progenitor cells) – that formed most of the tissue –, and other large cells (Fig. **4A-I).** As shown in Figs. **4A** to **4I**, the SCs mainly exhibit different shapes and sizes with circular nuclei. This type of cell is furtherly divided into two main types: the neighboring/surrounding cells (that hypothetically might supply the dividing cells with the membranes or any cytoplasmic organelles, Fig. **4F**), which showed cell lengths ranging from 4.96*8.27 µm to 5.97*19.82 µm (and their cytoplasm is highly loaded with membranes and vacuoles & others with blebbing nuclear envelope, Figs. **4C&E**). The nuclei of this type showed thin heterochromatin underneath the nuclear envelope with some patches alternated with the euchromatin (as shown in Figs. **4A-G**). The second type of these small cells is the defence cells that resemble the human neutrophil, with thickened peripheral heterochromatin (as shown in Figs. **4H & I**), and length = 4.58*6.65µm.

The large cells are also divided into two subtypes defined as which as secretory granular and secretory mucous cells (Figs. **4B, C, D, & F**). The obtained measurement of the secretory mucous cells as shown in Figs. **4C & D** recorded 16.57*11.18 µm.

Meanwhile, the secretory granular cells showed a length of 7.73*8.30 µm. As shown in Figure **4H**, the outermost cells detached from the outermost and escaped to act as free coelomocytes. The free coelomocytes might scavenge the surrounding as shown in Figure **4J.**

3-**The autotomy induction of *Holothuria arenicola:***

This experiment was handled for 3 weeks as shown in **Table "1"**, starting from the day of collecting the adult animals from the seawater. In this experimental trial, the only group that induced autotomy and divided was the fourth group, and the first result was recorded from the sixth day till the fifteenth day. All the animals of the 4th group were successfully divided, using the proper supplementation of the seawater and sediment imitating the natural habitat of the sea cucumber. The 2nd and 3rd groups were left for one week additionally, just in case of obtaining division, but it did not successfully accomplish. Unfortunately, we start to notice the animal deaths among these two groups (2nd and 3rd groups) of the experiment; so we decided to cease the experiment to reduce the mortality rate among the experimental groups, hence the main purpose is to duplicate the number and not vice versa.



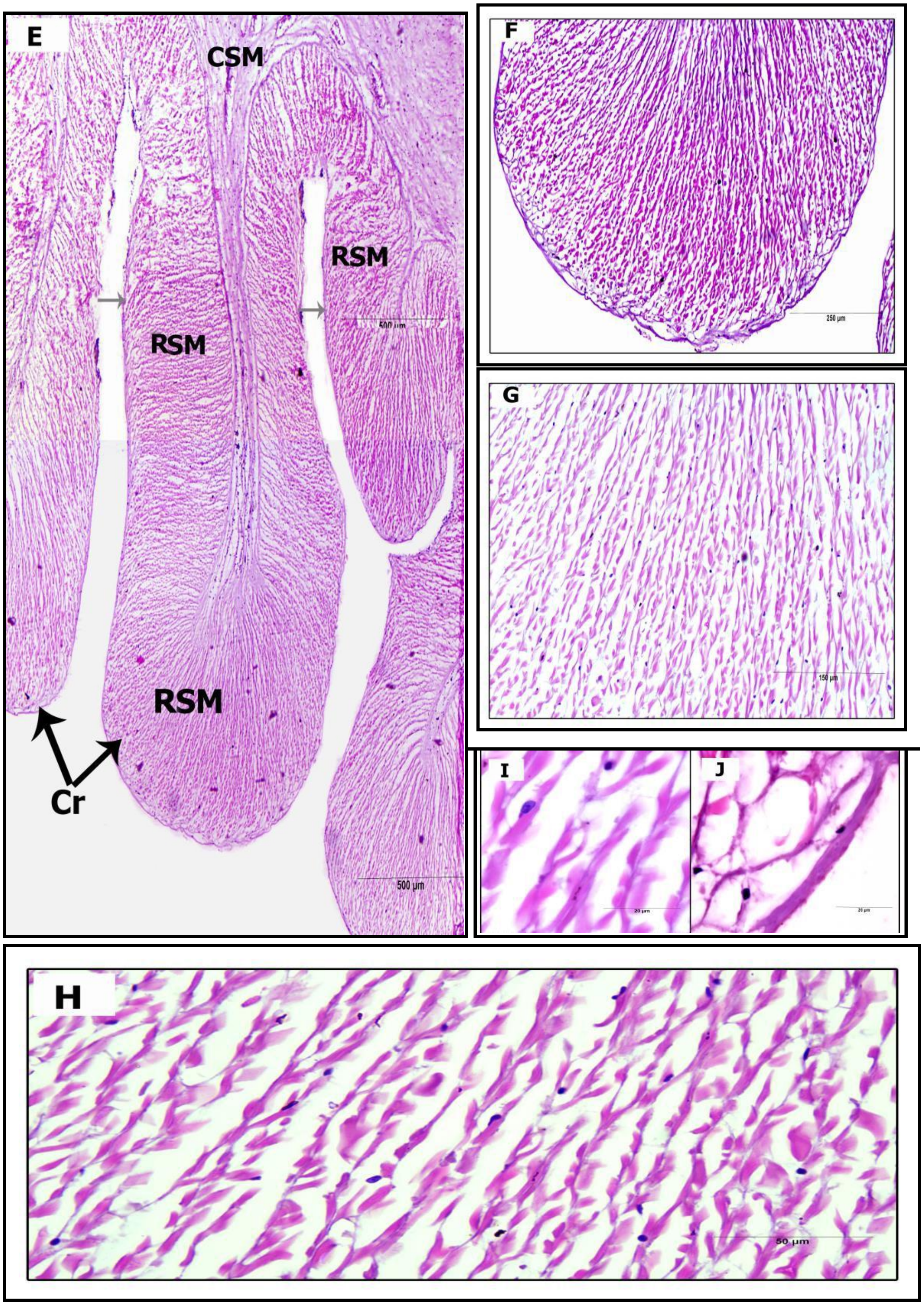
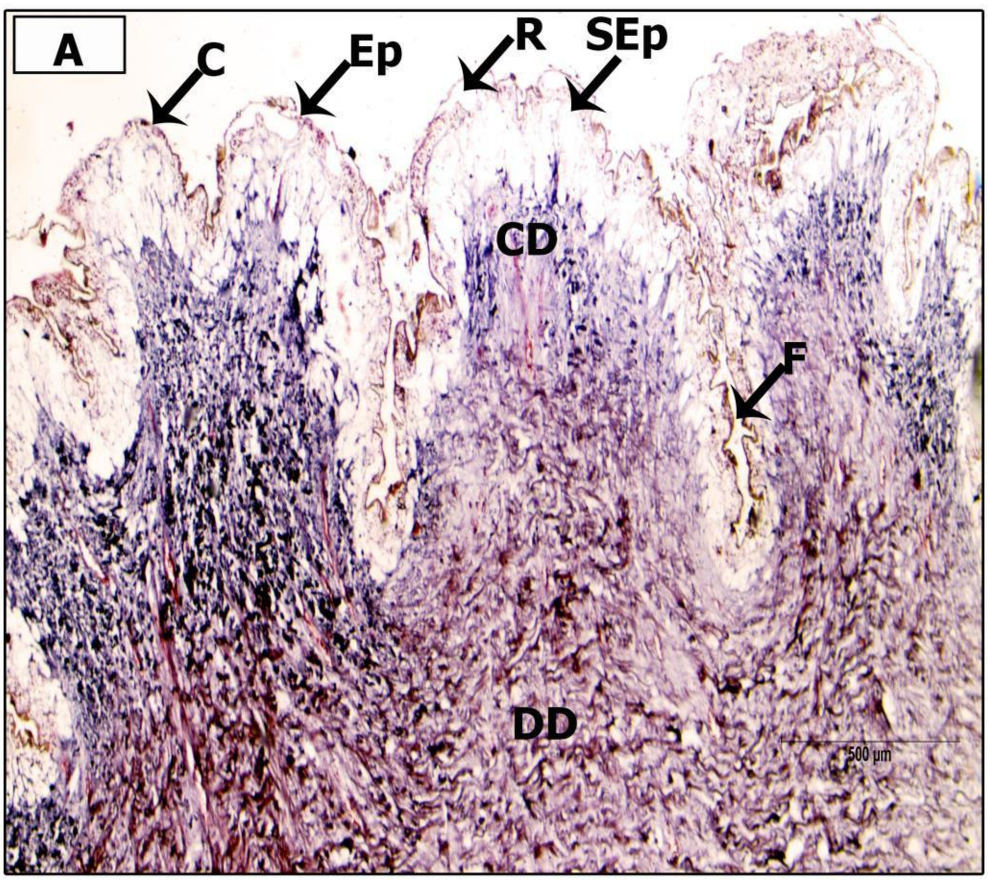
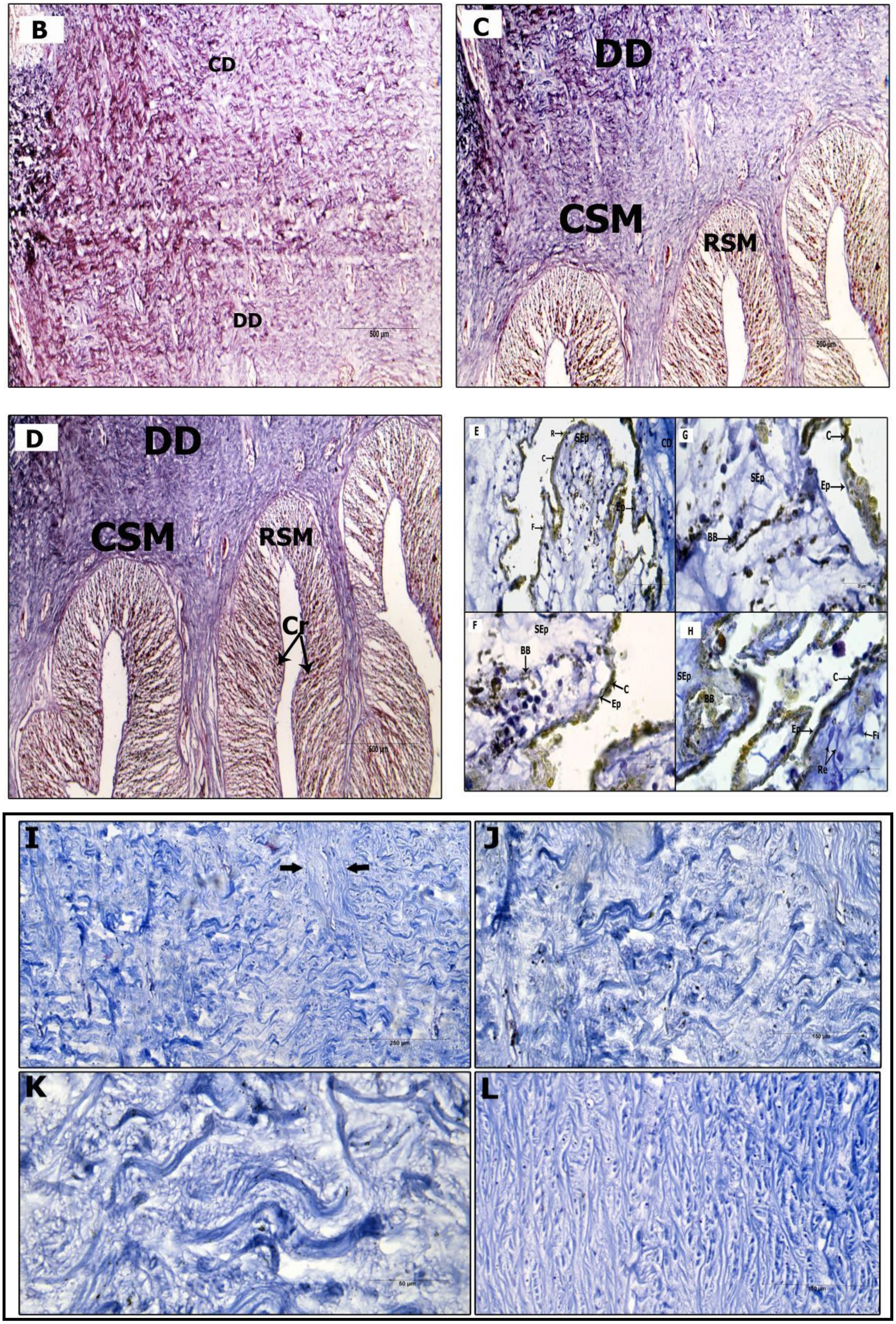


Fig. 2(A-J): Photomicrographs represent sections of the body wall of the sea cucumber "*Holothuria arenicola*" stained with H&E. A-D) shows that the outermost layer is composed of ridges "R", and furrows "F", and known as cuticle "C". The latter is covered by spicules. A single epidermis "Ep" layer lies underneath the cuticle and is followed by the sub-epidermis "SEp", which is composed of connective tissue. Then, the dermis is differentiated into condensed dermis "CD" and deeper dermis "DD". Underneath the dermis lies a layer of circular smooth muscle "CSM". C&D represents the muscular layers of the body wall which is composed of CSM followed by crypts "Cr" of radial striated muscles "RSM". X 40. E) shows a merge of Photomicrographs C&D, to illustrate the finger-like projections "crypts of the muscular system of the body wall of the sea cucumber, in which the CSM penetrates its central axis perpendicularly, and runs inside, and the striated muscles arrange radially around its axis. X 40. F-J) shows different magnifications of the radial arrangement of the striated muscles, *i. e.* RSM. **F)** shows the mesothelial cells (peritoneum) that line contact with the coelom and the coelomic fluid. **H&I**) illustrated the peripheral nuclei of the striated muscles (violet stain), and the spaces in between the pieces of the striated muscles. allow its characteristic mechanical movement of contraction and relaxation. **J)** Illustrates high magnification of RSM and shows the thickness of the crypt lining. X 100, 200, 400, 1000, & 1000; respectively.

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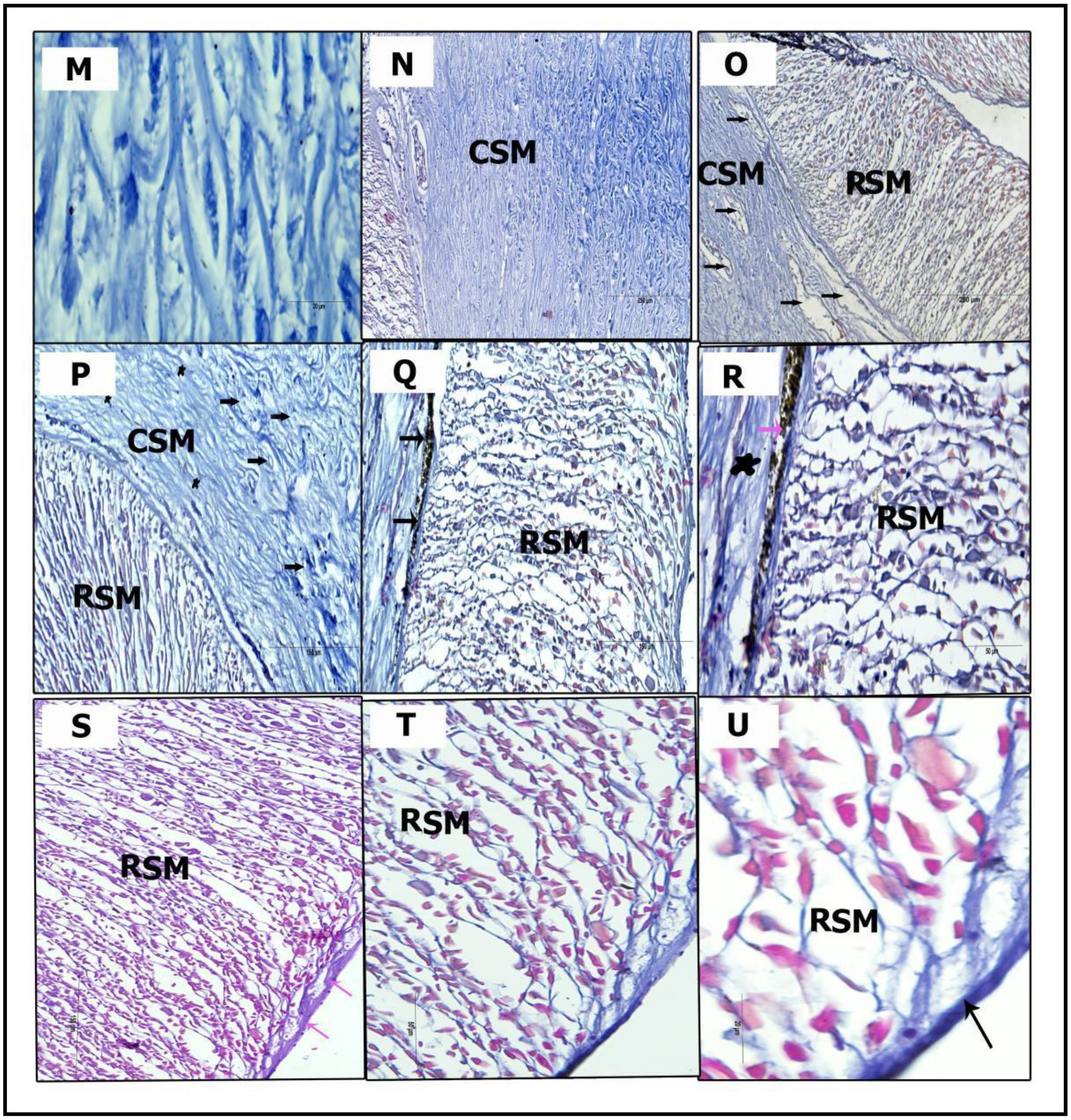
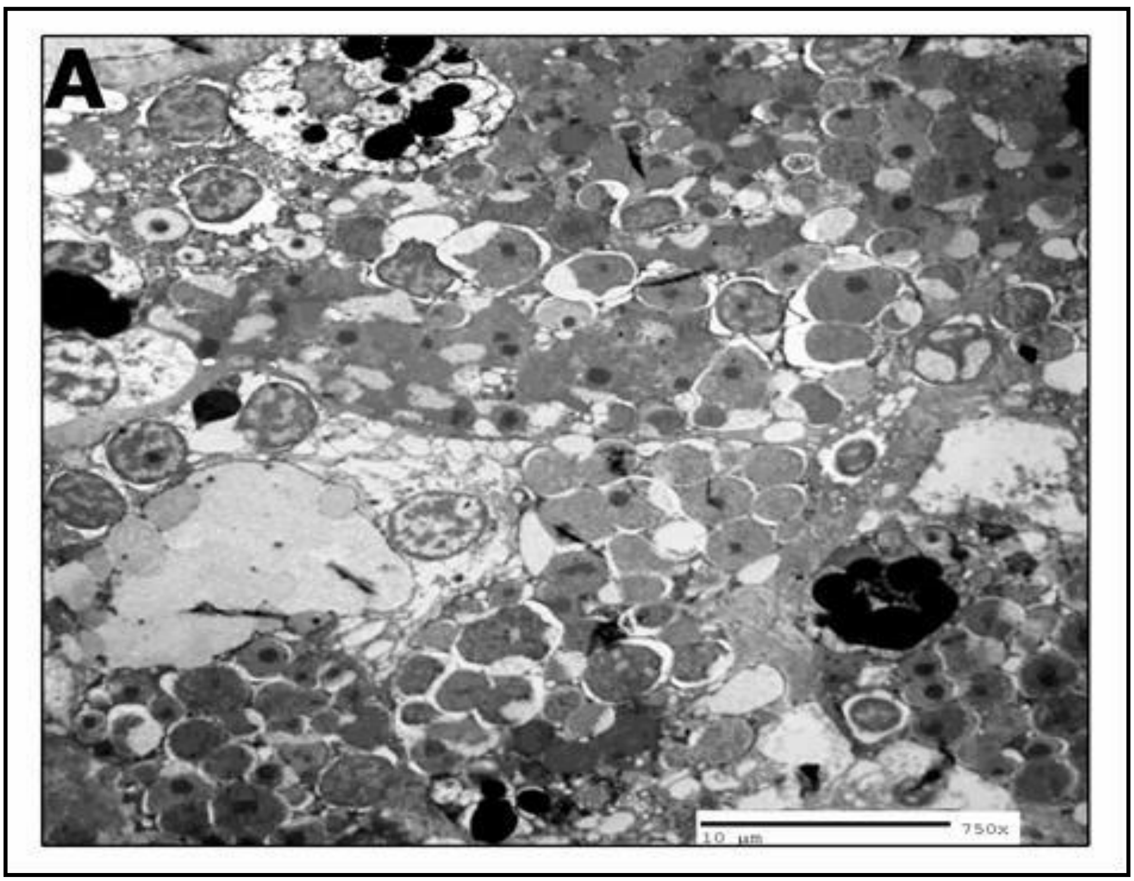


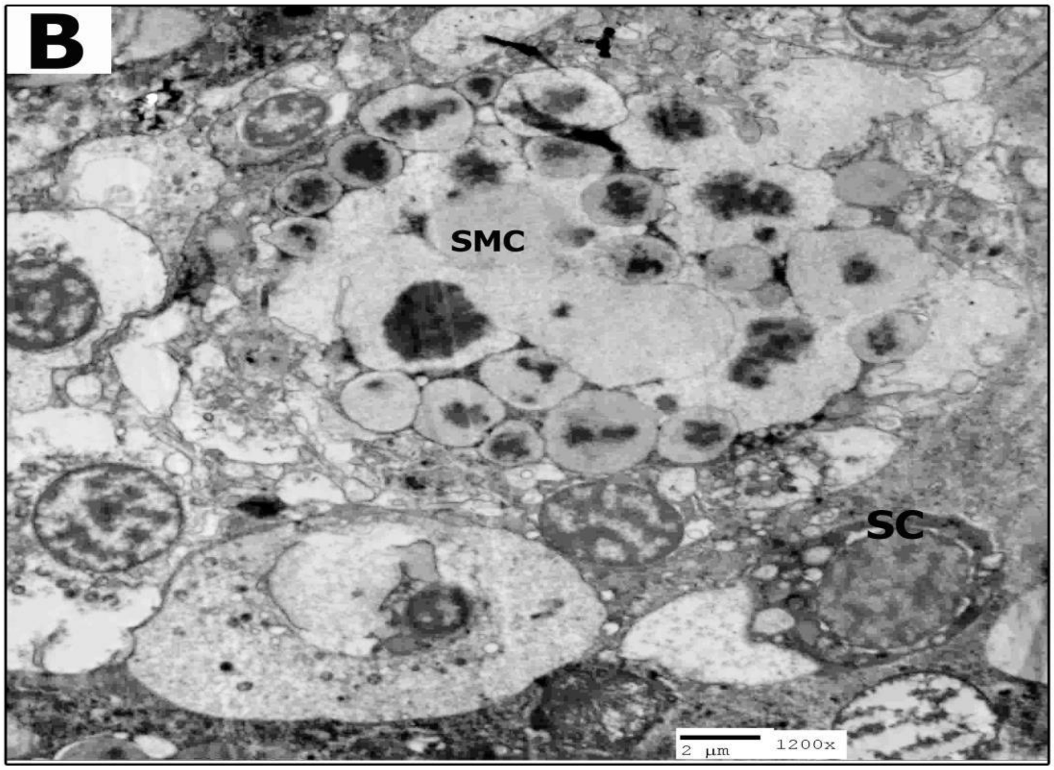
Fig. 3(A-U): Photomicrographs represent sections of the body wall of the sea cucumber "*Holothuria arenicola*" stained with MTC. A-D) shows the layers of the body wall. X 40. E-H) Focus on the integumentary structure of the sea cucumber starting from the outermost cuticle "C", the epidermis "Ep", to the sub-epidermal layer "SEp". The latter exhibit reticulocytes "Re", Fibrocytes "Fi", and the brown bodies "BB" (that might be found in pouch). X 1000. I-M) shows different magnifications of the dermis layer and muscular layers. The dermis layer is differentiated into condensed dermis "CD" and deeper dermis "DD". Both layers exhibit reticular connective tissue, fibrous CT, and elastic fibres, except that the deeper dermis is highly-occluded by elastic fibres. The dermis layer is followed by the circular smooth muscle "CSM" layer, but these muscles might be found occasionally parallel to the dermis and perpendicular to it (arrows in "I"). X 100, 200, 400, 200, & 1000. N-R) illustrates the muscular layers, which are represented by circular smooth muscles ("CSM", and "*" in Figs. P&R), and radial striated muscles "RSM". The latter are found in crypts. In Figs. O, Q, and R; many pouches are found which might be empty as shown in O, or occluded with the brown bodies. In Fig. P, the arrows are pointed to the condensed dermis. X 100,100, 200, 200, & 400. S-U) focuses on the RSM, in which the striated muscles are found as pieces. Figure "U" shows the mesothelial cells (peritoneum, arrow) that line contact with the coelom and the coelomic fluid. X 200, 400, & 1000.

The animal groups and the area of the body induced for autonomies were demonstrated in **Figures 5(A-D),** and **Table "1"**. After finishing the experiment, the total numbers of the surviving sea cucumber were returned to their original habitats at Ras Sedr, Gulf of Suez, Red Sea, Egypt.

Table (1): Illustrate the total number of groups of the sea cucumber" Holothuria *arenicola"* with their average body lengths, survival and mortality rates, and the net results of 3-weeks experiment.

Parameters summary / **Groups**	Autotomy-induction	Body length	Average body length	Mortality rate	Net result no.
The control group	------------	15, 14, 17, 19, 18, 15 cm	16.3	N/A	6
The 2nd gp	"X" Did not divide	16, 15, 14, 15, 16, 15 cm	15.2	2	4
The 3rd gp	"X "Did not divide	18, 16, 17, 15, 20, 15 cm	16.8	3	3
The 4th gp	"√ "Divided successfully	22, 21, 19, 18, 23, 17 cm	20	None	12
Total	24	24	24	5	25

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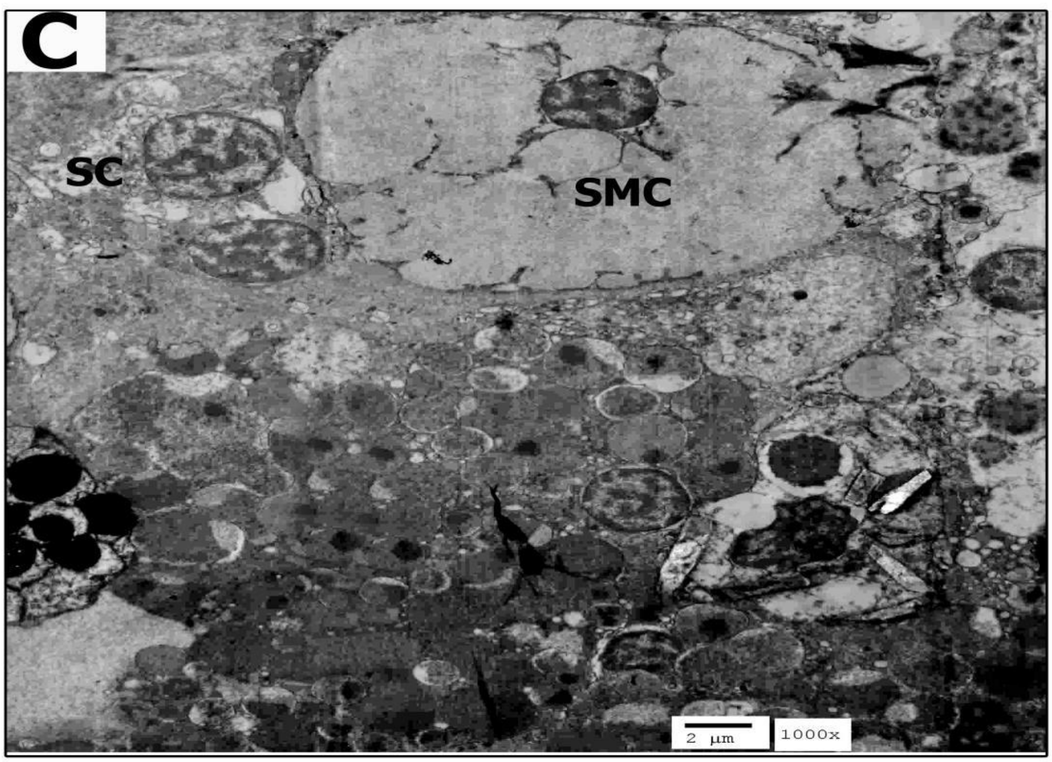
C
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SMC
2 µm
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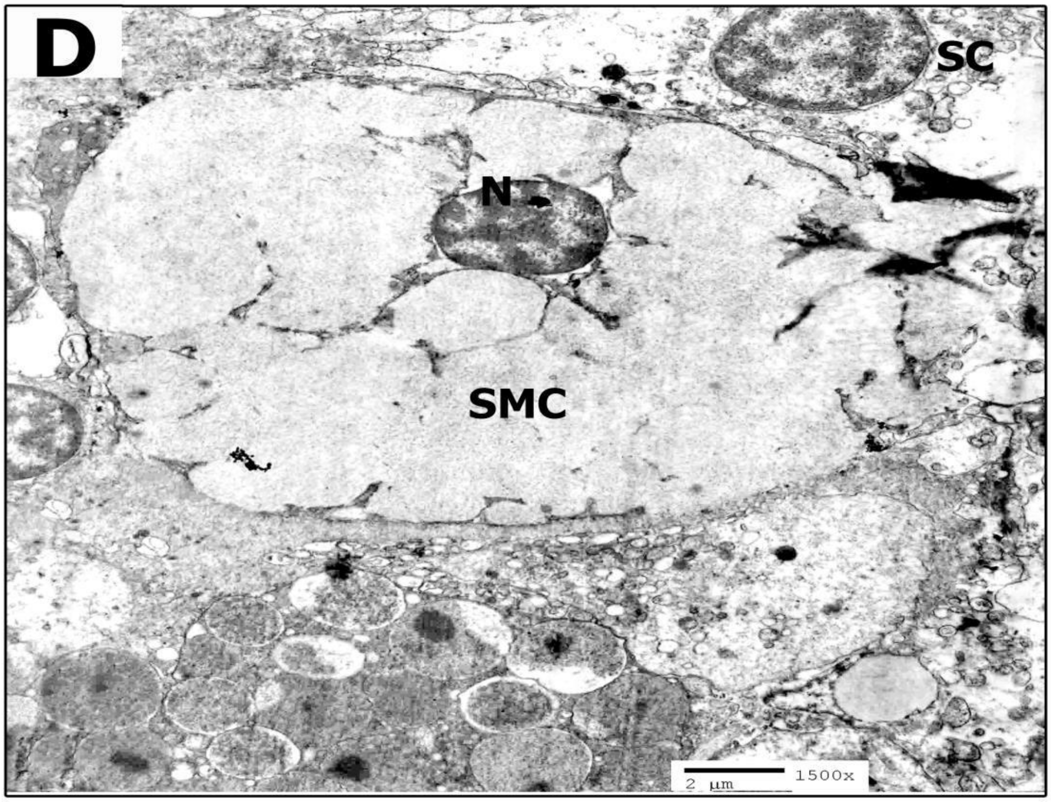
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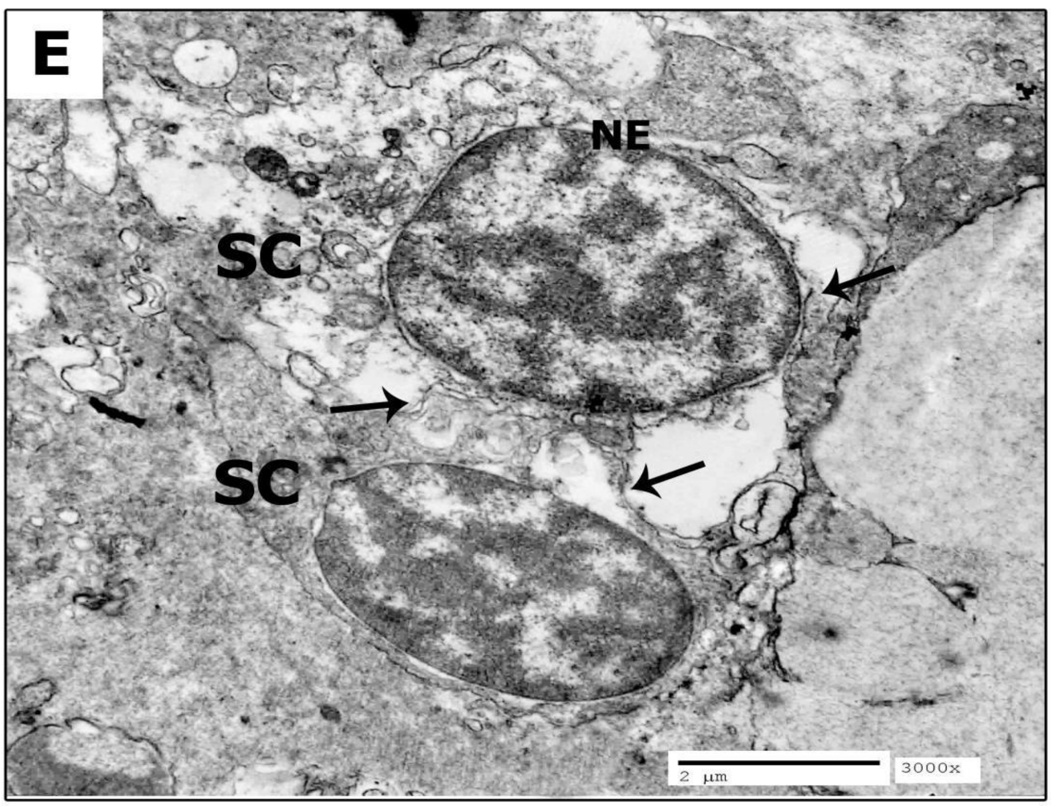
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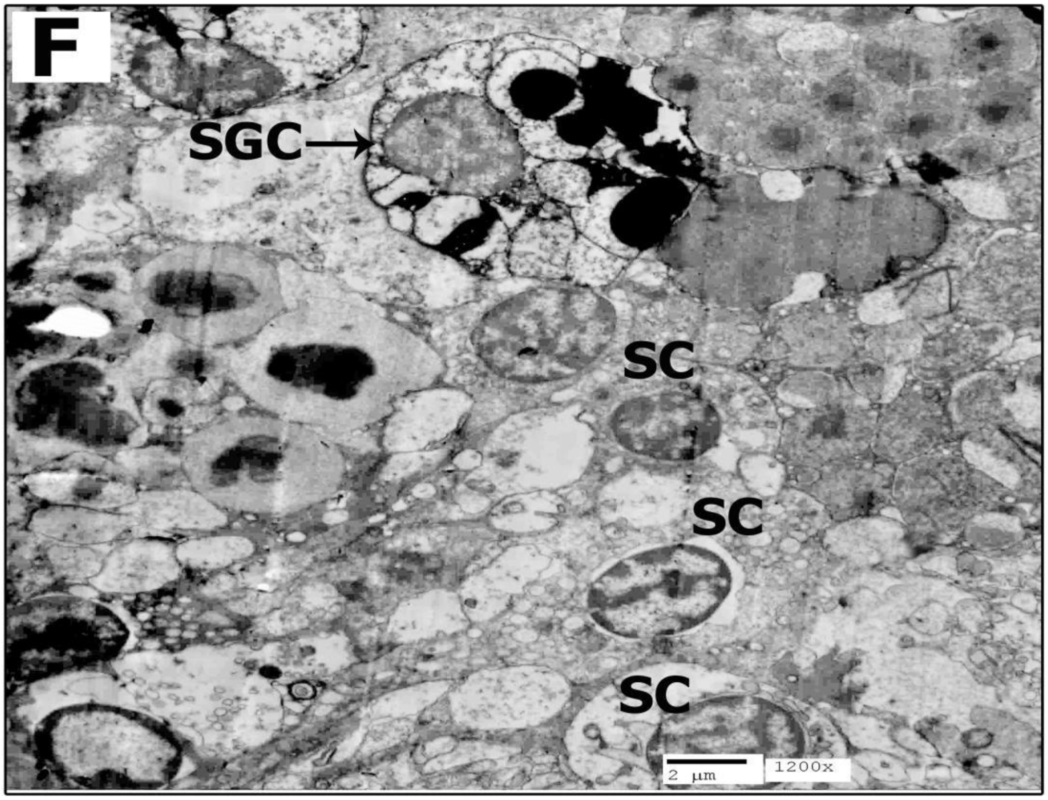
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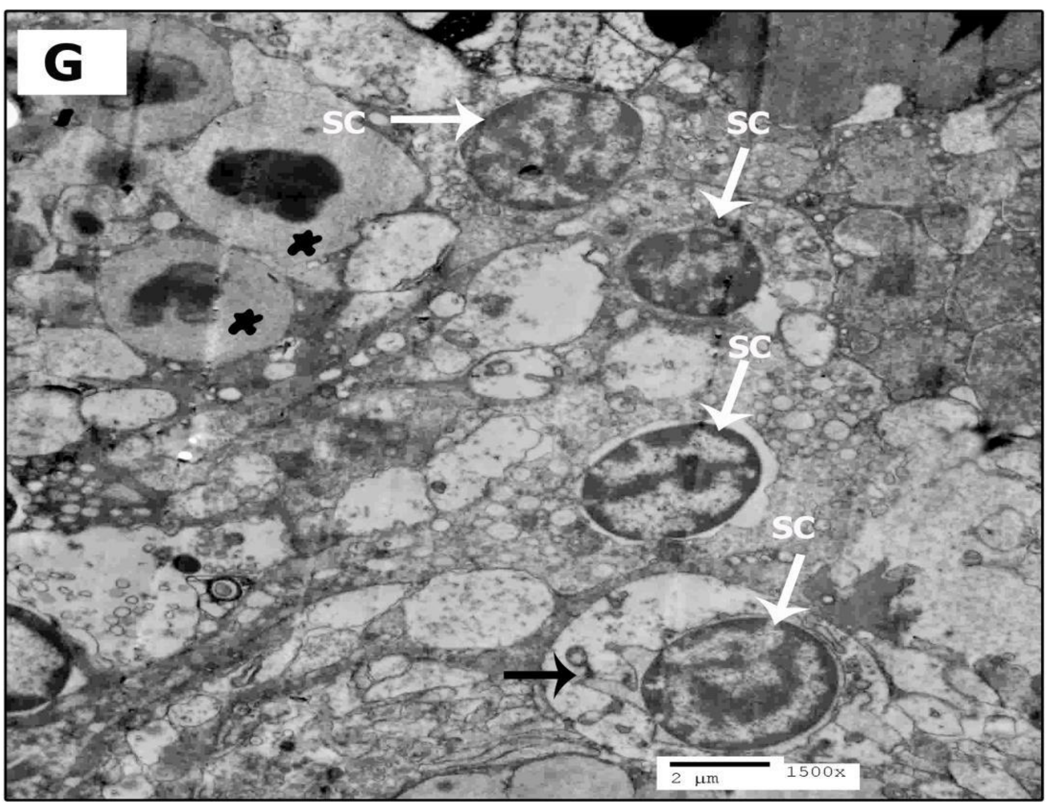
G
SC
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2 μm
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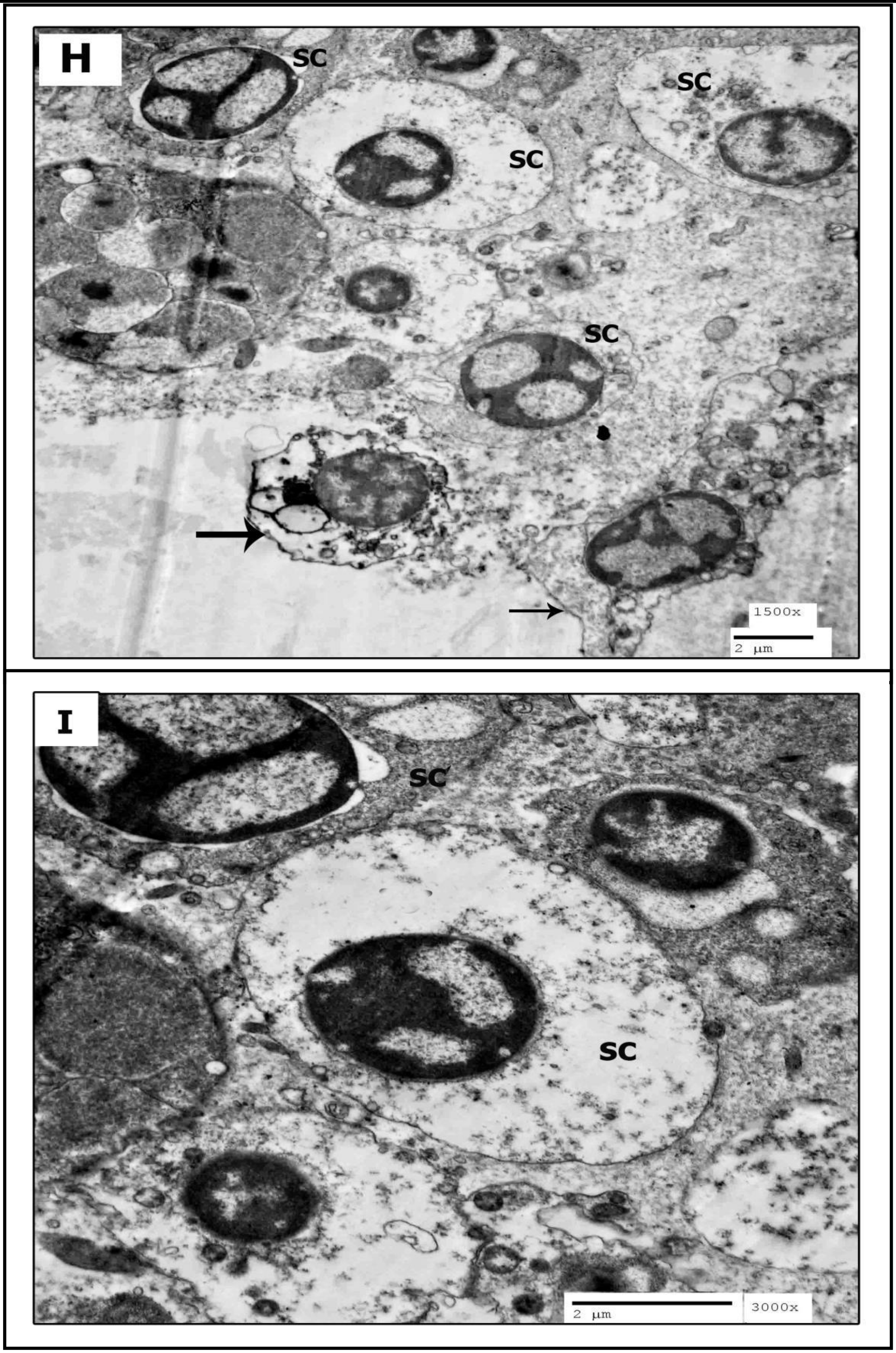
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2 µm
3000x

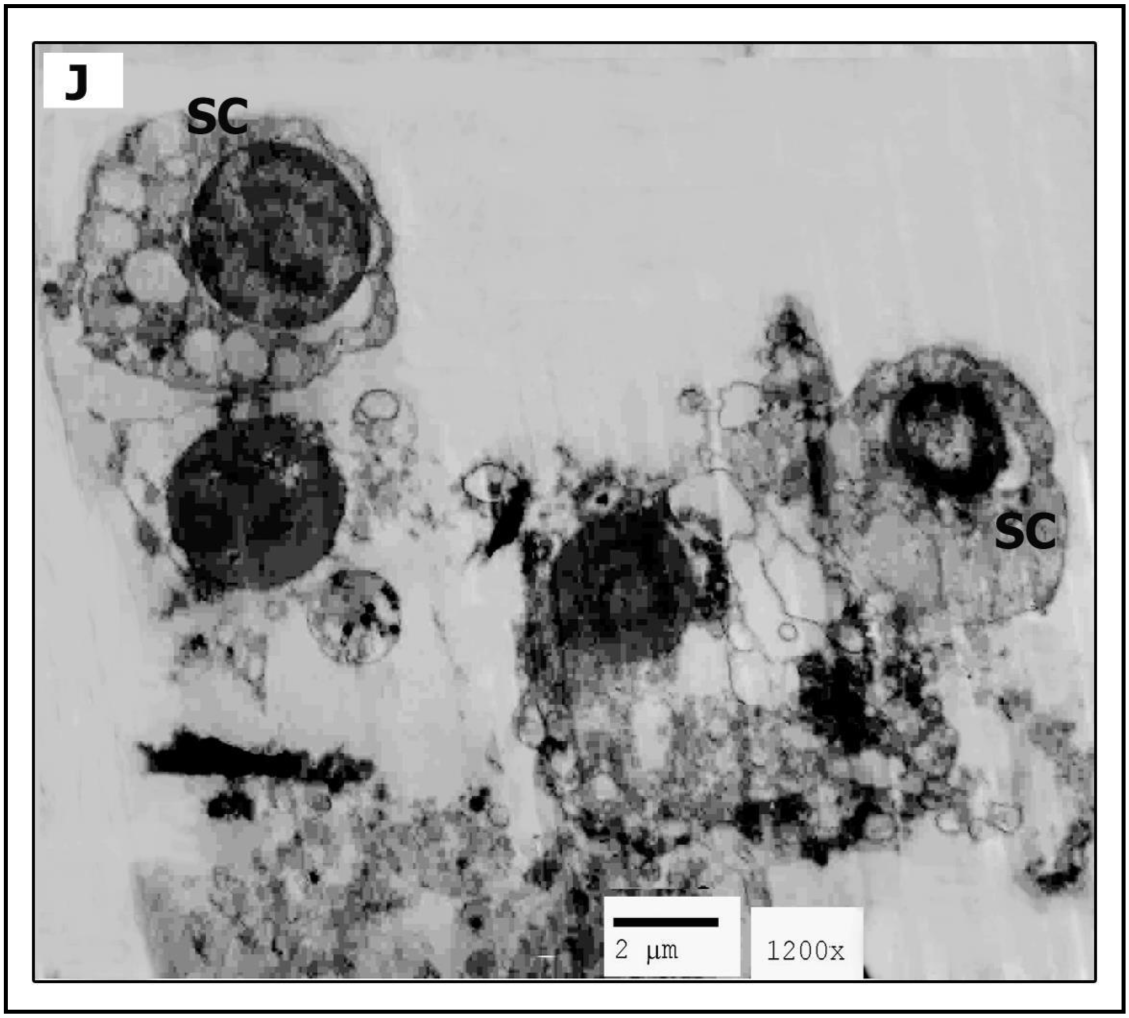

Fig. **4(A-J)**: Electron micrographs of the coelomic epithelium **(A-I)** and free coelomocytes **(J)** of the sea cucumber "*Holothuria arenicola*". The most dominant cells in the coelomic epithelium are the small cells "SC"; which may also be known as progenitor cells. **4A-C)** represent mainly the two subtypes of the large cells; which are illustrated as secretory granular "SGC" and secretory mucus cells "SMC"). In addition to **D). E)** shows SC. **F)** shows SC which also is known as the neighboring cells, and shows nuclear envelope (NE, arrows) blebbing, in addition to cytoplasmic vacuoles. **F&G)** show numerous SCs with extensive lengths "SC" and their cytoplasm is highly loaded with membranes and vacuoles. **H&I)** shows the 2nd type "SC" which resembles the human neutrophils. The nuclei of these cells exhibit highly condensed heterochromatin, which is marginal with centrally-located euchromatin. Also, figure **H** shows the outermost coelomocytes (arrows), which detach from the coelomic epithelium and migrate to the coelomic fluids. **J)** represents the free coelomocytes that scavenge some cellular debris in the area.

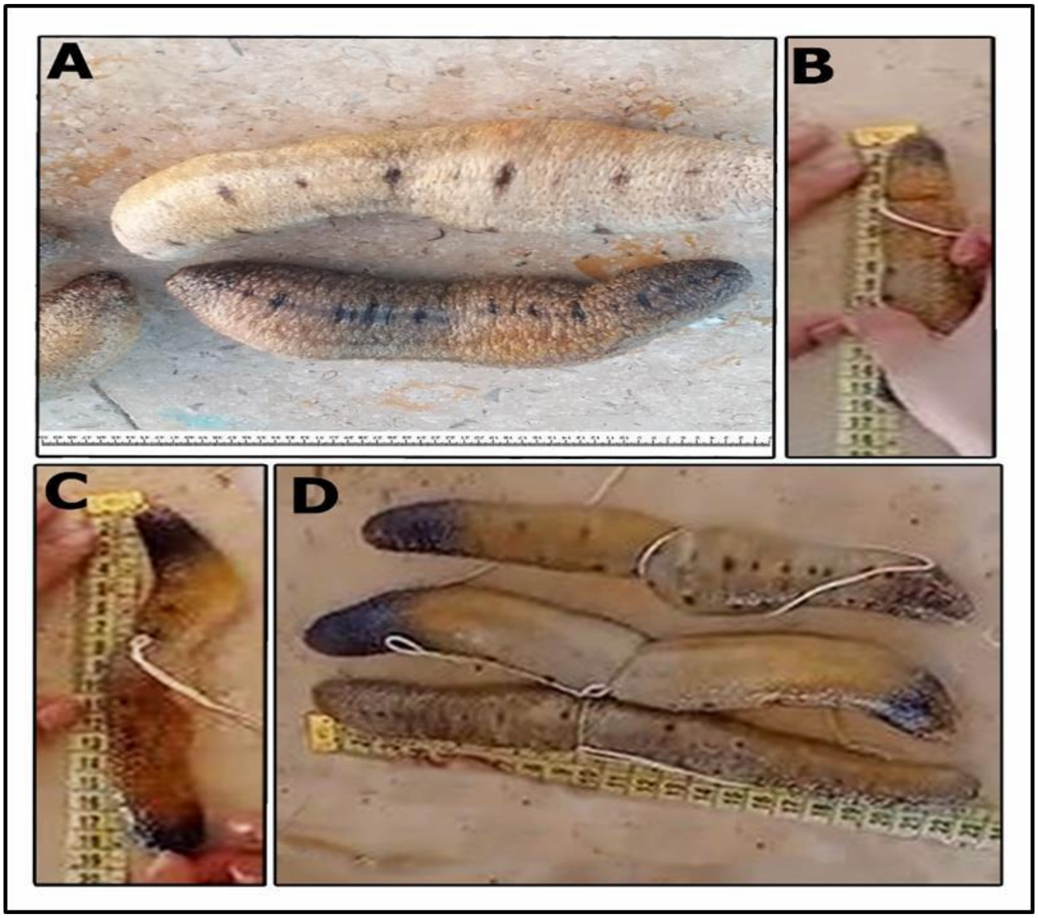

Fig. 5(A-D): Photos showing the individual animal groups used for autotomy induction. Also, the photos show the remarkable cream colour of *Holothuria arenicola*, having parallel bands of black dots down its back. **A)** represents the control group. **B)** shows the initiated ligation at ¼ of the total body length. **C)** illustrates the initiated ligation at ⅓ of the whole body length. **D)** shows the initiated ligation at ½ of the whole body of the animal.

B-**Chemical compositions of *n*-hexane fraction obtained from sea cucumber coelom using GC/MS**

Six major chemicals were identified using GC/MS analysis of the n-hexane fraction isolated from sea cucumber coelom: 4(E)-4-(2,6,6-trimethylcyclohex-1-enyl)-3-buten-2-one, n-heptadecane, n-hexadecanoic acid, oleic acid, octadecanoic acid, and supraene. Compounds derived from fatty acids have been shown to make up the bulk of the n-hexane fraction in the coelom (Table 2). Figure **"6"** is a schematic depicting the most common chemical components of the n-hexane fraction.

Table 2: Chemical compositions of *n*-hexane fraction obtained from **Sea cucumber** coelom using GC/MS supplied withRtx-5MS column.

No.	Compounds[	R_I		% Composition	References
		Measured	Reported		
1.	(E)-4-(2,6,6-Trimethylcyclohex-1-enyl)-3-buten-2-one	1491	14.91	5.21	MS, RI
2.	*n*-Heptadecane	1704	1711	5.97	MS, RI
3.	*n*-Hexadecanoic acid	1960	1964	12.20	MS, RI
4.	Oleic Acid	2131	2140	7.9	MS, RI
5.	Octadecanoic acid	2172	2170	8.41	MS, RI
6.	Supraene	2803	2817	19.49	MS, RI

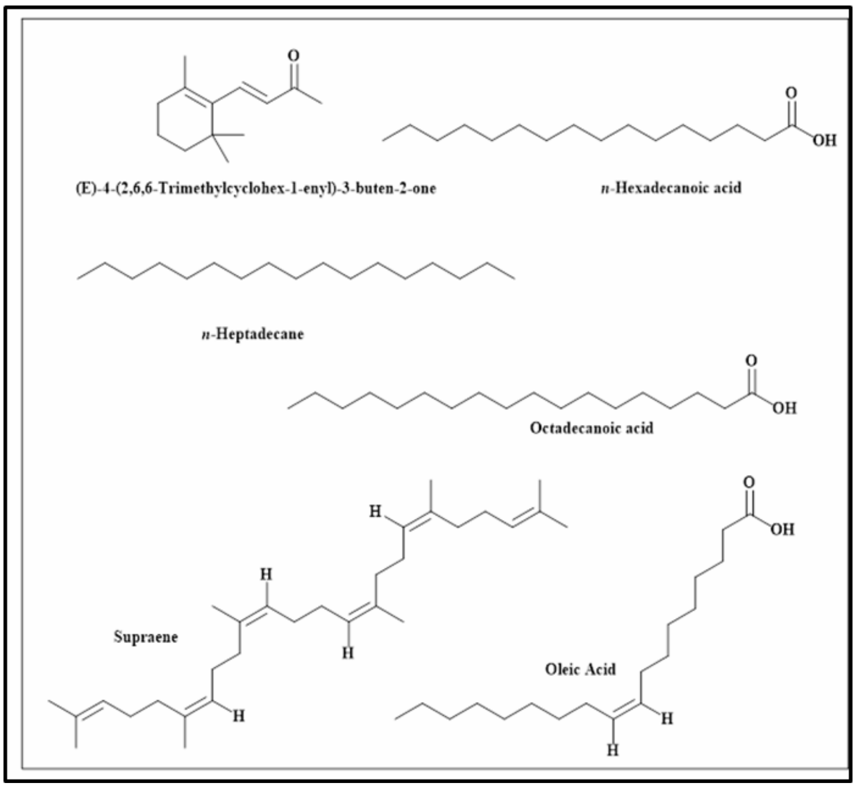

Figure 6: Scheme showing the chemical compositions of *n*- hexane fraction obtained from the **sea cucumber** coelom using GC/MS supplied with Rtx- 5MS column

In silico molecular docking studies:

According to the data in Table 3, all of the chemicals tested had significant inhibitory potential, although octadecanoic acid had the greatest binding energy (G) inside the active site of glycogen synthase kinase3- protein, followed by n-hexadecanoic acid, n-heptadecane, and oleic acid. At the glycogen synthase kinase3- protein binding site (shown in Figure **7A**), octadecanoic acid makes two conventional H-bonds with Tyr134 and Val135, whereas n-hexadecanoic acid forms two conventional H-bonds with Asp200 and Lys85 (Fig. **7B**). Specifically, oleic acid interacts with Gln72, yr134, and Lys60 through three typical H-bonds (Fig. **7C**). In addition, every chemical promoted Van der Waals contact between the amino acid residues.

Table 3: Coelomic fluid from the sea cucumber *"Holothuria arenicola"* was analysed for the free binding energies (kcal/mol) of its main components in the n-hexane fraction.

Compound	**Glycogen synthase kinase3-*β* protein**	**Number of formed Hydrogen bonds**	**Number of other formed bonds**
(E)-4-(2,6,6-Trimethylcyclohex-1-enyl)-3-buten-2-one	3.78	1;Val135	3 ; Val70, Ile62
n-Heptadecane	-38.56	-	1 ; Ile62
n-Hexadecanoic acid	-46.81	2; Asp200, Lys85	-
Oleic acid	-32.30	3; Gln72, yr134, Lys60	-
Octadecanoic acid	-48.04	2; Tyr134, Val135,	-
Supraene	88.94	-	5; Tyr134, Val70, Phe67, Ala83, Leu188

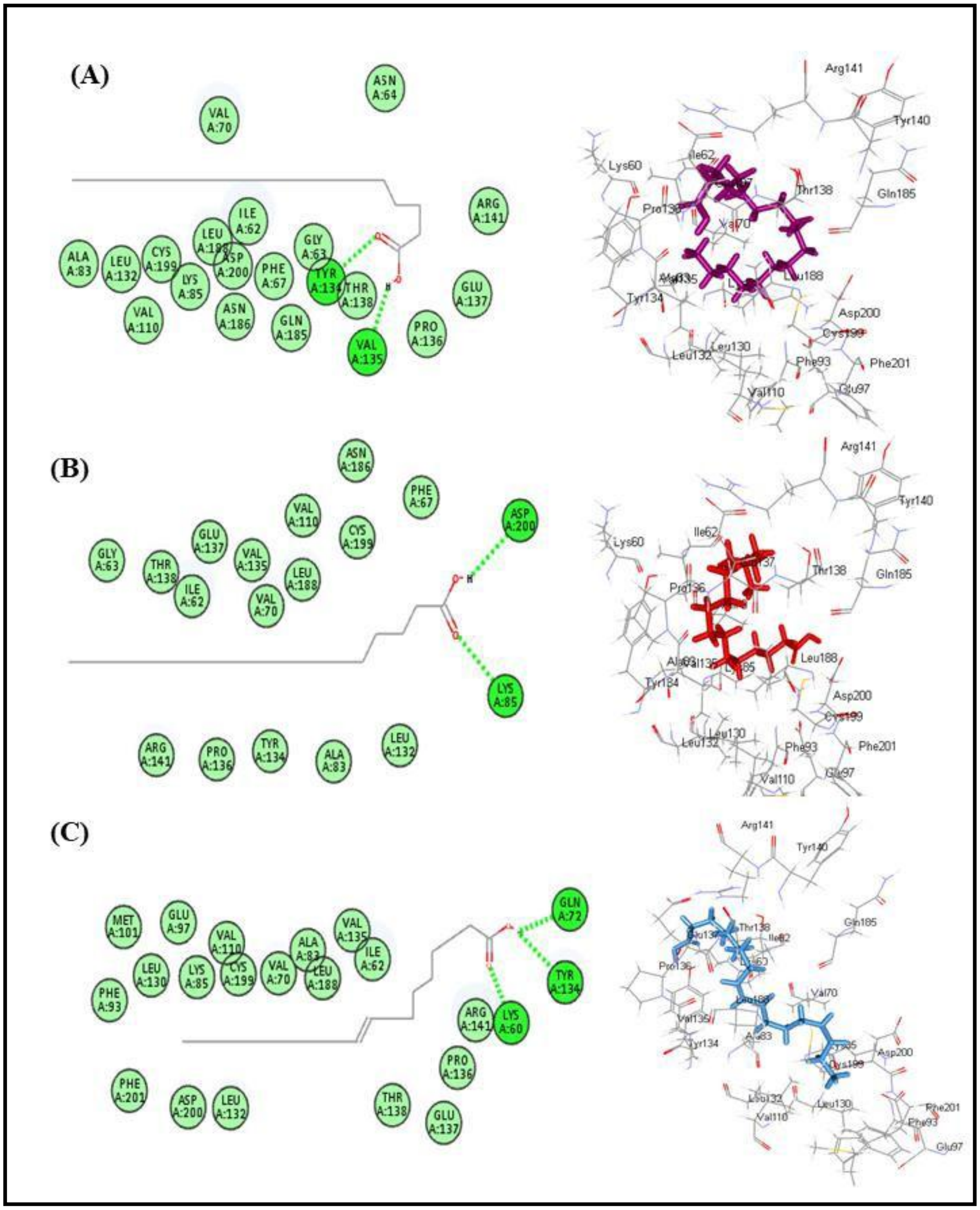

Figure 7 2D and 3D binding modes of octadecanoic acid (A), n-hexadecanoic acid (B), and oleic acid (C), within the active sites of glycogen synthase kinase3- β protein (GSK3-β protein).

DISCUSSION

Many previous studies proved that sea cucumber is considered an attractive source of nutraceutical and pharmaceutical drugs; especially *Holothuria arenicola;* the targeted sea cucumber of this study **(Ardestani *et al.;* 2004, Moskaug *et al.;* 2005, Althunibat *et al.;* 2009, and Fahmy, 2018).** In addition, the antioxidant effect shown by *H. arenicola*, including the elevation of GSH and the activity of GST, CAT, and SOD antioxidant enzymes, may be responsible for its anti-ulcerogenic actions **(Park *et al.*, 2008; Kim and Wijesekara, 2010 and Fahmy, 2015)**.

In this work, the collected species of interest was identified as *Holothuria arenicola* depending on the animal morphology that is confirmed microscopically by examining its ossicles as shown in **Figure 1(A-J);** this agrees with the data published by **Pourvali *et al.* (2014); and Eisapour *et al.* (2021)**. As far as we know, no study examined the layers of the body wall of the sea cucumber *Holothuria arenicola* except us in Egypt, all of the published papers are concerned with the ecological distribution, physiological and pharmacological parameters (**Ibrahim, 2012; Omar *et al.*, 2013; Fahmy,2015; Eissa *et al.*, 2017; and Fahmy, 2018;**). As illustrated in this work (Figs. 2A-J, and Figs. 3A-U), the body wall is composed of 5 main layers differentiated as **a)** the outermost Cuticle (characterized by ossicles), **b)** The epidermis, **c)** The dermis and superficial dermis, **d)** The circular smooth muscle, and **e)** The longitudinal skeletal muscles (which are shown as crypts). All of the aforementioned layers agree partially with those published by **Guerrero and Forero (2018)**; which is the only published work concerned with the body wall layers but on different species of the sea cucumber.

The coelomic epithelium and free coelomocytes were also been studied in this work using TEM. The examination of the coelomic epithelium of the sea cucumber using the TEM showed that it is mainly composed of two types of cells, numerous small cells (progenitor cells) – that form most of the tissue –, and other large cells (Figures **4A-I**). The small cells are furtherly divided into 2 main types: The first type of these small cells is SC or the neighboring/surrounding cells (that hypothetically might supply the surrounding dividing coelomocytes with the membranes or any cytoplasmic organelles). The nuclei of this type showed thin heterochromatin underneath the nuclear envelope with some patches alternated with the euchromatin (as shown in Figures **4A-G**). The 2nd type of "SCs" or defence cells resemble the human neutrophil. The hypothetical complementary relation between the small cells and other coelomocytes might be explained based on the published theory by **Ramkumar & Baum (2016), and Carlton *et al.*, (2020).** In their theory, cells undergo rapid changes in volume and surface topology during mitosis. Cells need to be able to shape, transport, and remove membranes since there isn't enough time for *de novo* membrane production to accommodate these changes. Additionally, new membranes may be supplied from internal stores by trafficking processes like exocytosis or endocytic recycling, or by plasma membrane modification, for instance *via* membrane blebbing, as stated by **Boucrot & Kirchhausen (2007), Kiyomitsu & Cheeseman (2013), and Carlton *et al.*, (2020).**

The large cells were also divided into two subtypes defined as secretory granular and secretory mucous cells (Figs. 4B, C, D, & F). The outermost cells of the coelomic epithelium detach from the outermost and escaped to act as free coelomocytes. The free coelomocytes might scavenge the surrounding as shown in Figure **4J**. **Abdel-Ghaffar and Youssef (2022)** previously supposed and concluded that All coelomocytes are differentiated from the young or progenitor coelomocytes, which then might harbor different secretory materials and turned into secretory granular and secretory mucous coelomocytes.

As it is mentioned in the introduction, the price falls between €43 and €348. Inexplicably, the price of high-quality species like *Holothuria (Metriatyla) scabra* is more than €1593/kg ($1800/lb) **(Purcell *et al.*, 2018).** For such crucial economic aforementioned reasons, in addition to the pharmacological importance together with the consideration of sea cucumber as one of the available untapped resources, it gives us the enthusiasm to design this study and artificially-induced-autotomy in the sea cucumber *H. arenicola.* Sea cucumber fishing was prohibited in Egypt and listed as endangered species, due to overfishing. Although the above decision was right at this time, but not acceptable in our consideration because this decision did not follow the rule of the ultimate usage of the available untapped resources. So; we investigate the ability of numerical increase of sea cucumbers through the artificial-induced autotomy. *H. arenicola* is known for its nutritional and pharmacological importance, as well as the medical importance of its coelomic extract **(Dai *et al.*, 2016; Mohamed *et al.*, 2020; and Abdel-Ghaffar and Yossef, 2022).** From all of these aforementioned points of view, the investment in sea cucumber shows several benefits, in addition; as a marine organism, its cultivation does not affect the Egyptian water resources. Finally, the volume of transactions in the global market generates foreign currency equal to 150 million dollars from ten countries led by China, and we look forward to entering that global market soon for the country beneficiary as a national project and get hard currency.

The great market value of fish and other marine products prompted an in-depth discussion of holothurians' system of asexual reproduction **(Dolmatov, 2014)**. Researchers are also trying to harness holothurians' regenerative capability and fission capacity to create cultivation techniques and enhance natural populations due to their great economic potential **(Reichenbach and Holloway, 1995; Reichenbach *et al.*, 1996; Purwati and Dwiono, 2005; Laxminarayana, 2006; Razek *et al.*, 2007; and Purwati *et al.*, 2009)**. In this work, we are concerned with asexual reproduction – because sexual reproduction is done naturally from spring to summer **(Tuwo and Conand, 1992; Conand,1993; Despalatović *et al.*, 2004; Navarro *et al.*, 2011; Kazanidis *et al.*, 2014; Santos *et al.*, 2017; and Venâncio *et al.*,2022)**, and for a large period of time during the year, the adults were left without any further reproduction. In this work, the experiment was handled for 3 weeks as shown in Table “1” and Figure **5(A-D).** The fourth group (in which the body was initiated for constriction by ligation in the middle of the body) was the only successful group in the division. The success of division might be referred to: Firstly, the mechanism of artificial autotomy depends on the integrated mechanism primarily of the nervous system.

Dolmatov (2014) claimed that no research has been done into the cellular and molecular processes of fission in holothurians or the role that the nervous system plays in its control. **Hoekstra *et al.* (2012)** published data consistent with the hypothesis that central nervous system triggers in the centre of the body are responsible for the successful division. The sea cucumber's mode of action in asexual division is dependent on a number of specialized cell types located in its buccal tentacles, esophageal area, and close to its radial nerve cords. Unipolar and bipolar cells in the radial nerve cords show large processes in close contact with muscle and other cells of the body wall, whereas sensory-like cells in the tentacles transmit processes toward the circumoral nerve ring. *Homo sapiens* also have a special capacity in that their connective tissue can change the mechanical properties on its own under the influence of different stimuli **(Wilkie, 2001; and 2005).** This kind of connective tissue is also known as catch connective tissue **(Wilkie, 1984)** or changeable collagenous tissue **(Motokawa, 1984).** This quality seems to be crucial for the fission process **(Mladenov and Burke, 1994; Uthicke, 2001; Motokawa and Tsuchi, 2003; and Motokawa *et al.*, 2012).** The features of the sea cucumber's nervous system and body wall discussed above are taken together to explain why *H. arenicola* is artificially divided into just four subgroups.

Additionally, in this study, the coelomic fluid of *Holothuria arenicola* contains a variety of compounds, including fatty acids, amino acids, peptides, proteins, carbohydrates, and lipids. The six main *n*-hexane derivatives compounds identified in the study are all fatty acid derivatives. Fatty acids are a major component of the cell membrane, and they play a role in a variety of cellular processes, including cell growth, differentiation, and signaling. The reason these six compounds were identified in the coelomic fluid is due to their solubility in the fluid. Fatty acids are relatively non-polar compounds, and they are therefore soluble in the non-polar coelomic fluid. The other compounds found in the coelomic fluid, such as amino acids, peptides, proteins, carbohydrates, and lipids, are also soluble in the fluid. The fact that these six compounds are the major identified compounds in the coelom *n*-hexane derivatives suggests that they may be important for the biological functions of the coelomic fluid. The study's findings suggest that these compounds may be involved in wound healing, as they were found to bind to the glycogen synthase kinase3-β protein, which is involved in the regulation of cell growth and differentiation. The study findings suggest that the coelomic fluid of *Holothuria arenicola* is a potential source of new wound healing agents **(Tables 2 and 3; Fig. 6).**

The glycogen synthase kinase3-β protein is a key regulator of cell growth and differentiation. It is involved in a number of cellular processes, including the formation of new blood vessels, the growth of new cells, and the repair of damaged tissue. Octadecanoic acid, also known as stearic acid, is a fatty acid that is found in the coelomic fluid of *Holothuria arenicola*. It is a relatively large molecule, and it has a long, straight chain of carbon atoms. This shape makes it a good fit for the active site of the glycogen synthase kinase3-β protein. The other fatty acids that were identified in the coelomic fluid, such as n-hexadecanoic acid, n-heptadecane, and oleic acid, are also relatively large molecules. However, they have slightly different shapes than octadecanoic acid. This means that they do not fit as well into the active site of the glycogen synthase kinase3-β protein. The finding that octadecanoic acid has the best binding affinity for the glycogen synthase kinase3-β protein suggests that it may be able to regulate the activity of this protein. (previously reported by **Harish *et al.*, 2008; Naika *et al.*, 2015; and Aksoy *et al.*, 2021).** This could lead to the promotion of wound healing, as the glycogen synthase kinase3-β protein is involved in the regulation of cell growth and differentiation. (Tables 2& 3, Figs. **7A-C**). The protein docking studies were performed using the C-Docker protocol and Discovery Studio 4.5 software. The active site of the glycogen synthase kinase3-β protein was modeled using the crystal structure of the protein. The six *n*- hexane derivatives compounds were docked into the active site of the protein. The binding affinity of each compound was determined based on the energy of the docked complex.

CONCLUSION

Histologically; the body wall of the sea cucumber's "*Holothuria arenicola*" is composed of five layers. Ultrastructurally; the coelomic epithelium small (progenitor) cells (coelomocytes) seem to be hypothetically differentiated into two main types: the neighboring/surrounding cells (that might supply the neighbouring dividing cells with the membranes or any cytoplasmic organelles, hypothetically *via* exocytosis or endocytic recycling trafficking events or by plasma membrane remodeling), and defence cells that resemble the human neutrophils. Experimentally, the artificially-induced autotomy of the half-body-length ligated group is the only successful group that divides completely. Chemically, we found the presence of bioactive GSK3-β protein in the sea cucumber-extracted coelomic fluid which makes them a valuable resource for wound healing treatment especially in diabetics. Finally, the sea cucumber "*Holothuria arenicola*" is considered a gift for Egypt, due to its proven significant potential for economic and medical applications, both criteria of significance are concomitant with the sustainable developmental goals and Vision of Egypt 2030.

Abbreviations:

H&E: Haematoxylin and Eosin. **MTC**: Mallory trichrome. **CSM**: Circular smooth muscles. **RSM**: Radial striated muscles. **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. Abdel-Ghaffar WH, Youssef FS, and Attia MS wrote the discussion equally in the area of interest.

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Competing interest statement
The authors declare no conflict of interest.
Availability of data and materials:
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Ethical approval:

This study follows guidelines for the care and use of experimental animals established by the Committee for the purpose of control and supervision of experiments on animals. Animal procedures were also made in accordance with the Faculty of Science protocol, at Ain Shams University. Also, this research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

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