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Microscopical and Chemical Analyses of the Coelomic Fluid and the Coelomocytes Extracted from the Sea Biscuits "*Clypeaster humilis*".

Wafaa H. Abdel-Ghaffar[1]*, Fadia S. Youssef[2]
[1] Zoology Department, Faculty of Science, **A**in **S**hams **U**niversity, Cairo, Egypt
[2] Pharmacognosy Department, Faculty of Pharmacy, **A**in **S**hams **U**niversity, Cairo, Egypt
***Corresponding Author:** wafaahassan@sci.asu.edu.eg **&** dr.wafaah2017@gmail.com

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ABSTRACT

Echinoderms received great attention in various scientific applications due to their unusual regenerative characteristics. *Clypeaster humilis* is the animal of choice in this study. Most of the published scientific research papers are concerned with the morphology of the animal, larvae and internal organs; nothing dealt with the cellular or chemical constituents of the animal coelomic fluid. Although the coelomic fluid (haemocoel) represents a vital component filled with many important constituents, few publications were published concerning this issue. *Clypeaster humilis* is also known as sea biscuits/sand dollars. This invertebrate animal was collected from Hurghada, Red Sea, Egypt. This study brings new knowledge of recording and documenting the obtained scientific data concerned with one of the poorly-studied phyla of the Egyptian fauna. The coelomic fluid of the sand dollar was chemically analyzed, and the obtained data revealed the presence of glycogen synthase kinase3-*β* protein by protein docking, which is essential for wound healing. In addition, the ultrastructural investigations detected two types of cells; namely, young and mature coelomocytes, and the latter is divided into secretory granular and secretory mucous cells, respectively. The coelomic fluid extracted from *C. humilis* exhibits glycogen synthase kinase3-*β* protein, which could be a promising constituent for many medicinal applications including wound healing in diabetics. 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.

INTRODUCTION

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 therapy molecules. The involvement of free-living coelomocytes in the regeneration processes of different echinoderms is undeniably crucial. **Smith (1991)**, **Smith and Davidson (1994),** and **Kudryavtsev and Polevshchikov (2004)** postulated that, the coelomocytes are considered a primitive system of innate immunity compared to the fact that the evolutionary link between coelomocytes and the vertebrate immune system may be traced back to a common ancestor.

Despite the importance of coelomocytes' organization, many species of echinoderms including *Clypeaster* sp. (Sea biscuit) haven't been studied yet, and the cellular constituents of its coelomic fluid have not been identified. In addition, the diverse data confused the researcher due to controversial studies and data **(Boolootain & Guise, 1958; Hetzel, 1963; Fontaine & Lambert, 1975; Kaneshiro & Karp, 1980; Korenbaum, 1989; Edds, 1993; Kozlova *et al.*, 2006; Pinsino *et al.*, 2007)**. First, the free-living coelomocytes system displays diverse organization in different echinoderm classes; second, the descriptions, structures and the classifications of coelomocytes vary depending on the authors' approach **(Gorshkov *et al.*, 2009)**.

To the best of our knowledge, few publications were generally published with respect to the family Clypeasteroidae, specifically on *Clypeaster* sp., and the topics of publication ranged from discovering new species, fossils, geographical distribution, the internal and external structures and its larval stages **(Suryanti *et al.*, 2016; Buwono & Fitri, 2017; Martín-Cao-Romero *et al.*, 2017; Armstrong & Grosberg, 2018; Mihaljević & Rosenblatt, 2018; Tsaparas *et al.*, 2018; Cambronero-Solano *et al.*, 2019; Lavtizar & Okamura, 2019; Mancosu & Nebelsick, 2019; Perricone & Collin, 2019; Collin *et al.*, 2020; Bencomo *et al.*, 2021; Lu Remindima *et al.*, 2021; Mooi & Noordenburg,** 2021**)**.

Tissue regeneration is a crucial regulatory phenomenon with broad biological ramifications throughout the animal world **(Die *et al.*, 2016)**. Scar repair is the standard method of healing for mammals such as humans; however, invertebrates like sea stars, Asteroidea and planarians can renew almost every component of their bodies. Starfish with normal developmental traits have remarkable healing capacity even among echinodermatous invertebrates. In most animals, the regenerative potential is restricted to vertebrates' wound repair, but humans are an exception. Based on the aforementioned trial by **Die *et al.* (2016)** about the usage of tissue extract from starfish for regeneration/wound healing together with the fact stated 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 pushes us to study its characteristics and how to use it optimally for human benefits. Furthermore, molecular docking is included in our consideration and approach as one of the most widely utilized methods in structure-based drug design owing to its capacity to anticipate the binding-confirmation of small molecule ligands to the correct target binding site. Binding behavior characterization is crucial for rational drug design and for understanding the underlying biochemical processes **(Kitchen *et al.*, 2004; Mostashari-Rad *et al.*, 2019)**.

The current study target was to describe and identify the basic cellular structure of the obtained coelomocytes of *Clypeaster humilis* using light (for semithin sections) and transmission electron microscope (TEM). In addition, it aimed at studying the chemical composition of the coelomic fluid of the sea cake using GC/MS analysis, analyzing the obtained data using protein docking, and evaluating the usage of such extract medically for human benefits.

MATERIALS AND METHODS

1- Materials

a- Chemicals

In this study, all of the used chemicals, solvents and reagents were of analytical and pure grade. 25% glutaraldehyde (100ml), osmic acid (EMS, 2g), xylene, acetone (HPLC), ethyl alc. (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 and toluidine blue were used in this study. In addition to the the uppe-mentioned items, the following laboratory supplies were utilized, including yellow tips, blue tips, gloves, 50ml wide plastic containers (tightly-sealed), 20L container, large ice backs, freon B-100, 9cm petri dish glass & 18cm- filter papers. Furthermore, 58*58*35cm tanks with cover (each tank contains a filter and oxygen pump for preserving the specimens in seawater during extraction) were used as well as sea salt.

b- Animals

The sea biscuits were collected from Hurghada, Red Sea, Egypt. *Clypeaster humilis* were identified by Prof. Dr. Ahmed Metwaly Helal (Zoology Department, Faculty of Science, Al-Azhar University "Boys-Branch") according to **Andreas (2018)**, based on the first identification of the specimen 1778 by the German biologist Nathanael Gottfried Leske.

2- Methodology

A. Samples collection, identification, classification and ecology

The sea dollars were collected from the coast of Hurghada, Red Sea, Egypt. Some ecological parameters were measured; related to the location of samples and their collection.

B. Coelomic fluid extraction procedure experimental design

The method of **Baveja *et al.* (2018, 2019)** was used in this study to prepare the coelomic fluid (CF) of the sea biscuits. A volume of 10cc or slightly-lesser had drawn out from the animal *via* a syringe. Each sea dollar's collected coelomic fluid was combined and then centrifuged at 10,000 rpm for 15 minutes to remove the sediment. After centrifugation, two components were obtained, the cellular components and the fluid (acellular). The cellular part was prepared for semithin and ultrathin investigations to identify the existing coelomocytes immediately after drawn by syringe and one hour after to record any difference in the coelomocytes (if any). The other fluid component (supernatant) was furtherly-analyzed using GC-MS, and furtherly analyzed by protein docking.

1- **The preparation of the separated 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)**.

- The semithin sections were photographed using a trinocular Leica research microscope (DM1000, LED, Light bright field), with Leica Flexcam C1 (ultrahigh resolution 4K/full HD, Digital Color Video Camera system). The analyses of the image morphometry were done using LAS X software.
- Finally, the stained grids were examined and photographed using a JEOL 1200 EX II electron microscope, E. M. Unit, the Faculty of Science, Ain Shams University.

2- Metabolic profiling of the n-hexane fraction of the tested samples using GC/MS analysis for the separated acellular coelomic fluid (supernatant).

Using a Restek Rtx-5MS (30 m 0.25 mm i.d. 0.25 m film thickness) capillary column and a Shimadzu MS, we performed GC/MS studies. The GC/MS used instrument was a Shimadzu GCMS-QP 2010 (Shimadzu Corporation, Koyoto, Japan). The column temperature was first set to 50°C for 3 minutes before being steadily increased from 50 to 300°C at a rate of 5°C/ min, followed by 10 minutes of isothermal maintenance at 300°C. An injector temperature of 280 degrees celsius was maintained, with an interface temperature of 220°C and an ion source temperature of 280°C. The standard carrier gas flow rate of helium was set at 1.37mL/ min. The diluted sample, with a concentration of 1% v/v was injected as a volume of 1 L using a split mode with a split ratio of 15:1. EI mode at 70 eV was used to capture the mass spectra from m/z 35 to m/z 500. The normalizing technique was used to determine the concentration of each compound by comparing the results of three separate chromatographic separations. Compounds were identified by comparing their retention indices to those of a series of n-alkanes (C8-C28) injected under identical conditions, and by comparing their mass spectra to those recorded in the Wiley Library database and the National Institute of Standards and Technology (NIST), as well as with the literature **(Youssef *et al.*, 2014; Ayoub *et al.*, 2015*;* Mamadalieva *et al.*, 2019; Youssef *et al.*, 2021)**. The run of the specimens GC/MS analysis was done in the Department of Pharmacognosy (GC/MS unit), Faculty of Pharmacy, Ain Shams University.

***In silico* molecular docking studies**

Molecular docking analysis was done on the major chemical constituents detected in the *n*-hexane fraction obtained from sea cake coelomic fluid using GC/MS on glycogen synthase kinase3-*β* protein (PDB ID 5K5N; 2.20 Å) that is considered among enzymes implicated in the process of wound healing. Using C-Docker protocol and Discovery Studio 4.5 (Accelrys Inc., San Diego, CA, USA), we performed the docking studies on this protein, which we obtained from the protein data bank **(Labib *et al.*, 2017; Thabet *et al.*, 2018; Talaat *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

3-A Animal identification, classification and ecological parameters

As shown in Fig. (1), the sand dollar/sea biscuit is pentagonal in shape, slightly longer than broad, median-sized and characterized by owning a thick margin. The petaloid portion of its surface protrudes above the remainder of the body. The animal had both an aboral and an oral surface, the latter of which is concave. Short primaries and short secondaries characterize its spines. The fresh specimens are yellowish or beige in color, whereas the petaloid portion is frequently a deeper brown. The hunted specimens were released after drawing the coelomic fluid with a syringe. The animal classification is as follows:

Kingdom	Animalia
Phylum	Echinodermata
Class	Echinoidea
Order	Clypeasteroida

Family	Clypeasteridae
Name	*Clypeaster humilis*
	C. humilis
sea biscuits/sand dollars	

Fig. 1. A photo of *C. humilis* showing the oral (on the right) and aboral (on the left) surfaces

In this study, many parameters were investigated, such as physical properties "temperature", (T °C), electrical conductivity (EC), dissolved oxygen (D.O), total dissolved solids (TDS), salinity (S), PH, and oxidation reduction potential (ORP)) in two seasons: winter 2021 and spring 2022, as shown in Table (1).

Table 1. The obtained data of the "temperature", (T °C), electrical conductivity (EC), dissolved oxygen (D.O), total dissolved solids (TDS), salinity (S), PH and oxidation reduction potential (ORP)) in two seasons: winter 2021 and spring 2022

Physical parameters	Season	T C°	EC	D.O ml/l	TDS ppm	S‰	PH	ORP
	Winter	14.4	63.8	13.5	38.65	41.3	7.5	315
	Spring	14.7	64.9	13.8	37.85	41.5	7.5	285

A global positioning system (GPS-GP80)

27°17'7.67"N	**33°46'22.02"E**

Fig. 2. A photo representing screen shoot of Google Earth positioning the site of the samples collection from the coast of Hurghada, Red Sea, Egypt

3B- The microscopical results

The Semithin sections

In the studied semithin specimens, the most dominant type of cells is the small cells (Fig. 3A, B), which represent most of the total cells. The small cells exhibit an almost cubical shape. Another type of cell being caught by these photomicrographs is the secretory cell. The latter cells are subdivided into two types, obviously varying in structure (Fig. 3A, B). The first type of secretory cells is the secretory mucous cells (SMC); the cytoplasm of this type is filled with several large vacuoles, which are lightly stained with toluidine blue, with a relatively small nucleus pushed aside to the periphery, occupying a basal composition (Fig. 3B). The second type of the secretory cells is known as the secretory granular cells (SGC). The cytoplasm of these cells is filled with rounded granules, which are intensively-stained darkly with toluidine blue semithin sections (Fig. 3A, B). One hour later, the coelomocytes plaque – at the site of injury – was examined, and detected a large number of small coelomocytes were in addition to SGCs and SMCs (Fig. 3B). At the site of the syringe from which the coelomic fluid was drawn, after one hour, a second drowning were applied to investigate the cells at the site of the hole formed by the syringe and found intensive cellular aggregations with mature coelomocytes larger than those obtained from the first drawn (Fig. 3B). These cells were identified similar to that done in the first drawn as young coelomocytes, secretory mucous cells (SMC) and secretory granular cells (SGC).

The ultrathin sections (the ultrastructure results of the cellular part of the coelomic fluid "coelomocytes" of Clypeaster humilis)

The examination of the ultrastructure of different types of coelomocytes prepared from the separated cellular part of the coelomic fluid of *C. humilis* that includes numerous young (small) cells and other mature (large) coelomocytes. The first drawn of the coelomic fluid by syringe revealed that the first type of coelomocytes, the young coelomocytes, exhibits a centrally-located nucleus, with prominent euchromatin (Fig. 4A). The heterochromatin is restricted to the rim of the nucleus and small patched inside it. The cytoplasm is highly-

loaded by numerous vacuoles in addition to other vacuoles including electron-dense materials, which might be lysosomes.

Numerous transverse sections of the cytoplasmic extensions atypical formula of microtubules inside these cytoplasmic extensions (Fig. 4A). The mature coelomocytes are subdivided into two types, which are defined as secretory granular and secretory mucous cells, as shown in Figs. (4B- D). As shown in Figs. (5A- F), these specimens were taken from the plaque site (or the site of the formed hole due to the syringe entrance for coelomic fluid drawn) one hour after injuring the sea cake to collect the coelomic fluid by the syringe. The young coelomocytes are shown interconnected with each other through cytoplasmic processes to form syncytium (Figs. 5A- D), which might be fused together to form larger coelomocytes to prevent the leakage of the cytoplasmic fluid with its cells. In Figs. (5E, F), the cells are shown highly loaded by extensive amounts of secretory granules and might represent the active site of forming secretory granular and mucous cells.

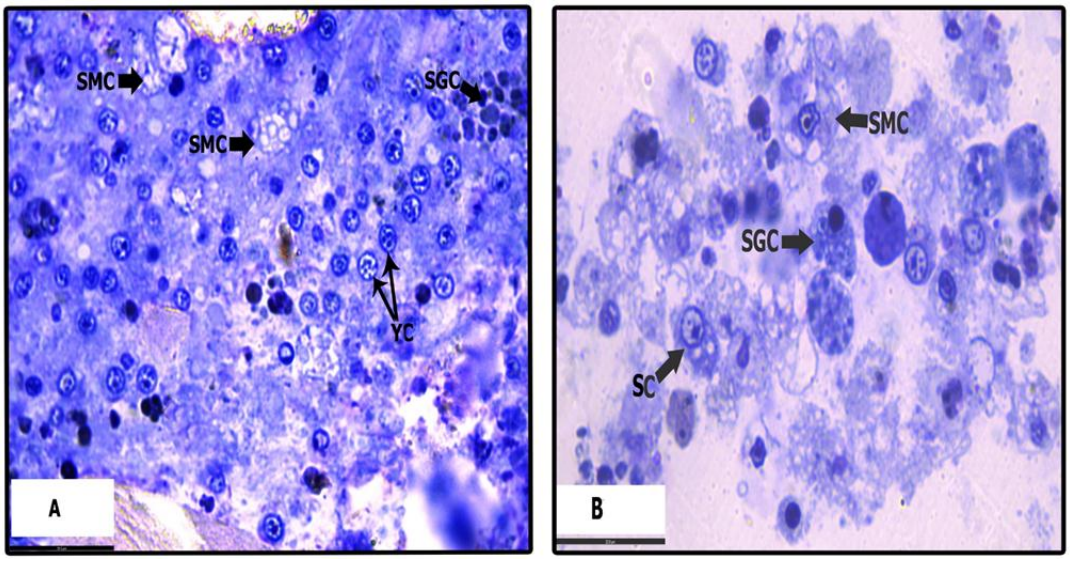

Fig. 3 (A, B). Photomicrographs of semithin sections of the cellular contents extracted from the coelomic fluid from *Clyoeaster humilis* processed, and stained with toluidine blue**. A)** showing the dominant cells that are known as the small cells (SC) or young coelomocytes. Other coelomocytes type is known as secretory cells differentiated into 2 subtypes: secretory mucous cells (SMC) and secretory granular cells (SGC). X 400. **B)** Showing the cells trapped at the site of extracting the coelomocytes by the syringe 1hr after; at the site of plaque to pan the coelom exudate. Numerous small coelomocytes were observed at the site destructed by the needle; in addition to the two types of mature coelomocytes SGC & SMC. Notes that these cells are larger than those trapped for the first time. X 1000.

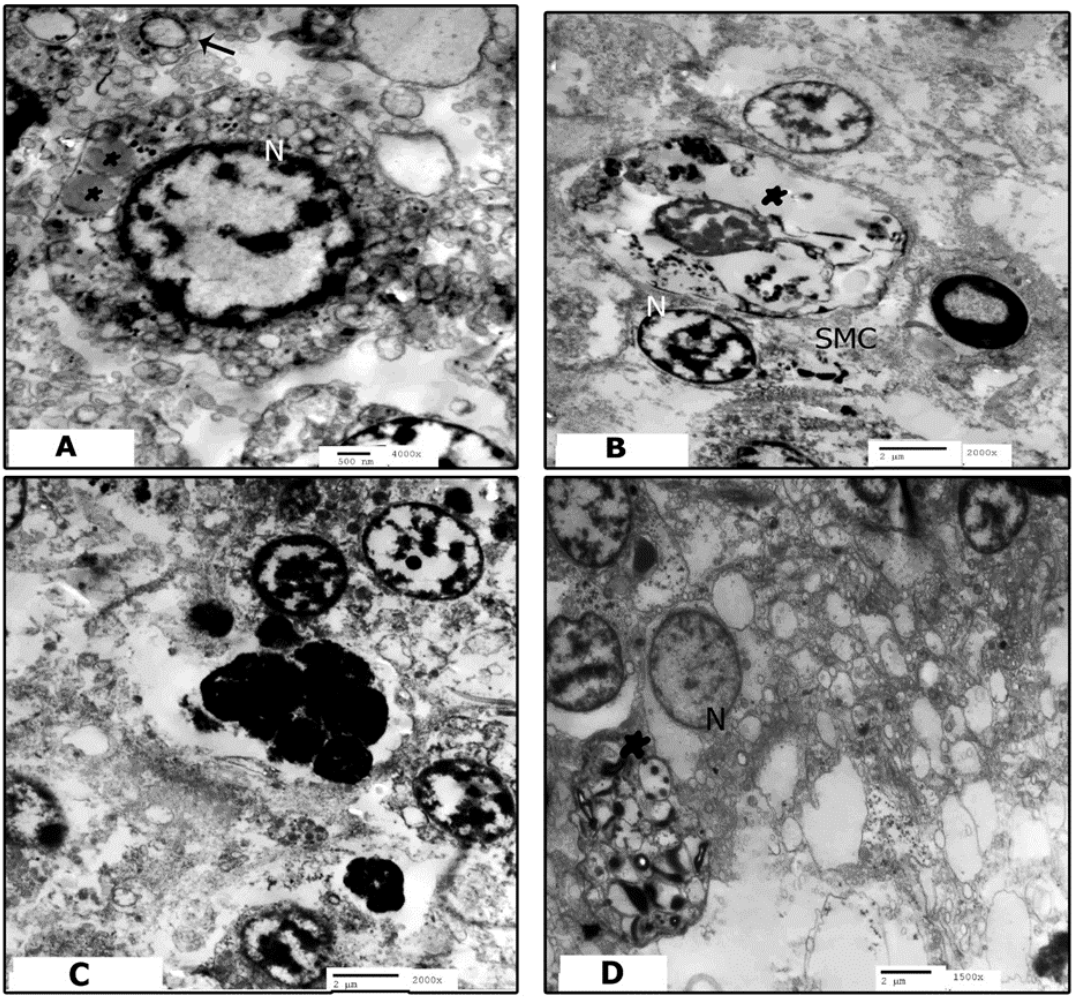

Fig. 4 (A-D). Photomicrographs of coelomocytes separated from the coelomic fluid extracted from *Clypeaster humilis*. **A)** showing numerous small cells, which are interconnected with each other through cytoplasmic processes. **B)** focusing on the ultrastructure of a small cell that exhibits a centrally-located nucleus (N), with prominent euchromatin; and the heterochromatin restricted to the rim of the nucleus and small [patched inside it. The cytoplasm is highly loaded by numerous vacuoles and lysosomes (*). Numerous t. s. of cytoplasmic extensions reveals no typical formula of microtubules (arrow). **C)** representing the active site of forming secretory granular and mucous cells (SGC, SMC). **D)** Sowing numerous types of secretory granular and mucous cells (SGC, SMC).

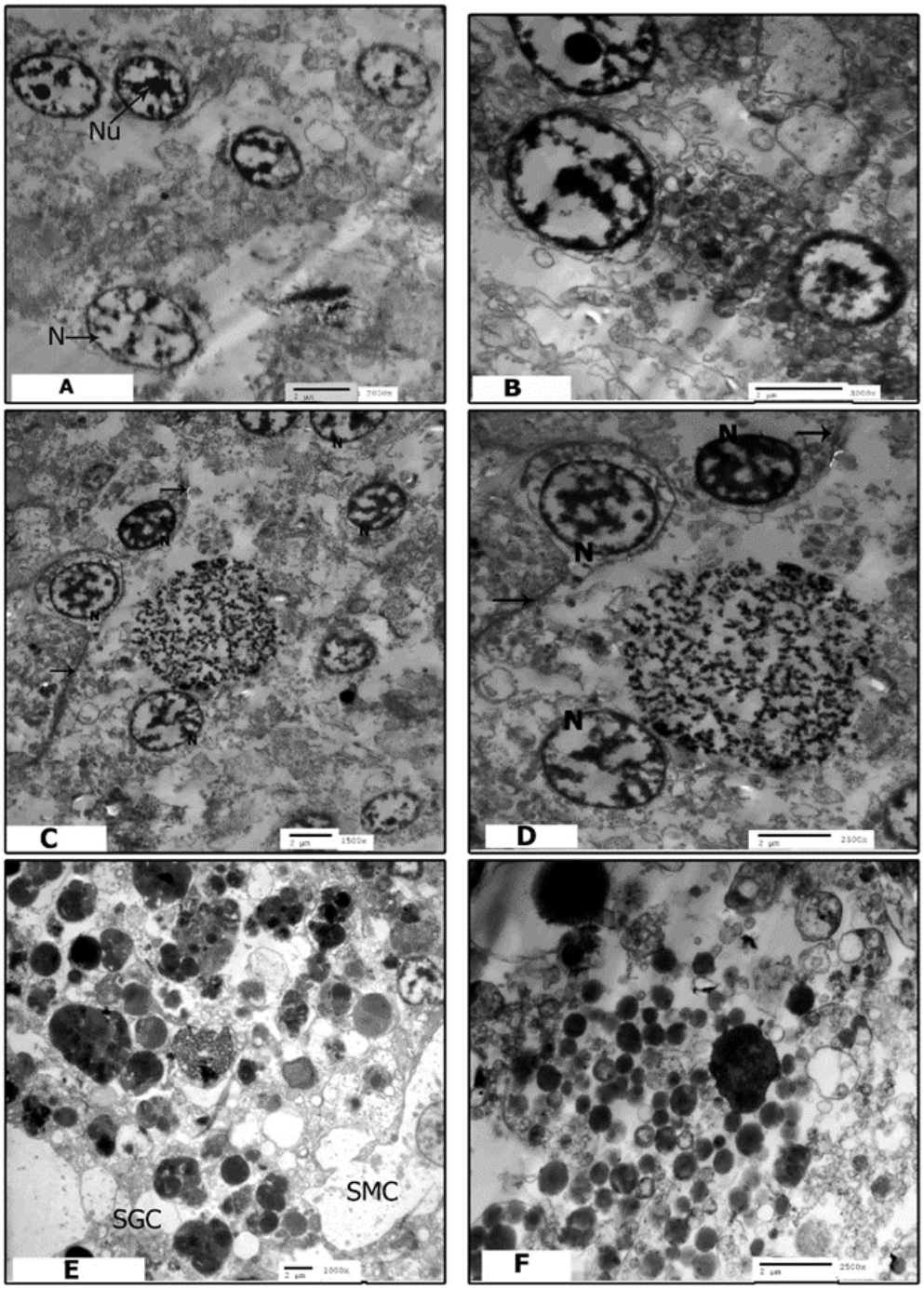

Fig. 5 (A-F). Photomicrographs of coelomocytes separated from the coelomic fluid extracted from *Clypeaster humilis* one hour after the 1st coelomic fluid was drawn. **(A-D)**: These specimens were taken from the plaque site, in which the young coelomocytes were interconnected with each other through cytoplasmic processes (arrows) to form a syncytium. **(E & F)** showed extensive amounts of secretory granules; and might represent the active site of forming secretory granular and mucous cells (SGS & SMC).

3C) Chemical compositions of n-hexane fraction obtained from sea cake coelomic fluid using GC/MS

GC/MS analysis of the *n*-hexane fraction obtained from sea cake coelom lead to the identification of seven compounds; namely, 4,6-dimethyldodecane, *n*-tridecane, *n*-tetradecane, *n*-heptadecane, *n*-octadecane, and Decanedioic acid, bis(2-ethylhexyl) ester that belong mainly to fatty acid derivatives (Table 2). A scheme showing the identified chemical conistiuents in the n-hexane fraction is illustrated in Fig. (6).

Table 2 Chemical compositions of *n*-hexane fraction obtained from sea cake coelom using GC/MS supplied withRtx-5MS column

No.	Compounds[f]	R_I		% Composition	References
		Measured	Reported		
1.	4,6-Dimethyldodecane	1276	1285	7.06	MS, RI
2.	*n*-Tridecane	1322	1300	2.70	MS, RI
3.	*n*-Tetradecane	1395	1400	8.09	MS, RI
4.	*n*-Hexadecane	1595	1600	10.07	MS, RI
5.	n-Heptadecane	1704	1700	4.25	MS, RI
6.	*n*-Octadecane	1786	1800	2.40	MS, RI
7.	Decanedioic acid, bis(2-ethylhexyl) ester	2803	2812	4.22	MS, RI

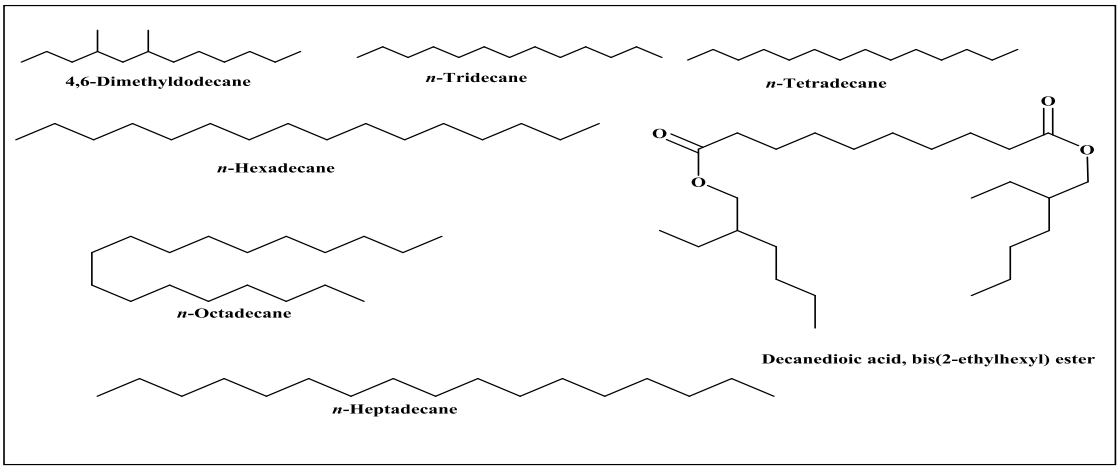

Fig. 6. Scheme showing the chemical compositions of *n*-hexane fraction obtained from sea cake "*C. humilis*" using GC/MS, supplied withRtx-5MS column

In silico molecular docking studies

Regarding sea cake secondary metabolites identified from the n-hexane fraction results presented in Table (3) show that decanedioic acid, bis(2-ethylhexyl) ester exhibited the best fitting within the active site of glycogen synthase kinase3-*β* protein, followed by *n*-octadecane and *n*-heptadecane, with free binding energies (Δ G) of -53.25, -40.42 and -38.56 Kcal/mol, respectively, exceeding in this approach the co-crystalised ligand (ΔG = -12.10 kcal/mol). One typical H-bond is formed between Arg141 and decanedioic acid, bis(2-ethylhexyl) ester, and five alkyl bonds are formed between Val110, Leu188, Leu132, Met101 and Ala83 (Fig. 7A). Four alkyls and -alkyl connections are formed between n-octadecane and Val110, Leu132, Met101, and Tyr140 (Fig. 7B), whereas n-heptadecane forms one alkyl bond with Ile32 and multiple Van der Waals contacts with the amino acid residues in the active sites (Fig. 7C).

Table 3 Free binding energies (kcal/mol) of major compounds in the *n*-hexane fraction obtained from sea cake coelom 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 ; Cys199, Leu188, Leu132, Tyr134, Ala83
n-Tridecane	-28.79	1:Ile32	1 ; Phe67
n-Tetradecane	-33.27	-	2 ; Cys199, Lys85
n-Hexadecane	-36.27	-	-
n-Heptadecane	**-38.56**	-	1:Ile32
n-Octadecane	**-40.42**	-	4; Val110, Leu132, Met101, Tyr140
Decanedioic acid, bis(2-ethylhexyl) ester	**-53.25**	1; Arg141	5; Val110, Leu188, Leu132, Met101, Ala83
Co-crystalised ligand (PF-367)	-12.10	2; Val135	8; Val70, Lys85, Ala83, Leu132, Leu188, Val110, Asp133

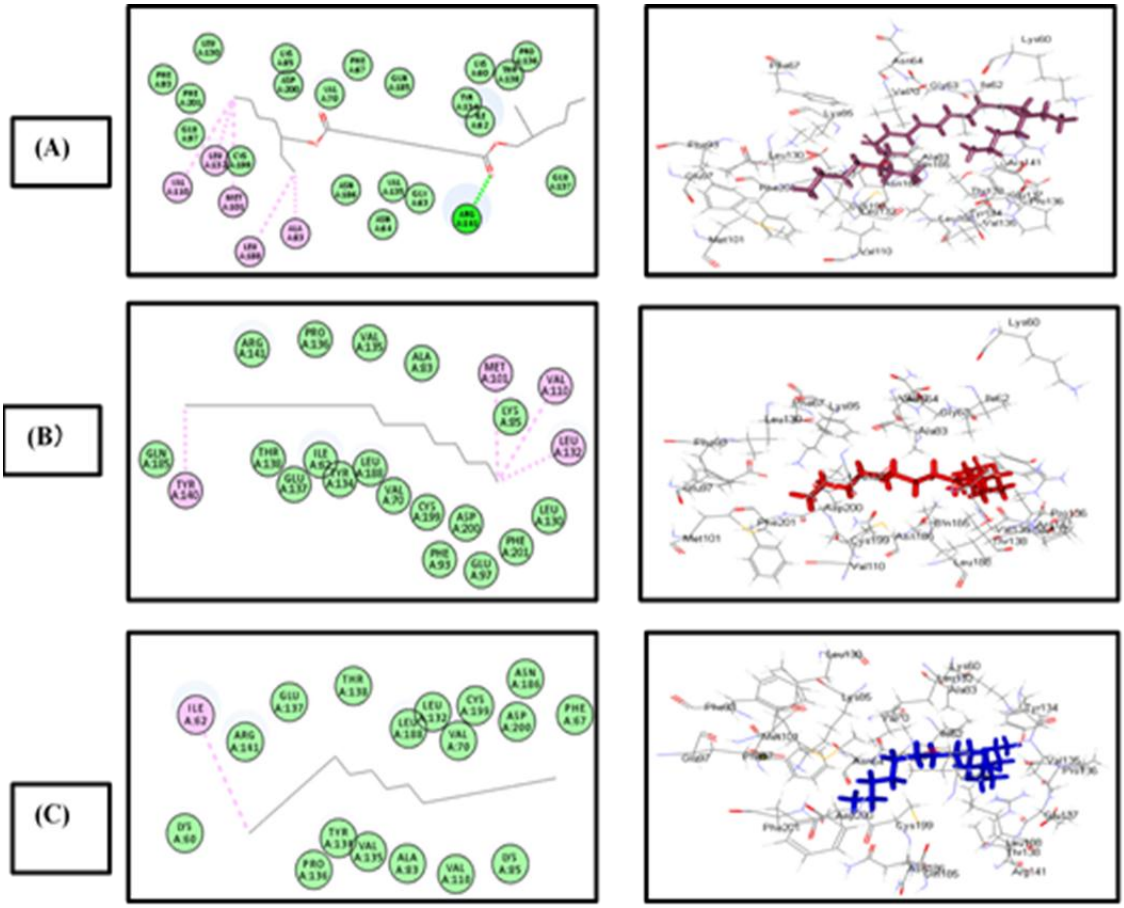

Fig. 7. 2D and 3D binding modes of decanedioic acid, bis(2-ethylhexyl) ester (A), *n*-octadecane (B) and n-heptadecane within the active sites of glycogen synthase kinase3-*β* protein

DISCUSSION

The worldwide specialists concerned with Echinodermata were based on the cell and molecular studies of the last decades for their regenerative properties; however in Egypt, nobody was found to be concerned with this phylum from a medical or cellular point of view. The echinoderms' importance is derived from their ability to restore any lost part of their bodies. The studies done on echinoderms were basically-carried out by comparing their diversity of regenerative phenomena on different animals on evolutionary bases; as previously-mentioned by **Dolmatov (1999)**, **Candia Carnevali and Bonasoro (2001)**, **Thorndyke *et al*. (2001)** and **Candia Carnevali (2006)**.

Numerous publications dealt with the fine organizations of various organs in different echinoderms including *Clypeaster* species **(Bargmann and von Hehn, 1968; Cobb, 1978; Walker, 1979; Schoenmakers *et al.*, 1981; Martinez *et al.*, 1991),** but no other publication subjected to the electron microscopic analysis of sea cake coelomocytes, or even the essential role of its coelomic fluid till now. Most of the popular published data are

concerned with insights into the paleoenvironment of sea biscuits as a fossil, its diversity as a living organism, or even the newly recorded species **(Suryanti *et al.*, 2016; Buwono & Fitri, 2017; Martín-Cao-Romero *et al.*, 2017; Armstrong & Grosberg, 2018; Mihaljević & Rosenblatt, 2018; Tsaparas *et al.*, 2018; Cambronero-Solano *et al.*, 2019; Lavtizar & Okamura, 2019; Mancosu & Nebelsick, 2019; Perricone & Collin, 2019; Collin *et al.*, 2020; Bencomo *et al.*, 2021; Lu Remindima *et al.*, 2021; Mooi & Noordenburg, 2021)**.

Based on previous publications, the most popular examined coelomocytes of this phylum is the sea stars. Microscopically, many authors estimated the most popular cells that lined the coelomic cavity which are known as the coelomic epithelial cells to others, which are floating on the coelomic fluid and are known as the coelomocytes. The latter cells are furtherly-differentiated into young coelomocytes (Figs. 3, 4 and 5) – and also known as small coelomocytes regarding their small size – and mature coelomocytes, which are larger in size. The mature coelomocytes are in turn subdivided into secretory granular and secretory mucous cells. As a matter of fact, in this work, the examined coelomocytes of *Clypeaster humilis* were compared to the coelomocytes identified in sea stars or sea cucumbers (no other work was published addressing sea cake coelomocytes, as well as the cells seem to be almost identical in this phylum); we own the privilege of the first publication on the coelomic fluid of this marine animal cellular content. It is worth mentioning that the examined young coelomocytes lack the typical arrangement of the microtubules of the flagellum (Fig. 4A), compared to those obtained in the sea cucumber or starfish **(Xing *et al.*, 2008; Gorshkov *et al.*, 2009; Sharlaimova & Petukhova, 2016; Sharlaimova *et al.*, 2021)**.

Based on the obtained data, it was shown that the young coelomocytes are interconnected with each other through cytoplasmic processes to form a syncytium at the site of injury after one hour by the syringe; this finding agreed with the results published by **Sharlaimova and Petukhova (2016)**. These authors found that in cultured media of cells from two types of starfish, part of these cells joined together to form a syncytium.

For the mature coelomocytes, **Xing *et al.* (2008)** reported in their article (about Japanese spiky sea cucumbers) that the small cells are called lymphocytes, and progenitor cells which are called here 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 differentiated into secretory granular and mucous cells represent 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 the quantity. Moreover, these small coelomocytes which then transformed to mature coelomocytes bear and deliver the clotting materials to the site of injury to form a plaque or clot as in humans, as shown in Fig. (5E, F). According to **Hillier and Vacquier (2003)** and **Xing *et al.* (2008)**, clotting is thought to be a defense mechanism that the animal forms to avoid the loss of its coelomic fluid. At the same time, according to literature, protein factors that promote cell migration and proliferation are often found in the coelomic fluid of injured starfish (Fig. 3A- B), which explains the coelomocytes with larger sizes in photomicrograph

(3B) and the electron micrographs (5A- D), in which the young coelomocytes tend to form a syncytium. Side by side, collectively; by mentioning the protein factors that activate the cell migration to form a plaque or clot, it is worth mentioning that one of these protein factors in the coelomic fluid extracted from *C. humilis* might be glycogen synthase kinase3-β protein, which is obtained by the protein docking. Additionally, it might be considered as a promising constituent for many medicinal applications including wound healing, especially in diabetic patients. In **2021**, **Soleimani *et al.*** worked on the coelomic fluid of a certain type of sea urchin and listed it as a new perspective for medicinal antioxidants, which amplifies the critical importance of the coelomic fluid of this phylum if we collect the findings of this work with ours.

In this study, the coelomic fluid was extracted from *Clypester humilis* by a syringe and then centrifuged, leading to the identification of seven compounds; namely, 4,6-dimethyldodecane, *n*-tridecane, *n*-tetradecane, *n*-heptadecane, *n*-octadecane and Decanedioic acid, bis(2-ethylhexyl) ester that belong mainly to fatty acid derivatives (Table 2 & Fig. 6). Regarding sea cake secondary metabolites identified from the n-hexane fraction results and presented in Table (3), it was shown that decanedioic acid, bis(2-ethylhexyl) ester exhibited the best fitting within the active site of glycogen synthase kinase3-*β* protein followed by *n*-octadecane and *n*-heptadecane. To this finding, the coelomic fluid of the sea cake could be used as an effective or potent agent for wound healing **(Harish *et al.*, 2008; Naika *et al.*, 2015; Aksoy *et al.*, 2021)** due to its characteristic glycogen synthase kinase3-*β* protein, which is identified by the protein docking (Table 2, 3 & Fig. 7A- C) of this work.

Coelomocytes have at least three distinct roles in the body. To begin, they swiftly aggregate in the wall of the injured portion of the body, repairing the barrier that keeps the organism's internal medium from leaking into the outside **(Davidson, 1953; Smith, 1981; Candia Carnevali *et al.*, 1993; Moss *et al.*, 1998)**. Second, during the process of regeneration, coelomocytes engulf dead cells and invading microbes in the wound region through phagocytosis **(Heatfield & Travis, 1975; Mladenov *et al.*, 1989; Matranga, 1996)**. Finally, coelomocytes are responsible for the production of several important protective and regulatory chemicals. In addition, echinoderms are useful to humans as a food and medicinal source, and they play a vital role in the environment.

Finally and collectively, from the obtained data in this work, it might be crystal clear that the main purpose of this work was describing and differentiating the coelomocytes – which are in our opinion responsible for secreting glycogen synthase kinase3-*β* protein – together with the protein docking that closely serves the hypothetical theory and emphasizes it, in which the coelomic extract is the best choice to be used for a promising wound healing process in the nearest future for human benefits.

CONCLUSION

The coelomic fluid extracted from *C. humilis* exhibits glycogen synthase kinase3-*β* protein, which could be considered a promising constituent for many medicinal applications including wound healing in diabetics. All coelomocytes are differentiated from the young or progenitor coelomocytes, which then harbor different secretory materials and turn into secretory granular and secretory mucous coelomocytes.

Abbreviations:
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.

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The authors declare no conflict of interest.

Availability of data and materials
The datasets supporting the conclusions of this article are included within the article.

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Declarations
Ethical approval and consent to participate:
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, Ain Shams University.

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