Egypt. J. Chem. **Vol. 67**, No. 8 pp. 95 - 107 (2024)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Study of the chemical composition and anesthetic effect of Cladophora *glomerata* extract on the physiological and hematological aspects of *Biomphalaria alexandrina* snails as biological models

Shereen M. Mansour,[a] Eman A. Morsi,[b] Ayman S. Mohamed,[c] Sara S.M. Sayed[d]*

[a] *Medical Malacology Department, Theodor Bilharz Research Institute, Giza, Egypt*
[b] *Medicinal Chemistry Department, Theodor Bilharz Research Institute, Giza, Egypt*
[c] *Zoology Department, Faculty of Science, Cairo University, Egypt*
[d] *Environmental Research Department, Theodor Bilharz Research Institute, Giza, Egypt*

Abstract

Algae produce numerous biologically active substances, some of which could be turned into brand-new medications. Thus, the current work utilized *Biomphalariaalexandrina* snails as a biological model to assess the anesthetic effect of *Cladophoraglomerata*methanolic extract. After 24 h of exposure followed by 24 h as a recovery period, the results indicated that *C. glomerata* extract was safe for snails up to 500 mg/L. The anesthetic effect was seen with snails subjected to 500 mg/l, reaching 100%, while the relaxation effect was observed with snails subjected to 100 and 300 mg/ L, recording 13.3 and 66.6% of the exposed snails, respectively. The total hemocyte count was directly proportional to the concentrations of algal extract after 1 h, however, it returned to normal after 20 h. Exposure of snails to different concentrations of the algal extract led to several rapid changes in the shape and content of hemocytes and stimulated hemocyte division after one hour of exposure. Acetylcholinesterase activity (AChE) and Lactate dehydrogenase (LDH) levels significantly decreased in all treated snails. GC-MS results exhibited that *C. glomerata* extract contained 16 chemical substances, which might possess anesthetic, antioxidant, and anti-inflammatory activities and stimulate cell proliferation.

Keywords:Anesthesia ; *C. glomerata* ; hemocytes ; *B. alexandrina* ; GC mass

1. Introduction

Many medicines are being made from natural ingredients **[1]**.Previous research proposed that phytochemical-rich herbal plant preparations might be turned into medicines that are relaxing, antinociceptive, analgesic, local anesthetic, and general anesthetic **[2,3,4]**. *Aconitum ferox* wall, for example, contains diterpenoid alkaloids and has been used as an analgesic, anti-inflammatory, sedative, and anti-rheumatic **[2]**.Mesaconitine, hypaconitine, lappaconitine (diterpenoid-ester alkaloids), benzaconine, and benzoylaconine are present in *Aconitum chasmanthum* and have been utilized as sedatives and anesthetics **[3]**, and *Ceropegiajuncea*Roxb has pyridine alkaloid, cerpegin, and lupeol, a triterpene, have shown as promising analgesic, local anesthetic, and topically anesthetic properties **[4]**. These potential phytochemical-based anesthetics could offer alternative options for patients who may have adverse reactions to conventional anesthesia. However, further research and clinical trials are needed to fully explore the efficacy and safety of these phytochemical compounds in anesthesia **[5]**.

Nowadays, our planet needs to turn environmental problems into opportunities. As a result, several beneficial uses of macroalgal biomass have been emphasized, including pharmaceutical, nutraceutical, and cosmetic applications, fertilizers and bio-stimulants for plant growth, food additives, indicators of environmental pollution, sorbents of toxic metal ions from wastewater, and raw materials for the production of biofuels **[6]**.

Cladophora algae has the potential to help environmental problems and maintain human health

*Corresponding author e-mail: eco_gut@yahoo.com.; (Sara S.M. Sayed).
Received date 11 November 2023; revised date 05 February 2024; accepted date 07 February 2024
DOI: 10.21608/EJCHEM.2024.247872.8853

and well-being in the face of new difficulties, even though it is frequently seen as a nuisance alga in natural water. The macroscopic green algae family contains over 183 species, and *Cladophoraglomerata* is one of the most prevalent freshwater green algae. It has filamentous forms and develops rapidly on a variety of substrates **[7]**. In pharmaceutical applications, *Cladophora*species contain active substances that display a wide range of activities, including antioxidant and antitumor properties **[8]**, antibacterial and antidiabetic properties **[9,10]**, anti-ulcer, hypotensive, and analgesic activity **[11,7]**. As a result, algal extracts are now a source of many chemicals for a healthy existence **[12]**.

Since gastropods have huge, uniquely identifiable nerve cells, they can be employed as model organisms for anaesthetics investigations on the cellular network and behavior**[12]**. To test the anesthetic effect, researchers have applied two behavioral criteria: (a) the withdrawal response to stimulation of the arms and siphon because withdrawal responses are used to test the depth of anesthesia in humans and other animals and are diminished in a dose-dependent manner by inhalational anesthetics in the pulmonatemollusk *Lymnaeastagnalis* **[13]** and (b) color change, which is common in cephalopods and is known to be under central motor control **[14]**.

Other indicators include Lactate dehydrogenase (LDH), which is a crucial enzyme for cellular respiration that transforms glucose into cellular energy and is present in all tissues **[15,16]**. Also, Acetylcholinesterase (AChE), which is a membrane-resident enzyme that participates in the termination of nervous impulse transmission by promoting the hydrolysis of the neurotransmitter acetylcholine to coordinate the nervous system **[17]**, and nerve conduction processes at the junction of the nerve ending of muscle **[18]**. Furthermore, the nerve ending produces the AChE that is released at the myoneural junction during an action potential in living organisms. After that, it diffuses via the gap between the muscle and the nerve. It is found in the membranes of both vertebrate and invertebrate animals, but invertebrates frequently have highly polymorphic cholinesterase enzymes (ChE) **[17]**. Acetylcholinesterase is quite sensitive to the various chemicals that inhibit it. The inactivation of AChE causes acetylcholine accumulation in synapses and ongoing stimulation of cholinergic receptors, resulting in altered neurotransmission and paralysis **[19]**. Therefore, the present work aims to study the potential anesthetic properties of *Cladophoraglomerata* methanol extract on the physiological and hematological aspects of *Biomphalariaalexandrina* snails as a biological model, supported by studying the chemical constituents of *C. glomerata* methanol extract.

2. Materials and Methods

2.1 Collection and identification of the alga

During the summer of 2019, *Cladophora glomerata* (L.) (Kützing, 1843) (Specimen number :L.4122502) was obtained from the freshwater pond's surface in Giza, Egypt. It was then transported to the laboratory, cleaned, washed, and identified in the department of botany at Ain Shams University.

2.2 Algal extraction

The alga was cleaned with fresh water to remove any contaminants. The biomass (70 g) was immersed in 1.5 L of methanol for one week at room temperature. Then, it was filtrated through filter paper 1.5 cm , and the solvent was evaporated using an evaporator (BUCHI, Switzerland) and kept in a fridge at 4°C until analysis.

2.3 Preliminary Phytochemical Screening

According to the method of **Abdel-Hady et al. [20]**, phytochemical evaluation of *C. glomerata* extract was conducted to determine the qualitative contents of carbohydrates, alkaloids, saponins, flavonoids, tannins, sterols, terpenes, and phenolic compounds.

2.4 GC-MS analysis

According to the procedure described by **Elangovan et al. [21]**, the crude methanol extract of the algae was subjected to GC-MS analysis utilizing a Thermo Scientific TRACE 1310 Series Gas Chromatograph on Helium as a carrier gas in the TG-SQC column. The sample was insinuated in split mode with a constant flow of 1.5ml/min, the mass spectral range was (40-1000Hz), the mass transfer line temperature was 3000c, and the ion source temperature was 3000c. The components were identified by comparing their mass spectra and retention time.

2.5 Effect of *C. glomerata* extract on snails

2.5.1 Mortality and relaxation

Adult *B. alexandrina* snails (8 mm ±1) were subjected to various concentrations (100, 300, 500, 700, and 1000 mg/L) of *C. glomerata* extract for 20 h. The snails were checked after 2, 4, 7, 10, 15, and 20 h, and the relaxation and anesthetic percentages were noted. Each dose was tested in three replicates with 15 snails/L in each replicate. Another set of snails of the same size was kept in dechlorinated water as a control group. After 20 h, they recovered in dechlorinated water, and the percentages of the mortalities were recorded. The relaxation effect was recorded as snails slowly withdrew their soft parts inside their shells. Meanwhile, the anesthetic effect was recorded when snails lost the ability to pull out during exposure.

2.5.2 Haematological analysis

2.5.2.1 Collection of hemolymph

The hemolymph from each group was obtained from 5-7 snails/group by a cardiac puncture in the soft part. The *B. alexandrina* snails were cleaned with 70% alcohol and dried **[22]**. For the hematological analysis, 700 µl of hemolymph were taken in a 1.5 ml Eppendorf tube.

2.5.2.2 Count and differentiation of hemocytes

Twenty microlitres of hemolymph were used, and the number of cells in each tested group was counted in three replicates by diluting freshly collected hemolymph in a leucocyte count solution of 1:20 ratio. For differential hemocyte investigation, hemocyte monolayers were prepared by placing ten µl of hemolymph on a glass slide and allowing hemocytes to adhere to the glass slide **Zelck and Becker [23]**. Hemocytes were dehydrated with methanol for 5 min at room temperature and stained **[24]**. Differential hemocyte counts were recorded in each treated and control group.

2.5.3 Activities of enzymes

2.5.3.1 Acetylcholinesterase (AChE)

Each snail's soft part was homogenized in 0.1 M phosphate buffer (pH 8.0) using a polytron homogenizer and centrifuged for 20 min. Using acetylthiocholine iodide as the substrate, the supernatants were utilized to quantify AchE activity **[25]**. The activity of AChE was measured in micromoles of substrate hydrolyzed per milligram of protein hydrolyzed per minute. Bovine serum albumin (BSA) was utilized as a reference, and activities were adjusted to total protein **[26]**.

2.5.3.2 Lactate dehydrogenase (LDH)

The activity of lactate dehydrogenase was assessed using**Bais and Philcox's[27]**methodology in the homogenates of snail tissue.

2.6 Statistical analysis

The significant variations in granulocytes, hyalinocytes and amoebocytes percentages were tested using the chi-square test between the control and the experimental groups after three periods. Two way ANOVA was used to compare the differences in total hemocyte count values between all treated groups at different exposure intervals and using different concentrationsof *C. glomerata* extract at $P<0.05$. Meanwhile, One way ANOVA was performed to evaluate the significant differences in total hemocyte count between all treatments and control groups at exposure time and concentrations separately at $P< 0.05$. Data of LDH and AchE activities were analyzed using one-way ANOVA to evaluate significant differences between the control and treatment groups. All statistical analyses were carried out using the SPSS computer program (version 17).

3. Results and Discussion

As shown in **Table 1**, the current methanolic extract of *Cladophoraglomerata* lacked tannins but contained high amount of alkaloids, flavonoids, terpenoids and phenols and moderate amount of carbohydrates and saponins. These findings support earlier research on *C. glomerata***[28, 29]**, and they are relatively in line with **Petchsomrit et al. [30]**, who claimed that the extract of *C. glomerata* is a good source of phenolic compounds, chlorophylls, carotenoids, fatty acids, and antioxidants. According to **Al Jaber et al. [31]**, *C. glomerata*methanolic extract is free of phenols while containing alkaloids, flavonoids, tannins, saponins, glycosides, triterpenoids, and sterols. Recent researches have shown that flavonoids, alkaloids, and saponins have broad therapeutic properties. They are commonly known for their antioxidant and anti-inflammatory effects, inhibiting pro-inflammatory cytokines and anti-cancer by promoting apoptosis in cancer cellsby producing caspases and immunomodulatory activities **[32, 33]**. Furthermore, terpenoids, alkaloids, and flavonoids are present in *C. glomerata* in high amounts which have been shown as a local anesthetic, general anesthetic, antinociceptive, analgesic, or sedative characteristics **[1, 5].** This observation supports the results of phytochemical screening of *C. glomerata*, which may have anesthetic, antioxidant, and anti-inflammatory activities.

The *C. glomerata*methanolic extract bioactive substances were detected using GC-MS analysis by comparing their mass spectrum fragmentation patterns to those reported in the Wiley and NIST Mass Spectrallibraries. The retention periods of these compounds are shown in **(Fig. 1).** A total of 16 compounds were determined, where Oleic acid (27.72%), methyl palmitate (19.63%), cis-vaccenic acid (14.83%), methyl linoleate (12.31%), palmitic acid (8.40%), and methyl stearate (6.68%) were the most prevalent substances **(Fig. 2)** and **(Table 2)**.

Chemical substances	Results
Carbohydrates	+
Alkaloids	++
Flavonoids	++
Saponins	+
Phenols	++
Tannins	-
Sterols and or/Triterpenes	++

(++): high amount, (+): moderate amount, (-): Absent.

Table1 Bioactive compounds in the methanolic extract of *Cladophoraglomerata*

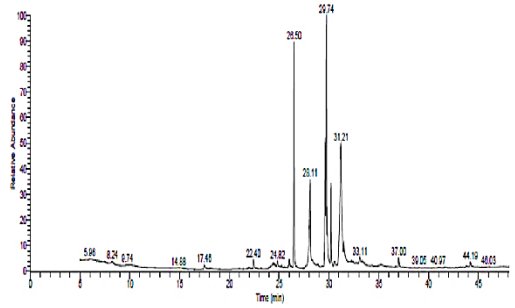

Fig. 1 The chromatogram of GC-Mass spectrophotometry showed in methanolic extract of *C. glomerata*. Oleic acid constitutes a mono-unsaturated omega-9 fatty acid that occurs naturally in a variety of vegetables.

Oleic acid is a mono-unsaturated omega-9 fatty acid occurring naturally in various vegetables. It appears in the modulatory impacts of numerous physiological activities **[34].** Some studies have shown that oleic acid protects against bacterial, cancer, autoimmune, and inflammatory illnesses, which may be related to the suppression of proinflammatory cytokines and the activation of anti-inflammatory cytokines **[35, 36]**.

Besides, its ability to improve the immune response and its antioxidant activity against cellular oxidative stress, DNA damage, and immune system modulation **[35, 37]**. Also, it was reported that oleic acid can promote cell proliferation without any apoptosis and necrosis **[38, 39]**.

Methyl palmitate, also known as hexadecanoate methyl ester or palmitic acid's methyl ester, demonstrated high antioxidant activity by lowering oxidative stress indicators, and anti-inflammatory and anti-apoptotic activity **[40, 41, 42]**. Cis-Vaccenic acid is an omega-7 fatty acid and possesses anti-inflammatory, antioxidant, antibacterial activity, and hypolipidemic activities **[43, 44]**, and it induces erythroid differentiation **[45, 46]**. Methyl linoleate, a fatty acid, exhibits immunological activation, protection against pathogens, and antioxidant, anti-apoptotic, and anti-inflammatory properties **[47, 48]**.

All living organisms contain palmitic acid, which has immunomodulatory, anti-inflammatory, and antioxidant activities. It could confer pre-treatment of cells and protection of cell viability against oxidative stress **[49, 50]**.

Methyl stearate, a methyl ester of a saturated fatty acid, hasantifungal, anti-inflammatory and immunomodulatory effects.

It activates polymorphonuclear leucocytes, which aid in the fight against infection. It also has biological features such as suppression of lipid production and antioxidant action **[51, 52]**.

Results in **Table (3)** shows that the *C. glomerata* extract was safe on snails up to 500 mg/L after 24 h of exposure. The relaxation effect was observed only with snails exposed to 100 and 300 mg/L of *C. glomerata* extract, recorded (13.3 and 66.6 %, respectively).

On the other hand, the anesthesia effect was observed with snails exposed to 500 mg/L recorded 100 %. **Martins-Sousa et al. [22]**reported that Cetamine at 250 mg/ L was not harmful to the snails, the relaxation of 60 % of *Biomphalaria*sp. snails was only after 8 hr of exposure, and complete anesthesia was observed in 40% of exposed snails post 20 hours. **Winlow et al. [12]** declared that the ideal anesthetics should have no side effects, act as muscle relaxants, analgesics, and amnesics, and render the individual unconscious.

The safety result also may be attributed to *Cladophora* sp. having a variety of compounds such as alkaloids, flavonoids, and terpenoids that have been frequently reported to possess local and general anesthetic, antinociceptive, analgesic, or sedative properties **[1, 5]**.

In relaxed snails, the headfoot region slowly withdrawn reflex when touched with a single needle and no change in color of the soft part. while the anesthetized snails showed redness of the soft parts after 1 h of exposure **(Fig. 3a)**, and the snails lost withdrawal reflex after 7 h **(Fig. 3b)**. The snail's group exposed to 500 mg/L of *C. glomerata* extract showed 100% relaxation and complete anesthesia after 4 and 7 h, respectively **(Table 4)**.

Because their relatively simple neural networks mediate well-defined and simple behaviors, molluscs are considered suitable models for anesthetic modes of action that preceded and established the foundation for future mammalian studies **[53]**.

Table 2 GC-MS analysis of major compounds in methanolic extract of *C. glomerata*

Peak no.	Rt.	Area %	MF	MW	Components	Compound Nature
1	17.45	0.48	$C_4H_{12}ClN$	109	Butan-2-amine hydrochloride	organic compound
2	22.40	1.06	$C_{15}H_{30}O_2$	242	Methyl myristate	fatty acid methyl ester
3	24.82	0.64	$C_{22}H_{30}N_2O_3$	370	Aspidospermidin-17-Ol, 1-acetyl-16-methoxy-	alkaloid
4	26.02	0.81	$C_{20}H_{30}O_2$	302	Arachidonic acid	Fatty acid
5	26.50	19.63	$C_{17}H_{34}O_2$	270	Methyl palmitate	fatty acid methyl ester
6	28.11	8.40	$C_{16}H_{32}O_2$	256	Palmitic acid	Fatty acid
7	29.60	12.31	$C_{19}H_{34}O_2$	294	Methyl linoleate	fatty acid methyl ester
8	29.74	27.72	$C_{19}H_{36}O_2$	296	Oleic acid	Fatty acid
9	29.83	3.28	$C_{19}H_{36}O_2$	296	Methyl elaidate	fatty acid methyl ester
10	30.20	6.68	$C_{19}H_{38}O_2$	298	Methyl stearate	fatty acid methyl ester
11	30.54	0.74	$C_{21}H_{34}O_2$	318	methyl arachidonate	fatty acid methyl ester
12	31.20	14.83	$C_{18}H_{31}ClO$	298	cis-Vaccenic acid	omega-7 fatty acid
13	31.50	1.24	$C_{17}H_{36}$	240	Heptadecane	alkane
14	33.10	0.71	$C_6H_{10}O_4$	146	Adipic acid /Hexanedioic acid	dicarboxylic acid
15	36.99	0.94	$C_{24}H_{38}O_4$	390	Diisooctyl phthalate	ester
16	44.19	0.55	$C_{29}H_{50}O$	414	(3b,24S)-Stigmast-5-En-3-Ol	beta-sitosterol
Total area %		100.02				

Rt.: retention time, **MF:** molecular formula, **MW:** molecular weight

Table 3 The percentages of survival rate, relaxation, and anesthesia of *B. alexandrina* snails exposed to *C. glomerata* extract for 20 h

Concentration (mg/L)	Survival rate (%)	No effect (%)	Relaxation (%)	Anesthesia (%)
Control	100	0	0	0
100	100	86.7	13.3	0
300	100	33.4	66.6	0
500	**100**	**0**	**0**	**100**
700	60	0	0	100
1000	0	-	-	-

Three types of hemocyte cells:hyalinocytes, amoebocytes, and granulocytes were observed in control*B. alexandrina* hemolymph**(Fig. 4)** by light microscopy. Amoebocytes contain clear pseudopodia and granulocytes have dense granules in the cytoplasm, while hyalinocytes have transparent cytoplasm **[54]**.**Mohammed et al. [55]** considered that immuno-cell responses and molecular traits of *B. alexandrina* snails are important biomarkers of environmental pollutants. In *Biomphalaria* snails, hemocytes serve as the first line of defense and are involved in phagocytosis, cytotoxic responses **[56]**, and produce soluble substances such as antimicrobial peptides and agglutinins**[57, 58]**.

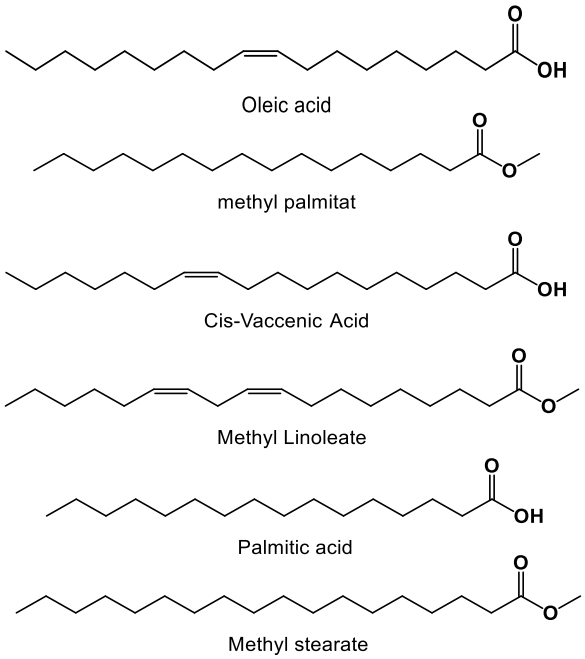

Fig. 2 The structure of major constituents of in methanolic extract of *C. glomerata*.

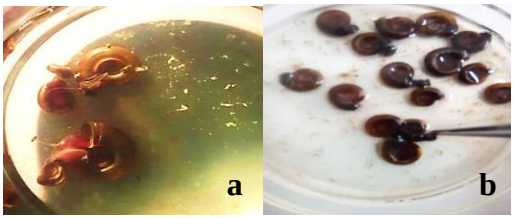

Fig. 3 Photos of snails in relaxation and anesthetic states after exposure to *C. glomerata extract*. **(a)** Snails showing redness of their soft parts as a result of increased hemocyte cells after one hour of exposure to 500 mg/L of *C. glomerata* extract, **(b)** snails unable to withdrawal reflex when touched after 7 h post exposure to 500 mg/L of *C. glomerata* extract.

Table 4 The percentages of relaxation, anesthesia, and mortality of *B. alexandrina* snails exposed to 500 mg/L of *C. glomerata* extract for different timeintervals

Exposure time (h)	Relaxation %	Anesthesia %	mortality (%)
2	26.6	0	0
4	100	0	0
7	0	100	0

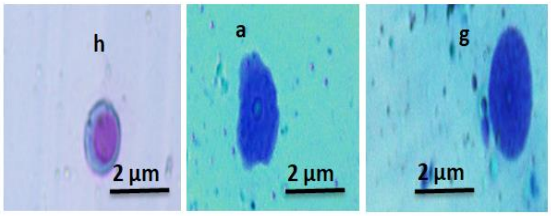

Fig. 4 Light photomicrograph of hemocytes from control *B. alexandrina* snail showing a hyalinocytes (h), amoebocytes(a) and granulocytes (g).

Snails exposed to 500 mg/L of *C. glomerata* extract witnessed several abrupt alterations in the composition and shape of hemocytes. Additionally, this concentration promotes hemocyte proliferation in cases where the cell has many nuclei and divided cytoplasm **(Fig. 5)**, which may be attributed to the presnce of oleic acid that can promote cell proliferation **[39].** More pseudopodia were extended by amoebocytes**(Fig. 6)**. Granulocytes exhabited their filopodia and denser, rough granules **(Fig. 7)**. Cell-to-cell clumping was caused by the hemocytes expanding several pseudopodia or filopodia**[59]**. The snails regarded cell-cell clumping as an immune protection mechanism. By forming a biological plug at the location of the lesion, invertebrate hemocyte aggregation reduced accidental blood bleeding and prevented the entry of infectious bacteria **[60]**. Additionally, it has been noted that granulocytes with rough granules occasionally transport metabolic chemicals from the digestive glands or may participate in the aggregation process **[61, 62, 63]**.

Also, it was found that 1.5 and 2% of the urethane solution induced the blood cells to withdraw their pseudopodia and detach from the slide surface**[64]**. The current work found that the algal extract's effect was more pronounced when snails were treated with 500 mg/l after one hour post-exposure. However, after 20 hours of exposure, all detected had returned to normal levels. These results may be attributed to palmitic acid found in the algal extract, which has immunomodulatory, inflammatory, and antioxidant effects **[49]**, and it may provide pre-treatment to cells and cell viability protection against oxidative stress **[50]**.

Although the granulocyte percentage of all treated snails was lower than that of the control group during the whole exposure period **(Table 5)**, the percentages of hyalinocytes and amoebocytes were greater than those of the control group. In gastropods, granulocytes perform the most phagocytosis using their dense granules, which contain enzymes responsible for the lysis of extracellular substances **[65, 66]**. Hyalinocytes participate in tissue healing, coagulation, and immunological response, whereas amoebocytes perform phagocytosis and encapsulation reactions**[67]**. Meanwhile, **Osman et al. [68]**indicated that hyalinocytes perform phagocytosis but to a lesser extent than granulocytes.

Table 5 Effect of *C. glomerata* methanol extract on granulocytes, hyalinocytes and amoebocytes percentages of *B. alexandrina*at different time of exposure (h).

Time (hour)	Granulocytes			Hyalinocytes			Amoebocytes		
	1 h	2 h	20 h	1 h	2 h	20 h	1 h	2 h	20 h
Control	74	76	73	19	21	22	7	8	8
100 mg/L	50***	55**	48***	26	33*	40***	14	8	12
300 mg/ L	45***	65	48***	40***	26	23	15*	9	16*
500 mg/ L	36***	59	64	48***	31*	22	16*	10	14

The asterisks indicated significant difference at (*$p<0.05$, **$p<0.01$ & ***$p<0.001$) in hemocytes percentages between each treated group and control at each exposure time.

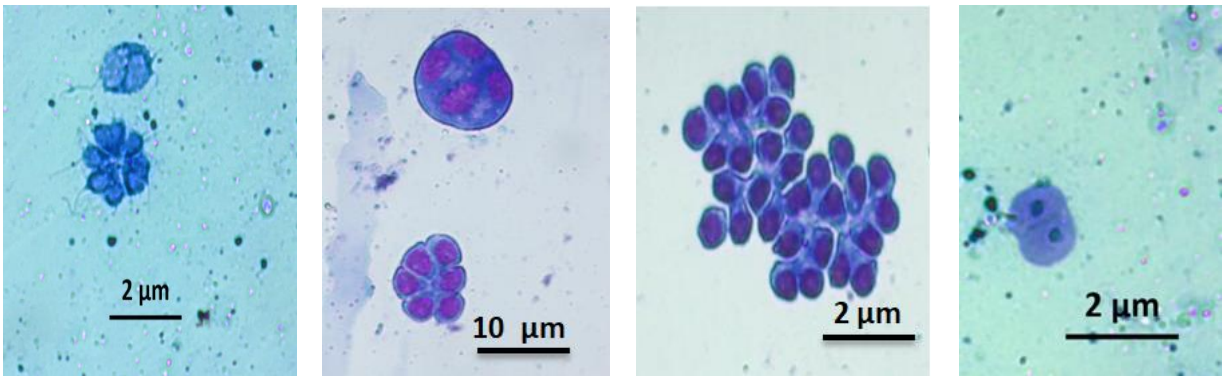

Fig. 5 Photomicrograph of hemocytes from *B. alexandrina* snails exposed to 500 mg/l of *C. glomerata* for 1 hr showing hyalinocytes division and aggregation.

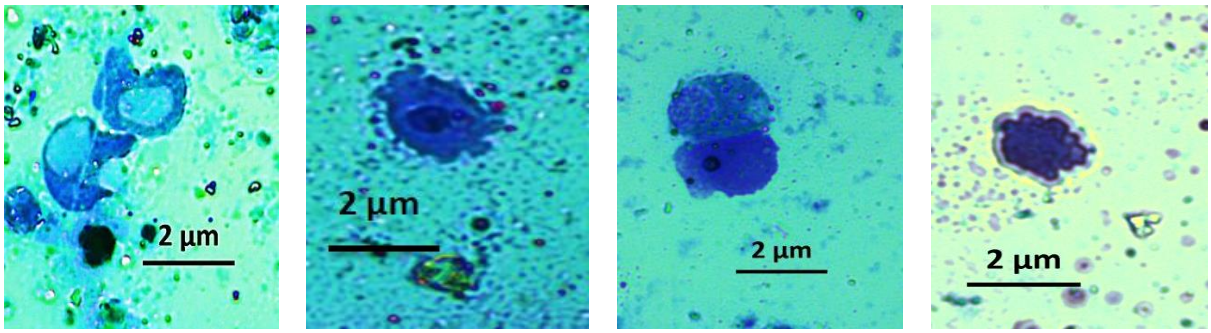

Fig. 6 Photomicrograph of hemocytes from *B. alexandrina* snails exposed to 500 mg/l of *C. glomerata* for 1 hr showing amoebocytes extending several pseudopodia.

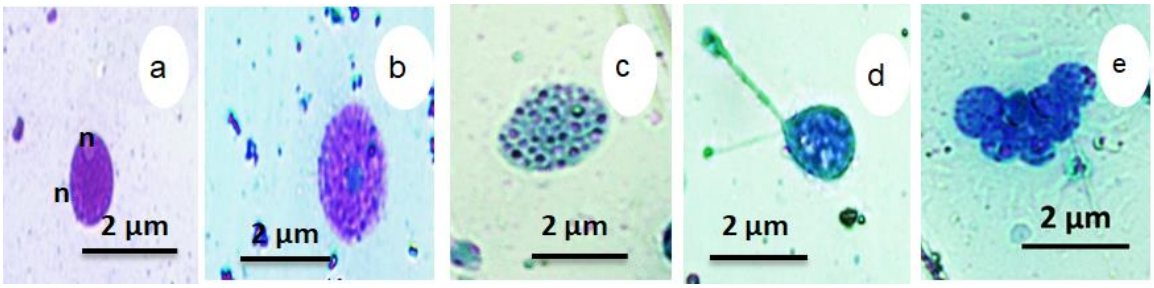

Fig. 7 Photomicrograph of hemocytes from *B. alexandrina* snails exposed to 500 mg/l of *C. glomerata* for 1 hr showing (a) granulocyte with 2 nuclei (n) (b) granulocyte with more dens granules (c) granulocyte with coarse granules (d) granulocyte with filopodia(e) aggregated granulocytes.

According to statistical analysis, the total hemocyte count of exposed snails was significantly affected by the varied *C. glomerata* methanol extract concentrations and the exposure duration, as shown in **Fig. (8)**. The total hemocyte count was directly proportional to algal extract concentrations after 1 hour of exposure. Compared to the control group, the snail group subjected to 500 mg/L of *C. glomerata* extract displayed a greater hemocyte count. After 20 hours of exposure, it then decreased to ordinary level **(Fig. 8)**. These findings partially match those of **Granath and Yoshino [64]**, who found that after two hours of exposure to urethane (ethyl carbamate), the total number of hemocytes in *Biomphalariaglabrata* snails increased threefold but then decreased to control levels after 12 and 24 hours. They attributed their findings to urethane's stimulation of mitotic activity in the organ that produces hemocytes, which releases many cells into circulation. The possibility that the medication causes circulating hemocytes to divide is also remote, given that urethane-induced *in vitro* cell culture did not effect on the number of cells present.*C. glomerata* extract may stimulate the mitotic activity of circulating hemocytes and produce a large number of cells in the hemolymph of the treated snails due to the presence of oleic acid, which has previously been shown to promote cell proliferation without apoptosis or necrosis **[38, 39]**.

Acetylcholinesterase (AChE) enzyme primarily found in muscles and nerves at postsynaptic neuromuscular junctions. Acetylcholine (ACh), a naturally occurring neurotransmitter, is instantly hydrolyzed into acetic acid and choline**[69]**. The present results showed that a noticeable significant decrease in acetylcholinesterase (AChE) activities in all treated snails with *C. glomerata* extract compared to the control group after 1 h of exposure (F=6.913, df=3,p=0.006) as shown in **Fig. (9)**. However, a gradual insignificant decrease was observed after 2 h of exposure (F=2.617, df=3,p=0.09) **(Fig. 9)**.

On the other hand, AChE concentrations showed significant decrease in treated snails with 100 and 300 mg/ L of *C. glomerata* extract after 20 h of the exposure and insignificant decrease was observed in treated snails with 500 mg/ L at the same time compared to the control group (F=4.748, df=3,p=0.021) **(Fig. 9)**. The observed decrease in AChE in the present work might be due to the fact that *C. glomerata* extract contains terpenoids, alkaloids, and flavonoids, which have frequently been reported to have local and general anesthetic, antinociceptive, analgesic, or sedative properties **[1, 5].** Also, **Woodall et al. [70]**observed that propofol (an intravenous anesthetic) inhibited only the postsynaptic acetylcholine response. **Naruo et al. [71]**discovered that the effects of volatile anesthetic, sevoflurane, inhibited the response of the postsynaptic nicotinic acetylcholine receptor. Lactate dehydrogenase (LDH) is an essential enzyme because it regenerates NAD+ and allows for continued carbon

flow through the glycolytic pathway to support anaerobic ATP synthesis **[72]**. This process can be significant in organisms exposed to hypoxic or anoxic conditions for extended periods. In the present work, LDH levels dramatically decreased in all treated snails with *C. glomerata* methanol extract after 2 and 20 h of exposure compared to the control group **(Fig. 10)**.

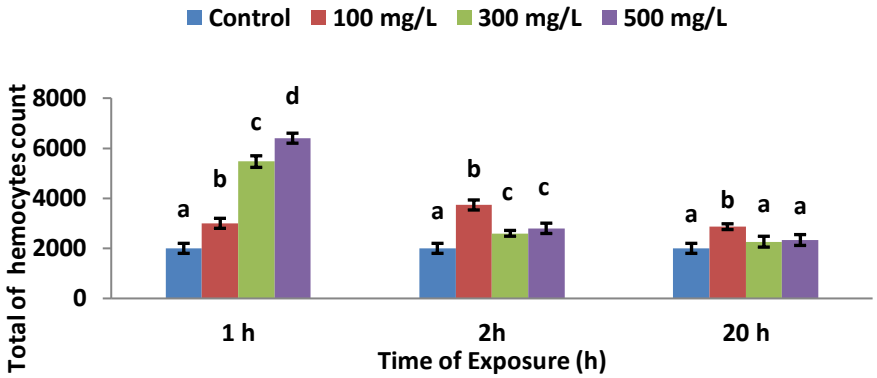

Fig. 8 Effect of *C. glomerata* methanol extract on total hemocytes count from *B. alexandrina.* Different letters in each period indicate significance between the control and treatment groups at $P < 0.05$, using (one-way ANOVA).

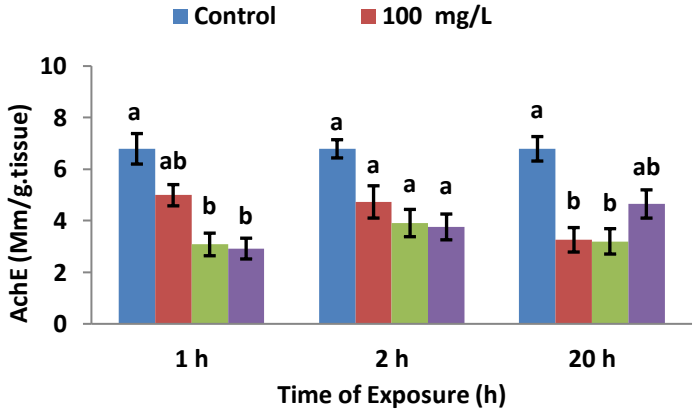

Fig. 9 Effect of *C.glomerata* methanol extract on acetyl cholinesterase (AChE) concentration (Mm/g. tissue) in *B. alexandrina* at different exposure times. Different letters in each period indicate significance between the control and treatment groups at $P < 0.05$, using (one-way ANOVA).

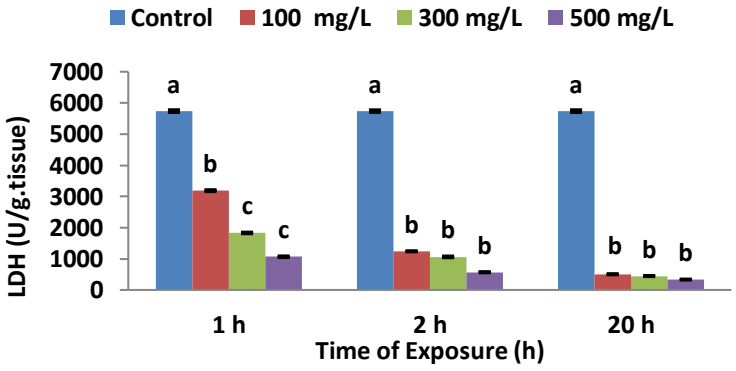

Fig. 10 Effect of *C. glomerata* methanol extract on Lactate dehydrogenase concentration (LDH) (U/g. tissue) in *B. alexandrina* at different exposure times. Different letters in each period indicate significance between the control and treatment groups at $P < 0.05$, using (one-way ANOVA).

However, low levels of LDH are uncommon and not regarded as dangerous **[73]**. This means there is no damage occurring in treated snail tissue. This result may be attributed to the presence of fatty acids found in *C. glomerata* methanol extract. Also, this result is matched by **Sepulveda and Pak[74]**, who prepared a lipid emulsion formulation containing (linoleic acid, oleic acid, palmitic acid, alpha-linolenic acid, and stearic acid) and reported these fatty acids can reduce anesthetic toxicity. On the other hand, **Mustafa et al. [75]** observed a significant increase in LDH activity in both experiments of anesthesia and surgery at 24 hr post-injection of the anesthetic regime, with a significantly increased heart rate and decrease in respiratory rate, and attributed this increase to the muscular damage due to surgery. Additionally, a serum LDH isozyme concentration reflects several types of tissue injury. Depending on the degree of tissue injury, the enzyme can remain elevated in the bloodstream for up to 7 days. Also, organ damage results in significant cell death and cytoplasm loss, which raises serum LDH levels **[76]**.

4. Conclusions

Cladophoraglomerata extract was safe on *Biomphalariaalexandrina* snails. The relaxation effect was observed with snails exposed to 100 and 300 mg/ L of the extract, recording at 13.3 and 66.6 %, respectively, while the anesthesia effect was observed with snails exposed to 500 mg/ L, reaching 100%. The total hemocyte count was directly proportional to the concentrations of *C. glomerata* extract after 1 h, and it returned to normal level after 20 h of exposure. A noticeable decrease was recorded in activities of AChE and LDH levels in all treated snails after different times and this may be due to terpenoids, alkaloids, and flavonoid compounds in the algal extract. After one hour of exposure, *C. glomerata* extract caused several fast changes in the morphology and composition of hemocytes and stimulated hemocyte proliferation, which may be attributable to oleic acid in the extract. According to GC-MS results, the methanolic extract of *C. glomerata* contains substances that may be anesthetic, antioxidant, and anti-inflammatory, as well as promoting cell proliferation. However, recommended further analysis is needed to identify the activity and role of terpenoids, alkaloids, and flavonoids found in *C. glomerata*.

5. Conflict of interest

The authors have no conflicts of interest to declare relevant to this research article's content.

6. Acknowledgments

The authors express profound thanks to Dr. Abdullah A. Saber, Associate Professor of Phycology at the Department of Botany, Faculty of Science, Ain Shams University, Cairo, Egypt, for his help in identifying *Cladophoraglomerata* algae.

7. References

[1] Tsuchiya H (2017) Anesthetic agents of plant origin: a review of phytochemicals with anesthetic activity. Mol 22(8): 1369.https://doi.org/10.3390/molecules22081369 .

[2] Khare CP (2008) Indian medicinal plants: an illustrated dictionary. Springer Science & Business Media, LLC, New York, pp 14-15

[3] Zhao Y, Bu Q, Zhou Y, Lv L, Yan G, Chen B, Wang Li, Cen X (2010) Mechanism study of Aconitum-induced neurotoxicity in PC12 cells: Involvement of dopamine release and oxidative damage. NeuroToxicol 31 (6): 752–757. https://doi.org/10.1016/j.neuro.2010.06.005.

[4] Binish T (2018) Micropropagation of traditional medicinal plant *Ceropegiajuncea.* Ann Plant Sci7(2):1992-1996. https://doi.org/10.21746/aps.2018.7.2.2.

[5] Bhardwaj IN, Sharma M (2019) Herbal anesthetic agents: An overview on sources, uses and future perspectives. Asian J Pharm Pharmacol5(S1): 21-27. https://doi.org/10.31024/ajpp.2019.5.s1.7.

[6] Michalak L, Messyasz B (2021) Concise review of *Cladophora spp.*: macroalgae of commercial Interest. J App Phycol 33:133–166.https://doi.org/10.1007/s10811-020-02211-3.

[7] Munir M, Qureshi R, Bibi M, Khan AM (2019) Pharmaceutical aptitude of *Cladophora*: a comprehensive review. Algal Res 39: 101476. https://doi.org/10.1016/j.algal.2019.101476.

[8] Lezcano V, Fernández C, Parodi ER, Morelli S (2018) Antitumor and antioxidant activity of the freshwater macroalga*Cladophorasurera*. J App Phycol 30: 2913–292. https://doi.org/10.1007/s10811-018-1422-5.

[9] Soltani S, Saadatmand S, Khavarinejad R, Nejadsattari T (2011) Antioxidant and antibacterial activities of *Cladophoraglomerata* (L.) Kütz. in Caspian Sea Coast, Iran. Afri J Biotechnol 10: 7684–7689. https://doi.org/10.5897/AJB11.491

[10] Zbakh H, Chiheb I, Motilva V, Riadi H (2014) Antibacterial, cytotoxic and antioxidant potentials of *Cladophoraprolifera* (Roth) Kutzing collected from the Mediterranean coast of Morocco. Ameri J PhytomedClinTherap2: 1187–1199.

[11] Srimaroeng C, Ontawong A, Saowakon N, Vivithanaporn P, Pongchaidecha A, Amornlerdpison D, Soodvi S, Chatsudthipong V (2015) Antidiabetic and renoprotective effects of *Cladophoraglomerata*Kützing extract in experimental type 2 diabetic rats: a potential nutraceutical product for diabetic nephropathy. J Diab Res 320167–320115.https://doi.org/10.1155/2015/320167

[12] Winlow W, Polese G, Moghadam HF, Ahmed IA, Di Cosmo A (2018) Sense and Insensibility – An Appraisal of the Effects of Clinical Anesthetics on Gastropod and Cephalopod Molluscs as a Step to Improved Welfare of Cephalopods. Front Physio l 9: 1147. https://doi.org/10.3389/fphys.2018.01147.

[13] Girdlestone D, Cruickshank SG, Winlow W (1989) The actions of three volatile general anaesthetics on withdrawal responses of the pond-snail *Lymnaeastagnalis* (L.). Comp Bioch Physiol.C, Comp PharmacolToxicol92(1): 39-43.

[14] Holmes RS, Goldberg E (2009) Computational analyses of mammalian lactate dehydrogenases: human, mouse, opossum and platypus LDHs. Computbiolchem33(5): 379-85. https://doi.org/10.1016/j.compbiolchem.2009.07.006.

[15] Fournier D, Bride J, Hoffmann F, Karch F (1992) Acetylcholinesterase. Two types of modifications confer resistance to insecticide. J BiolChem 267 (20): 14270–14274.https://doi.org/10.1016/S0021-9258(19)49708-1.

[16] Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C, Viarengo A (2000) The use of biomarkers to assess the impact of pollution in coastal environment of the Iberian Peninsula: a practical approach. Sci Total Environ 247(2-3): 295-311.https://doi.org/10.1016/S0048-9697(99)00499-4.

[17] Bocquene´ G, Roig A, Fournier D (1997) Cholinesterases from the common oyster (Crassostreagigas): evidence for the presence of a soluble acetylcholinesterase insensitive to organophosphate and carbamate inhibitors. FebsLett 407(3): 261–266. https://doi.org/10.1016/S0014-5793(97)00339-6

[18] Gaitonde D, Sarkar A, Kaisary S, Silva CD, Dias C, Rao DP, Ray D, Nagarajan R, Patill D (2006) Acetylcholinesterase activities in marine snail (Croniacontracta) as a biomarker of neurotoxic contaminants along the Goa coast, West coast of India. Ecotoxicol 15(4): 353-358.

[19] Deidda I, Russo R, Bonaventura R, Costa C, Zito F, Lampiasi N (2021) Neurotoxicity in Marine Invertebrates: An Update. Biol10 (2): 161. https://doi.org/10.3390/biology10020161

[20] Fitriansyah SN, Aulifa DL, Febriani Y, Sapitri E (2018) Correlation of total phenolic, flavonoid and carotenoid content of Phyllanthusemblica extract from Bandung with DPPH scavenging activities. Pharmaco J 10(3). https://doi.org/10.5530/pj.2018.3.73.

[21] Abdel-Hady H, Morsi EA, El-wakil EA (2022) In-vitro Antimicrobial Potentialities of Phylunthusemblica Leaf Extract against Some Human Pathogens. Egypt J Chem 65(7): 701-707. https://doi.org/10.21608/EJCHEM.2021.109577.4998.

[22] Martins-Sousa RL, Negrão-Corrêa D, Bezerra FSM, Coelho PMZ (2001) Anesthesia of *Biomphalaria* spp. (Mollusca, Gastropoda): sodium pentobarbital is the drug of choice. Memórias do InstitutoOswaldo Cruz 96: 391-392. https://doi.org/10.1590/S0074-02762001000300199.

[23] Zelck U, Becker W (1992) *Biomphalariaglabrata*: influence of calcium, lectins, and plasma factors on in vitro phagocytic behavior of hemocytes of noninfected or *Schistosomamansoni*-infected snails. Experimparasitol 75(1):126–136.

[24] Barracco MA, Steil AA, Gargioni R (1993) Morphological characterization of the hemocytes of the pulmonate snail *Biomphalariatenagophila*. Memórias do InstitutoOswaldo Cruz 88(1):73–83.https://doi.org/10.1016/0014-4894(92)90128-W.

[25] Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochempharmacol *7*: 88–95.https://doi.org/10.1016/0006-2952(61)90145-9.

[26] Lowry OH, Rasebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J BiolChem 193: 265–275.

[27] Bais R, Philcox M (1994) Approved recommendation on IFCC methods for the measurement of catalytic concentration of enzymes. Part 8. IFCC Method for Lactate Dehydrogenase (l-Lactate: NAD+ Oxidoreductase, EC 1.1.1.27). International Federation of Clinical Chemistry (IFCC). In European journal of clinical chemistry and clinical biochemistry: J Forum EurClinChemSoci 32(8):639-55.

[28] Nami N, Ebadi SF, Otaghsara SFT, Baei M, Rahimi ES (2016) GC-MS analysis and preliminary test of phytochemical screening of

crude ethanolic extract of green algae, *CladophoraGlomerata* (L.) Kütz from Caspian Sea. Iranian J Org Chem8(4): 1899-1907.

[29] JV SA(2019) Preliminary phytochemical screening and GC-MS analysis of *Cladophoraglomerata*: green marine algae. Int J Basic ClinPharmacol8 (4).https://doi.org/10.18203/2319-2003.ijbcp20191108

[30] Petchsomrit A, Chanthathamrongsiri N, Jiangseubchatveera N, Manmuan S, Leelakanok N, Plianwong S,Siranonthana N, Sririrak T (2023) Extraction, antioxidant activity, and hydrogel formulation of marine *Cladophoraglomerata*. Algal Res71:103011.https://doi.org/10.1016/j.algal.2023.103011.

[31] Al-Jaber GT, Al-Ismaeel WN, Al-Ali AL (2021) The effect of Rhuscoriaria L. methanolic extract on cytotoxicity of *Cladophoraglomerata* L. Kützingmethanolic extract against human breast carcinoma MCF-7 cell line. Int J Pharmac Res13:12-8.https://doi.org/10.31838/ijpr/2021.13.02.008.

[32] Gokhale JP, Mahajan HS, Surana SJ (2019) Quercetin loaded nanoemulsion-based gel for rheumatoid arthritis: In vivo and in vitro studies. BiomPharmaco 112: 108622.https://doi.org/10.1016/j.biopha.2019.108622

[33] Santiago LÂM, Neto RNM, Santos Ataíde AC, Fonseca DCSC, Soares EFA, de Sá Sousa JC, Lima-Neto LG, Carvalho RC, de Sousa EM (2021) Flavonoids, alkaloids and saponins: Are these plant-derived compounds an alternative to the treatment of rheumatoid arthritis? A literature reviews. ClinPhytosci 7(58): 1-10.https://doi.org/10.1186/s40816-021-00291-3.

[34] Arsic A, Stojanovic A, Mikic M (2019) Oleic acid-health benefits and status in plasma phospholipids in the Serbian population. Serbian J ExperimClin Res 20(2): 3-8. https://doi.org/10.1515/sjecr-2017-0077.

[35] Sales-Campos H, Reis de Souza P, CremaPeghini B, Santana da Silva J, Ribeiro Cardoso C (2013) An overview of the modulatory effects of oleic acid in health and disease. Mini Rev Med Chem 13(2): 201-210. https://doi.org/10.2174/138955713804805193.

[36] Santa-María C, López-Enríquez S, Montserrat-de la Paz S, Geniz I, Reyes-Quiroz ME, Moreno M, Palomares F, Sobrino F, Alba G (2023) Update on Anti-Inflammatory Molecular Mechanisms Induced by Oleic Acid. Nutrients 15(1): 224. https://doi.org/10.3390/nu15010224.

[37] Carrillo Pérez C, CaviaCamarero MDM, Alonso de la Torre S (2012) Role of oleic acid in immune system; mechanism of action; a review. NutriciónHospitalaria 27 (4): 978-990. http://hdl.handle.net/10259.4/2523.

[38] Liu Z, Xiao Y, Yuan Y, Zhang X, Qin C, Xie J, Hao Y, Xu T, Wang X (2013) Effects of oleic acid on cell proliferation through an integrin-linked kinase signaling pathway in 786-O renal cell carcinoma cells. OncolLett 5(4): 1395-1399.https://doi.org/10.3892/ol.2013.1160.

[39] Belal SA, Sivakumar AS, Kang DR, Cho S, Choe HS, Shim KS (2018) Modulatory effect of linoleic and oleic acid on cell proliferation and lipid metabolism gene expressions in primary bovine satellite cells. Anim Cells Syst 22(5): 324-333.https://doi.org/10.1080/19768354.2018.1517824.

[40] Lee RHC, e Silva AC, Possoit HE, Lerner FM, Chen PY, Azizbayeva R, Citadin CT, Wu CY, Neumann JT, Lin HW (2019) Palmitic acid methyl ester is a novel neuroprotective agent against cardiac arrest. ProstaglLeuk Essen Fatty Acids 147*:* 6-14. https://doi.org/10.1016/j.plefa.2018.11.011.

[41] Saeed NM, El-Demerdash E, Abdel-Rahman HM, Algandaby MM, Al-Abbasi FA, Abdel-Naim AB (2012) Anti-inflammatory activity of methyl palmitate and ethyl palmitate in different experimental rat models. Toxicolapplpharmacol264(1): 84-93. https://doi.org/10.1016/j.taap.2012.07.020.

[42] Hamed AB, Mantawy EM, El-Bakly WM, Abdel-Mottaleb Y, Azab SS (2020) Putative anti-inflammatory, antioxidant, and anti-apoptotic roles of the natural tissue guardian methyl palmitate against isoproterenol-induced myocardial injury in rats. Future J PharmacSci 6: 1-14. https://doi.org/10.1186/s43094-020-00044-y.

[43] Anyasor GN, Funmilayo O, Odutola O, Olugbenga A, Oboutor EM (2014) Chemical constituents in n-butanol fractions of CastusaferkerGawl leaf and stem. J IntercuEthnopharmacol 3(2): 78. https://doi.org/10.5455/jice.20140112010648.

[44] Awonyemi OI, Abegunde MS, Olabiran TE (2020) Analysis of bioactive compounds from *Raphiataedigera* using gas chromatography-mass spectrometry.Eurasian ChemCommunic 2 (8): 933-944. https://doi.org/10.33945/SAMI/ECC.2020.8.10.

[45] Djoussé L, Matthan NR, Lichtenstein AH, Gaziano JM (2012) Red blood cell membrane concentration of cis-palmitoleic and cis-vaccenic acids and risk of coronary heart disease. Amer J Cardiol 110(4):539-544. https://doi.org/10.1016/j.amjcard.2012.04.027.

[46]Aimola IA, Inuwa HM, Nok AJ, Mamman AI, Bieker JJ (2016) Cis-vaccenic acid induces differentiation and up-regulates Gamma globin synthesis in K562, JK1 and transgenic mice erythroid progenitor Stem Cells. Eur J Pharmacol 776: 9-18. https://doi.org/10.1016/j.ejphar.2016.02.041

[47] Kolar MJ, Konduri S, Chang T, Wang H, McNerlin C, Ohlsson L, Härröd M, Siegel D, Saghatelian A (2019) Linoleic acid esters of hydroxy linoleic acids are anti-inflammatory lipids found in plants and mammals. J Biol

[48] Anderson SM, Cheesman HK, Peterson ND, Salisbury JE, Soukas AA, Pukkila-Worley R (2019) The fatty acid oleate is required for innate immune activation and pathogen defense in *Caenorhabditiselegans*. PLoSpathog15(6): e1007893.https://doi.org/10.1371/journal.ppat.1007893.

[49] Gori M, Altomare A, Cocca S, Solida E, Ribolsi M, Carotti S, Rainer A, Francesconi M, Morini S, Cicala M, Pier Luca Guarino M (2020) Palmitic acid affects intestinal epithelial barrier integrity and permeability in vitro. *Antioxidants9*(5):417. https://doi.org/10.3390/antiox9050417.

[50] Palomino OM, Giordani V, Chowen J, Fernández-Alfonso MS, Goya L (2022) Physiological doses of oleic and palmitic acids protect human endothelial cells from oxidative stress. Molecules 27(16): 5217. https://doi.org/10.3390/molecules27165217.

[51] Kim IS, Hwang CW, Yang WS, Kim CH (2021) Multiple antioxidative and bioactive molecules of oats (*Avenasativa* L.) in human health. Antioxidants 10(9): 1454. https://doi.org/10.3390/antiox10091454

[52] Elwekeel A, Hassan MH, Almutairi E, AlHammad M, Alwhbi F, Abdel-Bakky MS,Amin E, Mohamed EI (2023) Anti-Inflammatory, Antioxidant, GC-MS Profiling and Molecular Docking Analyses of Non-Polar Extracts from Five Salsola Species. Separations 10(2): 72. https://doi.org/10.3390/separations10020072

[53] Armstrong R, Riaz S, Hasan S, Iqbal F, Rice T, Syed N (2018) Mechanisms of anesthetic action and neurotoxicity: Lessons from molluscs. Front Physiol 8: 1138. https://doi.org/10.3389/fphys.2017.01138

[54] Mansour SM, Sayed SSM, Abdel-Wareth MT (2021) Effect of methyl gallate on immune response of *Biomphalariaalexandrina* (Ehrenberg, 1831) snails to infection with *Schistosomamansoni*(Sambon, 1907). Parasitol Res120(3): 1011-1023.https://doi.org/10.1007/s00436-020-07037-z

[55] Mohamed AH (2011) Sublethal toxicity of Roundup to immunological and molecular aspects of *Biomphalariaalexandrina* to *Schistosomamansoni* infection. Ecotoxicol Environ Saf74:754–760. https://doi.org/10.1016/j.ecoenv.2010.10.037

[56] Fried B (2016) An update on hemocytes in *Biomphalaria* snails. J HematolOncol Res2: 26(20). https://doi.org/10.14302/issn.2372-6601.jhor-14-401.

[57]Ottaviani E (2006) Molluscanimmunorecognition. Inver Surv J 3: 50–63

[58] Mitta G, Vandenbulcke F, Noël T, Romestand B, Beauvillain JC, Salzet M, Roch P (2000) Differential distribution and defence involvement of antimicrobial peptides in mussel. J Cell Sci 113: 2759–2769.https://doi.org/10.1242/jcs.113.15.2759.

[59] Mahilini HM, Rajendran A (2008) Categorization of hemocytes of three gastropod species *Trachea vittata* (Muller), *Pilaglobosa* (Swainson) and *Indoplanorbisexustus* (Dehays). Journal Inver Pathol97: 20–26. https://doi.org/10.1016/j.jip.2007.07.007

[60] Guria S, Parveen S, Goswami DS (2016) Alteration of morphology, phagocytic behaviour and aggregation of insect haemocytes exposed to contaminated food with arsenic and lead. Int J PharmTech Res 9:177–186

[61] Nakayama K, Nomoto AM, Nishijima M, Maruyama T (1997) Morphological and functional characterization of hemocytes in the Giant Clam *Tridacnacrocea*. J Inver Pathol69: 105–111. https://doi.org/10.1006/jipa.1996.4626

[62] Kheder SI (2020) Morphology and kinetics of susceptible and resistant *Biomphalariaalexandrina*hemocytes during the first week of exposure to *Schistosomamansoni*miracidia. ParasitolUni J 13 (3): 179–189. https://doi.org/10.21608/puj.2020.47046.1089

[63] Mansour SM, Taha RG, Youssef AA (2023) Assessment of Amphora coffeaeformis and *Scenedesmusdimorphus* algae as immunostimulant agents on *Biomphlariaalexandrina* snails against *Schistosomamansoni*. Biologia78:737–748https://doi.org/10.1007/s11756-022-01262-w.

[64] GranathJr WO, Yoshino TP (1985) *Biomphalariaglabrata* (Gastropoda): effect of urethane on the morphology and function of hemocytes, and on susceptibility to *Schistosomamansoni* (Trematoda). J Inver

Pathol 45(3), 324-330.https://doi.org/10.1016/0022-2011(85)90110-7

[65] Feng SY, Feng JS, Burke CN, Khairallah LH (1971) Light and electron microscopy of the leucocytes of *Crassostreavirginica* (Mollusca: Pelecypoda). ZeitschriftfürZellforschung und mikroskopischeAnatomie 120: 222–245.https://doi.org/10.1007/BF00335537

[66] Barçante TA, Barçante JMP, Fujiwara RT, Lima WS (2012) Analysis of circulating haemocytes from *Biomphalariaglabrata* following *Angiostrongylusvasorum* infection using fowcytometry. J Parasitol Res1–6. https://doi.org/10.1155/2012/314723

[67] Yoshino TP, Coustau C (2011) Immunobiology of *Biomphalaria*–Trematode Interactions. In: Toledo, R., Fried, B. (eds) *Biomphalaria* Snails and Larval Trematodes. Springer, New York, NY. pp 159-189.https://doi.org/10.1007/978-1-4419-7028-2_7

[68] Osman AM, Gomaa M, Saad AH (2003) *Biomphalariaalexandrina*: cellular responses of susceptible and resistant snails to *Schistosomamansoni* infection. J Egypt SocParasitol33 (3): 805–827.

[69] McHardy SF, Wang HYL, McCowen SV, Valdez MC (2017) Recent advances in acetylcholinesterase Inhibitors and Reactivators: an update on the patent literature (2012-2015). Expert Opinion Therap Patents 27(4): 455-476.https://doi.org/10.1080/13543776.2017.1272571

[70] Woodall AJ, Naruo H, Prince DJ, Feng ZP, Winlow W, Takasaki M, Syed NI (2003) Anesthetic treatment blocks synaptogenesis but not neuronal regeneration of cultured *Lymnaea* neurons. J Neurophysiol 90(4): 2232-2239.https://doi.org/10.1152/jn.00347.2003

[71] Naruo H, Onizuka S, Prince D, Takasaki M, Syed NI (2005) Sevoflurane blocks cholinergic synaptic transmission postsynaptically but does not affect short-term potentiation. J AmerSocAnesthesiol102(5): 920-928.https://doi.org/10.1097/00000542-200505000-00010

[72] Hochachka PW, Somero GN (2002) Biochemical adaptation: mechanism and process in physiological evolution.Oxford university press

[73] Farhana A, Lappin SL (2023) Biochemistry Lactate Dehydrogenase. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing PMID: 32491468.

[74] Sepulveda EA, Pak A (2024) Lipid Emulsion Therapy. [Updated 2022 Mar 11]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK549897/

[74]Mustafa MA, Sanhouri AA, Mohamed GA (2018) The Effect of Surgery and Anaesthesia in Clinical Parameters and Plasma Glucose Concentrations with LDH Enzyme in Donkey (*Equusasinus*). J AgriVeterinSci 19(2):1-11.

[76] Feng Y, Xiong Y, Qiao T, Li X, Jia L, Han Y (2018) Lactate dehydrogenase A: A key player in carcinogenesis and potential target in cancer therapy. Cancer med 7(12):6124-6136.https://doi.org/10.1002/cam4.1820