Damanhour Journal of Veterinary Sciences 9 (2), (2023) 9-16

Damanhour Journal of Veterinary Sciences

Journal homepage: https://djvs.journals.ekb.eg/

E-ISSN 2636-3003 | ISSN 2636-3011

Anti-inflammatory properties of *Holothuria atra* extract on lipopolysaccharide-induced inflammation of White blood cells

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Abstract

Holothuria atra is a marine invertebrate found in the tropical indo-pacific region. It contains potent bioactive compounds, which include lectin, steroidal saponins, and triterpene glycoside. The anti-inflammatory and antioxidant characteristics of *Holothuria atra* extract have been investigated *in-vitro* in this study. Ethyl acetate extract of *H. atra* was prepared by maceration methods, and then the phytochemical compositions were measured colorimetric and by using gas chromatography-mass spectrometry (GC-MS) Analysis. Hemolytic activity and anti-inflammatory properties of the extract were measured through cell culture protocol. The obtained results showed that the extract has a high phenol content (121.92±0.14 mg/g), and GC-MS analysis revealed that it contains, Oleic acid, 9-octadecenoic acid, methyl ester, and Octadecanoic acid.

Moreover, antioxidant properties were determined by assessing 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, ferric reducing power, hydroxyl radical scavenging, and American Board of Thoracic Surgery (ABTS) scavenging activity. Furthermore, it is concluded that *H. atra* inhibited the stimulated Lipopolysaccharide (LPS) - Inducible nitric oxide synthase (*iNOS)* gene expression and protein expression by 6.17±0.01-fold change and Cyclooxygenase-2 *(COX-2)* gene expression by 4.70±0.05-fold change. The crude extract of *H. atra* could be considered a good anti-inflammatory agent.

Keywords: Sea cucumbers; Antioxidant activity; DPPH; MTT assay; *COX-2*; *iNOS*

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Email: dr_hazemshaheen3010@gmail.com
P ISSN: 2636-3003
EISSN: 2636-2996
DOI: 10.21608/djvs.2022.157375.1090
Received: August 20, 2022; Received in revised form: September 09, 2022; Accepted: September 25, 2022
Editor-in-Chief:
Prof Dr/Ali H. El-Far (ali.elfar@damanhour.edu.eg)

1. Introduction

The significant impact of aquatic creatures as a source of splitting medicinal ingredients and medications is confirmed by global marine medicine. Genetic variation, or macro taxonomical variety, is substantially higher in marine and freshwater waterways than on land. Of the thirty-three classes of complex organisms, only eleven are discovered on land, thirty-one are located in freshwater, and seventeen are found in the oceans (Adrianov 2004). Many pharmacologically essential compounds, such as polyunsaturated fatty acids, glucosides, peptides, and polysaccharides, can be found in the sea environment creatures because of their great species diversity. Many marine species live in complex environments subject to extreme climates, and to adapt to these new exposures, they develop a variety of secondary (bio-active) products that are unique and do not exist in the terrestrial species. Also, studies investigating novel bioactive chemicals from the marine ecosystem have witnessed a somewhat unlimited field of research due to its enormous genetic variability (Plaza et al. 2008, Khotimchenko 2018).

Underwater bioactive molecules can be used as drug products as well as initial models for the advancement of substances with novel or enhanced pharmacological activities. Scientists have extracted about 27,000 chemical substances from marine species, many of which have medicinal properties. The molecules vary in complexity from simple linear peptides like dilatations to complicated macrocyclic polyethers like Halichondria B (Khotimchenko 2018). Many scientists believe that compared to any other species group, aquatic invertebrates, like a sponge, are a more effective source of new anticancer, antiviral, and anti-inflammatory compounds (Molinski et al. 2008).

Sea cucumbers are marine echinoderm that contains valuable nutrients and medicinal compounds. Sea cucumbers' bioactive compounds, which include antioxidant properties, antibacterial, and anti-tumor actions, offer promising biological and pharmacologic abilities (Wei et al. 2021). Sea cucumbers are reported to aid in the treatment of asthma, high blood pressure, joint pain, and bowel and urinary problems (Lee et al. 2019). Similarly, sea cucumber metabolites have been found to have antitumor, anti-clotting, anti-hypertension, anti-inflammatory, antibacterial, anti-oxidative, anti-mutagenic, anti-diabetic, anti-aging, and wound healing activities (Bordbar et al. 2011, Aminin et al. 2015).

These bioactivities are associated with various metabolites, including triterpenoid glycosides, proteins, collagen, mucopolysaccharides, carotenes, polyunsaturated oils, and phospholipids (Bahrami et al. 2016). *Holothuria atra* (Jaeger 1833) is a popular sea cucumber species in the Indian region, also known as black sea cucumber and lolly fish. According to (Nugroho et al. 2022a), *H. atra* has the highest level of antioxidants of any sea cucumber species. It resided on seagrass beds and rocky coral reefs (Lovatelli et al. 2021). Because of its innate immunity properties, black sea cucumber is typically consumed as nutraceutical.

Several sea cucumber species have provided metabolites with immunomodulatory properties (Lee et al. 2019). The extract of *H. atra* contained flavonoids, polyphenol components, terpenes, saponins, alkaloids, and other substances, in addition to the existence of 59 substances that was revealed through Gas chromatography. Anti-proliferative features of *H. atra* extracts were reported against Michigan Cancer Foundation-7 (MCF-7) cell lines. Extracts also inhibited Herpes simplex virus type 1 (HSV-1) and Herpes simplex virus type 2 (HSV-2) strains (Dhinakaran and Lipton 2014).

The immune system's response to potentially harmful stimuli such as pathogenic microbes, damaged tissue, dangerous chemicals, or irradiation is known as inflammation (Medzhitov 2010). It works by removing harmful stimuli and starting the healing process. Inflammation is thus a necessary defence mechanism for health (Chen et al. 2018). Over the past decade, a comprehensive study has found that most chronic conditions, including cancer, neurodegenerative disorders, diabetes, and immune disorders, are characterized by disturbances of multiple cell signaling pathways associated with inflammation (Senthilkumar and Kim 2013).

Finding natural antioxidants and anti-inflammatory agents to synthesize new drugs is gaining popularity (Mohamed et al. 2010). Anti-inflammatory medications are available to help control inflammation in the body. However, they frequently have side effects and are not always effective (Ghosh et al. 2015). Nonsteroidal anti-inflammatory drugs (NSAIDs) are pain relievers that assist in reducing inflammation. They are, in other words, anti-inflammatory medications and can cause minor gastrointestinal issues such as stomach upset, gas, diarrhea, and heart attack (Li et al. 2018.

The purpose of this study was to investigate the ethyl acetate extract of *H. atra* and evaluate its *in-vitro* antioxidant properties and anti-inflammatory features in order to use it as an effective natural anti-inflammatory source.

2. Materials and Methods

2.1. Sampling and identification of sea cucumber

Sea cucumber *H. atra* samples (Length 10 - 30 cm and total weight 300 - 700 g) were collected from Hurghada coastal along the Red Sea (27.28°N; 33.77°E), Egypt in March 2021. At the National Institute of Oceanography and Fisheries, Egypt, the specimens were identified under light microscopy using characteristic features and taxonomic references. The samples were cleaned from the sand and saved in an icebox before ship to the research institute and storage at -20 °C until utilize.

2.2. Extraction

The samples were washed with fresh water and shade dried at room temperature then cut into pieces of approximately 2-4 cm^3 then pulverize in a mixer grinder. The extraction was conducted three times by 48 hr. maceration using 99% ethyl acetate (1:4 w/v) followed by filtration through Whatman filter paper (No.1) at room temperature. The filtrate was concentrated under reduced pressure at 40 °C using a rotary evaporator, and the leftover sample has been freeze drying to drain water.

2.3. Gas chromatography-mass spectrometer (GC-MS) analysis

The GC-MS Agilent 7890A-5975C was used to identify various bioactive components using capillary column of (0.25mm 30mm 0.25µm film density). The oven's temperature remained constant at 70˚C for 2 minutes before being slowly risen from 20˚C/min to 305˚C and held for 1 minute. As a carrier gas, helium gas at a stream rate of 1.2 mL/min was used, and 1 µl of the sample had been injected for the GC-MS analysis. Based on retention time, active molecules from the extract were defined. The National Standards and Technology Laboratory database (NIST) was used to analyze the molar mass of the GC-MS.

2.4. Compound screening using thin layer chromatography (TLC)

TLC has been used to recognize the number and nature of the compounds in ethyl acetate extract utilizing aluminum plates infused with silica gel 60 F254 (20x20 cm). The formed spots were seen under an ultra-violet lamp at 254 nm to detect ultra-violet bioactive components with unsaturated systems (double bonds or benzene rings), then sprayed with sulfuric acid (10%) and heated on a hot plate to identify any other substances, and RF was determined as follows:

$$RF = \frac{\text{Distance of the sample (solute)from the origin}}{\text{Distance of the solvent from origin}}$$

2.5. Phytochemical analyses of ethyl acetate extract

2.5.1. Evaluation of total phenol content (TPC)

The Folin-Ciocalteu method were used to figure out the total phenolic content following (Dewanto et al. 2002). One ml of deionized water was employed to solubilize one mg of extract, and 200 µl of the soluble sample was combined with 600 µl purified water and 100 µl of the Folin-Ciocalteu reagent. After shaking the combination for 6 minutes, 2 ml of 2% Sodium carbonate (Na_2CO_3) was added. After being adapted to an end volume of 3 mL with purified water, the solution had been thoroughly mixed. The absorbance at 650 nm had been taken after thirty minutes in the dark, in contrast to the blank. A calibration curve has been drawn utilizing different concentrations of gallic acid (standard, from 0-100 mg/ml). TPC was estimated using the calibration curve-based liner formula and conveyed as Gallic acid equal (GAE)/mg dry weight: y = 0.0095x - 0.0409, R2 = 0.9953, where y represents absorbance, x represents concentration ((GAE) mg/g extract), and R2 represents the correlation. Every estimation was made in three replicates.

2.5.2. Determination of total flavonoid content (TFC)

In a modified colorimetric method described by (Sakanaka et al. 2005), the total flavonoid content of the *H. atra* extract was determined using catechol as a standard, with levels from 20 to 200 mg/ml. After five minutes, extract or standard solutions (250 µl) were combined with 1.25 ml distilled water, 75 µl of 5% sodium nitrite ($NaNO_2$) solution, and 150 µl of 10% aluminium chloride ($AlCl_3$) solution. 0.5 ml of 1 M Sodium hydroxide (NaOH) and 0.6 ml of purified water had been added after 6 minutes. After mixing all the contents, the absorbance at 510 nm was recorded. The flavonoid amount was determined using catechol equivalents (CE) with the standard curve and liner formula: Y = 0.0079 x - 0.109, R2 = 0.986, where y represents absorbance, X represents density (catechol equivalents) mg/g extract), and R2 represents the correlation. All measurements were taken three times.

2.6. Determination of the antioxidant effect of ethyl acetate extract

2.6.1 Determination of the scavenging activity of 1, 1-Diphenyl-2-picrylhydrazyl (DPPH)

The radical scavenging activity of *H. atra* extract was evaluated using the DPPH method (Brand-Williams et al. 1995) with a few alterations. A 0.2 mM DPPH solution in methanol had been prepared (0.0078 g/100 ml), and 1 ml of this radical solution was added to 1 ml of a sample or standard solution at different densities (1:1 V/V). A spectrophotometer was used to quantify the absorption coefficient at 517 nm after 30 min of incubation in darkness at room temperature. Ascorbic acid solutions with densities ranging from 5 to 200 µg /ml used as standards to establish a standard curve. The scavenging activity of DPPH has been expressed in mg ascorbic acid equivalents (AAE)/g dried sample. The following formula was used to determine the proportion of DPPH radical-scavenging activity:

$$DPPH\ radical\ scavenging\ activity\ (\%\ inhibition) = \frac{(\mathrm{Abs_{Control}} - \mathrm{Abs_{Sample}})}{\mathrm{Abs_{Control}}} \times 100$$

All the reagents except *H. atra* extract were added for control, and all determinations were done in triplicate, with the average values tabulated.

2.6.2. Ferric reducing power assay

In the reduction assessment, spectrophotometry method of (Ferreira et al. 2007) had been used. 1ml of *H. atra* extract was combined with 2.5 ml of phosphate buffer (0.2M, PH 6.6) and 2.5 ml of potassium ferricyanide in related solvents (1%). For twenty

minutes, this mixture was kept in water at 50˚C. After cooling, 2.5 ml of 10% tricolored acetic acid was added and centrifuged at 3000 rpm for 10 minutes. The upper part (2.5 mL) was mixed with deionized water (2.5 mL), and 0.5 mL of freshly prepared ferric chloride mixture (0.1%) for 10 minutes. At 700 nm, the absorbance was recorded. The control was made in the same way as the samples without the extract. The calibration curve was drawn with varying concentrations of ascorbic acid, and the reducing power was reported as EC_{50} (mg/ml), the efficient density at which the absorption is 0.5.

2.6.3. American Board of Thoracic Surgery (ABTS) radical scavenging assay

The 2, 2′- Azino-bis (3- ethylbenzothiazoline-6-sulfonic acid; ABTS) method was used to calculate the ABTS radical scavenging assay (Abdel-Razek et al. 2017). As a standard stock solution, the solutions had 7 mM ABTS and 2.4 mM potassium persulfate. After that, the solution mixture has been created by combining equal parts of the two stock solutions and letting them interact in the dark for 12 hours at room temperature. 1 mL of ABTS solution was mixed with 60 mL of methanol to obtain an absorption spectrum of 0.802 ± 0.005 parts at 734 nm. For each assay, a new ABTS solution was developed. After allowing *H. atra* extract or ascorbic acid (1 ml) to react with 1 ml of the ABTS solution for 7 minutes, the absorbance at 734 nm was determined using a spectrophotometer. The extract's ABTS scavenging capacity was compared to that of butylated hydroxyl toluene (BHT), as well as the inhibition percentage was determined by:

$$\text{ABTS radical scavenging activity (\%)} = \frac{(\text{Abscontrol} - \text{Abssample})}{(\text{Abscontrol})} \text{ x } 100$$

Abs control is the absorbance of the ABTS radical + methanol; Abs sample is the absorption coefficient of the ABTS radical + sample extract/standard.

2.6.4. Determination of hydroxyl radical scavenging activity

The scavenging of hydroxyl activity of the tested *H. atra* extract has been determined utilizing the salicylic acid method (Smirnoff and Cumbes 1989). 1ml of serial dilution of the sample or positive control ascorbic acid was added to 1ml of prepared salicylic acid, 1ml of prepared Ferrous Sulfate ($FeSO_4$), and 1ml of prepared Hydrogen Peroxide (H_2O_2). The mixture was then incubated for 60 minutes in a boiling water bath at 37˚C. The tubes were cooled, and the absorbance at 510 nm was measured with an optima spectrophotometer. A reduction in absorbance indicates an increase in hydroxyl free radical scavenging activity. The test was performed three times.

$$\text{Percentage of OH scavenging activity} = \frac{(\text{AC} - \text{AE})}{\text{AC}} \times 100$$

AC: The negative control absorbance means. AE: The extract absorbance means.

The results were expressed as IC_{50} and determined using data from the equation of the percentage of hydroxyl radical scavenging activity at each serial concentration of extracts by the Graph Pad Instate software.

2.7. Hemolytic activity assay

The experiment was conducted in 2mL microtubes with some modifications to the procedure described by (Farias et al. 2013). Two-fold serial dilution of *H. atra* extract with 0.9 percent Sodium chloride (NaCl) was organized and held, with concentrations varying from 1 to 1.9 μg/mL. The extract dilution was again introduced to a small micro tube comprising 900 μL of a 1% red blood cell suspension (human blood types or rabbit blood), which was then placed in an incubator at 37 degrees Celsius for 60 min. The tubes were then centrifuged for 5 min at 3,000 g. A 96-well plate was filled with supernatant (200 μL), and A microplate reader was utilized to measure absorbance at 540 nm. Cell suspensions of each human blood type or rabbit (100 μL) were mixed with distilled water or 0.9 percent NaCl (900 μL) to obtain an absorption spectrum for 100% and 0% autolysis. The following percentage of hemolysis has been calculated:

$$\text{\% hemolysis} = \frac{\text{Abs test}}{\text{Abs pc}} \times 100$$

Abs test is the Abs 540 of a 1% stock solution that resulted with sample test, and Abs pc is the Abs 540 of a 1% stock solution obtained through filtered water.

2.8. Anti-inflammatory activity

The extract's anti-inflammatory activity was tested *in-vitro* using the membrane stabilizing method of human red blood cells (RBCs) (Anandarajagopal 2013) with minor alterations. A healthy human volunteer's blood was collected and converted to anticoagulated centrifuge tubes and separated by centrifugation at 3000 rpm. Before being suspended in 10% normal saline, the crowded cells were washed with saline. 2 ml of hypotonic saline (0.25 % w/v NaCl), 1 ml of 0.15 M phosphate buffer (pH 7.4), 1 ml of sample solution (1 mg/ml) in sterile saline, and 0.5 ml of 10% RBCs in sterile saline comprised the stock solution (4-5 ml). As a control, 1 ml of isotonic saline had been instead of the sample solution. The mixes were kept for 30 minutes at 56 Celsius degrees, and the supernatant absorbance was recorded at 560 nm using a spectrometer after centrifugation at 3000 rpm for 20 minutes. The experiment was carried out three times. The control sample is entirely devoid of lyses. The following formula was used to calculate the % membrane stabilization:

$$\text{\% Protection} = 100 - \frac{\text{OD of test}}{\text{OD of control}} \text{x} 100$$

By estimating percentage inhibition instead of treatment density, the extract/drug concentration for 50% inhibition (IC_{50}) was calculated.

2.9. Cytotoxicity of the ethyl acetate extract using Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT assay)

In this assay, 200 µl of medium supplemented with 100,000 WBCs per well (96-well cell culture plate) were prepared with various concentrations of extract in Roswell Park Memorial Institute (RPMI) medium without fetal bovine serum. The plate was incubated for 72 hours in a Humid chamber (37˚C, 5% CO_2, and 90% moisture content). After 72 hours, each well received 20 µl of MTT solution, and the plates were left in a CO_2 incubator for 3 hours to allow the MTT to interact. After culturing, the plates were centrifuged for ten minutes at 1650 rpm, and the medium was thrown away. The precipitated particles (a byproduct of MTT) were dissolved in a 100 µl DMSO. The reading at 570 nm with a local optimum spectrophotometer was taken to identify a safe dose that causes 50% cell viability (Mosmann 1983).

$$\text{The \% viability} = \frac{\text{AT} - \text{Ab}}{\text{AC} - \text{Ab}} \text{ x } 100$$

AT = average absorbance of cell lines handled with varying densities of *H. atra* extract. AC denotes the mean absorbance of untreated control in a cell cultural context. Ab = average absorbance of *H. atra* extract-treated cells (RPMI without fetal bovine serum). A cytotoxicity test for the substance has been calculated using Graph Pad Instate software, and the percentage viability was estimated using serial dilutions of *H. atra* extract.

2.10. Detection of the presence of potent anti-inflammatory densities of therapies used in lipopolysaccharide-stimulated living person WBC culture

A 96-well plate was packed with 50µl of cell culture containing 100,000 living people's WBCs for this test. 50 µl of lipopolysaccharide was added to the plated cell lines and cultured in a humidified incubator to induce inflammation. The cells were collected within 24 hours. By centrifugation for five min at 1650 rpm, the supernatant was thrown away, and 200 µl of intensity (0.1 of the EC_{50} of the ethyl acetate extract in culture media, 1.15 ug/ml) was included. The positive control incorporated only cell lines

medium, whereas the remaining control cells were incubated with LPS. For an additional 72 hours, the plates were placed in a CO_2 incubator. MTT was used to assess cellular proliferation 72 hours after culturing (as previously illustrated). After centrifugation, pellets of treated and untreated cells were gathered and dissolved in phosphate buffer saline (PBS). Each suspension was dissolved in PBS containing 1X protease inhibitor in an ice chest for molecular assays (Mosmann 1983).

2.11. Determination of pro-inflammatory modalities at the expression level cyclooxygenase-2 (COX-2) gene and Inducible nitric oxide synthase (iNOS) gene expression levels using Real-time polymerase chain reaction (RT-PCR)

Firstly, RNA was extracted from both treated and untreated LPS-induced living person white blood cells. Then complementary DNA (cDNA) was reproduced from RNA extracted from treated and untreated LPS-induced living person white blood cells. Real-time PCR has developed into a well-established method for measuring gene expression (He et al. 2016). In PCR tubes, 12.5µl of 2X SYBR green stock solution and 5µl of complementary DNA were combined. For each *iNOS* or *COX-2* gene, use 0.5 µl of 10 pmoles/ml forward primer and 0.5 µl of pmoles/ml reverse primer (**Table 1**). To the reference tube, 0.5 µl of 10 pmoles/ml *β-actin* forward primer and 0.5 µl of 10 pmoles/ml *β-actin* reverse primer was added. Some other tubes were used by substituting the template with 1µl of nuclease-free water, as just a non-template control (NTC). (NTC) used to verify for reagent contamination or primer dimers. The tubes were gently mixed with 6.5µl of nuclease-free water without creating bubbles (bubbles interfere with fluorescence detection) and spun for a few seconds. Samples were placed in the cycler and the programmer was started as follows: 1 cycle of 95˚C for 10 minutes (initial denaturation), 40 cycles of denaturation at 95˚C for 15 seconds, annealing at 60˚C for thirty seconds, and annealing at 72 ˚C for 30 seconds (extension) using a kit according to (Salman 2013).

Table 1. Primers' sequences

Genes	Primers' sequences
iNOS	F: 5'-AGAAGGAAATGGCTGCAGAA-3'
	R: 5′-GCTCGGCTTCCAGTATTGAG-3′
COX-2	F: 5′-CCTGTGTTCCACCAGGAGAT-3
	R: 5′-CCCTGGCTAGTGCTTCAGAC-3′
β-actin	F: 5′-TGGTTACCAACTGGGACGACA-3′
	R: 5′-ACATCTGCTGGAAGGTGGAC-3

2.12. Statistical Analyses

The data are analyzed using the one-way ANOVA test and t-test. Contrast (polynomial), option (descriptive), and Post Hock's (LSD) multiple comparison tests (SPSS 16). All data were presented as mean ±standard deviation of the mean. A p-value < 0.05 was regarded as significant.

3. Results

3.1. GC-MS of H. atra extract:

Ethyl acetate extract was found to contain fatty acids and fatty acid esters including 2-Butanone, 4-hydroxy-, Acetic acid, Butanedioic acid, 2,3-bis(acetyloxy)-, [R-(R*, R*)]-, Heptane, 1-chloro-, tetrahydro-2-methoxy-6-methyl-,2H-Pyran-3,4,5-triol, n-Hexadecanoic acid, 9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E, E, E)-, 9-Octadecenoic acid, methyl ester, Oleic Acid, and Octadecanoic acid (**Figure 1**).

3.2. TLC of H. atra extract

TLC demonstrated several spots indicating terpenoids and steroid compounds with RF values of 0.93, 0.96, 0.62, 0.34, and 0.12 (**Figure 2**).

3.3. Phytochemical analysis of examined H. atra extract

Ethyl acetate extract has been subjected to standardized quantitative methods to investigate Phyto-ingredients with antioxidant properties, including total phenolic compounds and total flavonoid content as shown in **Table 2**.

3.4. The Antioxidant Effect of examined H. atra extract:

The *in-vitro* antioxidant capacity of the ethyl acetate extract was determined and the IC_{50} value of the ethyl acetate extract was frequently used to assess its scavenging activity. The smallest IC_{50} value indicates the highest radical scavenging activity. DPPH, ABTS, ferric reducing, and H_2O_2 scavenging free radicals' activities of ethyl acetate extract were illustrated in **Table 3**. At $p < 0.05$, the antioxidant capacity of the ascorbic acid levels also provided as a control sample was lower in the *H. atra* ethyl acetate extract.

3.5. The Hemolytic Effect of examined H. atra extract

The hemolytic activity of ethyl acetate extract against regular human erythrocytes was tested. **Figure 3** demonstrated that increasing the concentration of ethyl acetate extract from 10 mg/ml to 100 mg/ml raised the hemolytic % from 20 to 49.57%. The extract's measured hemolytic IC_{50} was 100.8±80.23 mg/ml.

3.6. Anti-inflammatory activity of H. atra extract:

Table **4** shows the anti-inflammatory action of ethyl acetate extract utilizing normal human erythrocytes. When the density of ethyl acetate extract was increased from 10 mg/ml to 100 mg/ml, the percentage of human red blood erythrocytes membrane stabilizing lowered from 80 to 50.43%. The extract's measured membrane stabilization IC_{50} value was 99.140 ±15 mg/ml.

3.7. MTT assay for cytotoxic activity and anti-inflammatory action

The safety of the tested extract was assessed using the MTT test and IC_{50} values resulting in 50% cell viability were calculated. The calculated IC_{50} value of the extract was 11.5 ug/ml. After that, 1.15 µg/ml (0.1 of IC_{50} value) concentration was used for the detection of anti-inflammatory action in a living person WBC culture, which was stimulated by lipopolysaccharides (LPS), which caused 91% cell viability compared with LPS treated cells (50% cell viability) and control cells (untreated cells) (100 % cell viability) as shown in **Table 5**.

3.8. Expression levels of cellular pro-oxidants or pro-inflammatory mediators

3.8.1 Expression level of COX-2 in normal, untreated, and treated inflammatory cells

The incubation of WBCs with LPS for 24 h upregulated *COX-2* expression (15.30 ±0.04) at $p \leq 0.05$ when compared with normal control cells (1.00±0.08). Treatment with ethyl acetate extract (4.70 ± 0.05) significantly down-regulated *COX-2* expression compared to LPS- stimulated WBCs as shown in **Table 5**.

3.8.2. Expression level of iNOS in LPS- stimulated WBCs treated with examined H. atra extract compared to control normal cells:

The incubation of WBCs with LPS for 24 h upregulated *iNOS* expression (10.53±0.02) at $p \leq 0.05$ in comparison with control cells (1.00±0.05). Treatment with ethyl acetate extract (6.17±0.01) significantly down-regulated *iNOS* expression compared to LPS-stimulated WBCs as shown in **Table 5**.

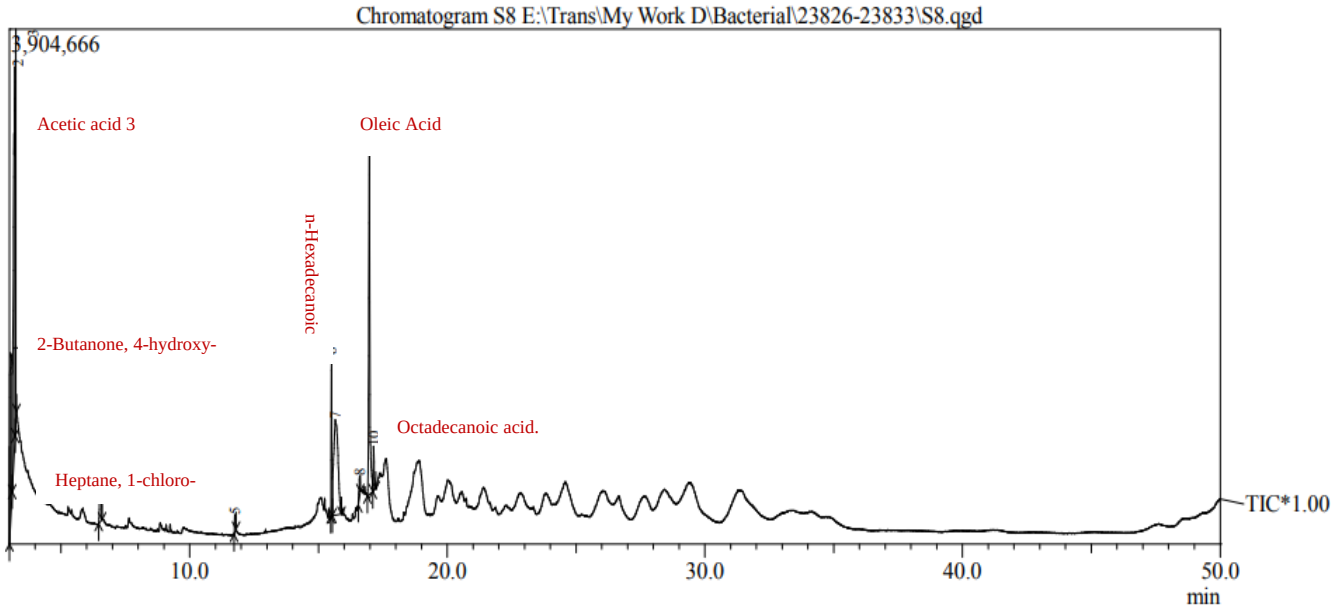

Figure 1: Chromatogram of the *H. atra* extract as investigated by GC-MS chromatography.

Figure 2. TLC of *H. atra* extract

Table 2. Total phenolic and total flavonoid contents of *H. atra* extract

Parameters	Concentration (mg/g)
Total phenolic content	121.92±0.14
Total flavonoid content	23.84±1.77

The values reported are the mean± SD of 3 replications. At $p < 0.05$, Total phenolic was expressed as gallic acid equivalents (GAE) mg/g sample. Total flavonoids were expressed as mg catechol/g sample.

Table 3. Different antioxidant activity assays of *H. atra* ethyl acetate extract using ascorbic acid as a positive control.

Parameter	Ethyl acetate extract IC_{50} (mg/mL)	Ascorbic acid IC_{50} (mg/mL)
DPPH	101.31±0.01[a]	3.44±0.01[b]
Ferric reducing power	61.65±0.01[a]	4.01±0.01[b]
ABTS	47.62±0.05[a]	7.73±0.01[b]
Hydroxyl radical	130.62±0.02[a]	23.99±0.01[b]

The values reported are the mean± SD of 3 replications. At $p < 0.05$, means within the same column with various lowercase letters differ significantly.

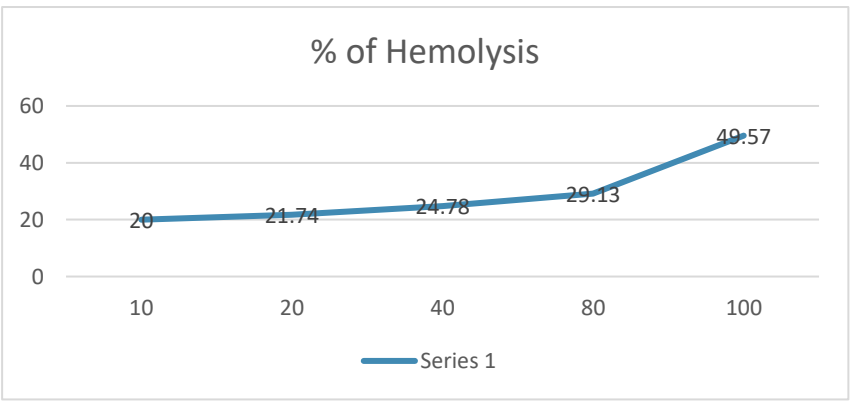

Figure 3. Hemolytic activity of *H. atra* extract

Table 4 Membrane stabilization activity of *H. atra* extract

Concentration (mg/ml)	RBCs membrane stabilization IC_{50} (mg/ml)
10	80.00
20	78.26
40	75.22
80	70.87
100	50.43
IC_{50} (mg/ml)	99.14±0.15

The values reported are the mean± SD of 3 replications. At $p < 0.05$, IC_{50} (mg/ml): Extract concentration that causes 50% RBCs membrane stabilization.

Table 5. Assessment of anti-inflammatory activity and Expression level of *COX-2* and *iNOS* in normal, untreated and treated inflammatory cells

	Concentration (µg/ml)	**Viability %**	***COX-2*** Fold change	***iNOS*** Fold change
Ethyl acetate extract	1.15±0.05 [b]	91±0. 00 [b]	4.70±0.05[b]	6.17±0.01[b]
LPS treated cells	20±0.02[a]	50±0. 00[C]	15.30±0.04[a]	10.53±0.02[a]
Control (Untreated cells)		100±0.00[a]	1.00±0.08[c]	1.00±0.05[c]

The values reported are the mean± SD of 3 replications. At $p < 0.05$, means within the same column with various lowercase letters differ significantly.

4. Discussion

The sea cucumber *Holothuria atra* collected from Egyptian Red Sea shores was studied for its antioxidant and anti-inflammatory properties on lipopolysaccharide-induced inflammation of White blood cells. Sea cucumbers are high in biologically active chemicals that have therapeutic properties. Their potential use in treating and preventing chronic diseases requires further exploration.

The current study's phytochemical investigation of *H. atra* demonstrated the presence of flavonoids and phenolic compounds, which could explain that *H. atra* is a common medicine for treating various diseases. In dealing with this discovery reported by (Sulardiono et al. 2020), *H.atra* contains a large number of flavonoids and phenolic compounds. Ethyl acetate was revealed to be capable of extracting bioactive compounds from *H. atra* including, alkaloids, flavonoids, glycosides, phenol hydroquinone, saponins, steroid, and triterpenoid (Sibero et al. 2019). (Murniasih et al. 2015) reported the presence of Flavones, terpenes, phenol, saponins, alkaloid, anthraquinonoid, and glycosides in *H.atra.* On the other hand (Sukmiwati et al. 2020) reported that *H.atra* sea cucumbers contained terpenoids, saponins, and phenols but did not contain flavonoids.

Because of their capacity to donate hydrogen atoms to free - radicals, flavonoid and phenolic compounds are powerful antioxidant components involved in free radical deactivation. They also have remarkable structural features for scavenging free radicals. A linear relation between total flavonoid, phenolic content, and antioxidant properties has been discovered in several studies (Aryal et al. 2019).

Furthermore, the GC-MS chromatogram of *H. atra* confirmed the existence of several important compounds with biological activity, including fatty acids and fatty acid esters. Such compounds were discovered using spectrometry and GC and have been reported to possess significant biological activities (Fraise et al. 2013). For example, 9-Octadecenoic acid and its methyl ester showed antimicrobial and anticancer activity (Muflihunna et al. 2021), n-Hexadecanoic acid, oleic Acid, and octadecanoic acid had antioxidant activity (Prakash et al. 2011).

In the present study, the antioxidant activity of *H. atra* extract was determined using several assays. The ethyl acetate extract's antioxidant properties revealed that the *H. atra* displayed a decreased ability to inhibit DPPH and reducing power assays, whereas mild ability to inhibit hydroxyl radical and ABTS, consistent with previous findings. The extract and tested saponins demonstrated low antioxidant activity in both the DPPH Radical scavenging and reducing power assays (Hawas et al. 2021). In contrast, (Dakrory et al. 2015) found that *H.atra* extract inhibited DPPH radicals from 81 to 94% in a dose-dependent manner when compared to ascorbic acid. In another study by (Nugroho et al. 2022b)**,** *H.atra* has the most antioxidant activity of any other species studied. These could be explained according to many factors, including polyphenols and flavonoid contents, genetic, environmental, biological, species characteristics, seasons, and geological factors, as well as sample preparation, extraction, and analysis method (Sobhy Darwish et al. 2021).

During the investigative process of *H. atra's* anti-inflammatory characteristics utilizing red blood cells assay, lysosomes lysed and spread their constituent enzymes, causing a variety of pathologies. Nonsteroidal anti-inflammatory drugs (NSAIDs) operate by either impairing or stabilizing lysosomal enzyme secretion. Because RBC membranes are similar to lysosomal membranes, inhibition of hypotonicity-induced RBC membrane lysis was used to assess the mechanism of anti-inflammatory activity of the tested *H. atra* extract due to its simplicity and reproducibility (Vadivu and Lakshmi 2008). According to our findings, ethyl acetate crude extract demonstrated anti-inflammatory activity, which is consistent with (Dhinakaran et al. 2014). More investigation into the anti-inflammatory properties of *H. atra* is required.

The cytotoxic impact of various doses of *H. atra* extract against living person WBCs was determined using MTT assay, which is based on the activity of the NAD(P) H-dependent oxidoreductase enzyme that converts MTT to formazan in viable cells (Mosmann 1983). The present study indicated the dose that caused 50% cell viability was 11.5 ug/ml, which was considered a low dose, so ethyl acetate extract was considered to cause inhibition rather than viability. This result was consistent with previous findings by (Isaac DHINAKARAN and Premnath LIPTON 2015), who reported that sea cucumber had been shown to inhibit the growth of human colon adenocarcinoma and induce apoptosis.

After treating the white blood cells with lipopolysaccharide (LPS), Toll-like receptors (TLRs) on the plasma membrane recognized and reacted to these inflammatory triggers, stimulating pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1), inducible enzymes like COX-2, and inducible nitric oxide synthase *iNOS*, and inflammatory markers like nitric oxide (NO) and Prostaglandin E2 (PGE2) (Himaya et al. 2010). This study explains the ability of *H. atra* to inhibit the LPS-induced expression of primary inflammatory mediators such as *iNOS/NO* and *COX-2/PGE2* by using 1.15 ug/ml of the extract as a safe dose that resulted in 91% percent viability.

Incubating WBCs with LPS for 24hrs has been shown to increase *COX-2* expression. It has also been found that the two COX forms are closely related, which are now recognized as *COX-1* and *COX-2*. Even though this enzyme converts arachidonate to prostaglandins via both isoforms (Simon 1999). *COX-2* upregulation and overexpression are primarily linked to inflammation, apoptosis loss, uncontrolled cell proliferation, growth, metastasis, neovascularization, and angiogenesis,

ultimately leading to cancer (Gandhi et al. 2017). The results of this study indicate that ethyl acetate extract can lower *COX-2* expression. In agreement with these results, according to (Abdelgawad et al. 2021), Phenolic compounds were found to have *COX* inhibitory properties.

Furthermore, incubating WBCs with LPS for 24 hours has been shown to increase *iNOS* expression. Inducible nitric oxide synthase (i*NOS*) is one of three key enzymes responsible for producing nitric oxide (NO) from the amino acid l-arginine. NO produced by *iNOS* is involved in a variety of physiological (e.g., blood pressure regulation, wound repair, and host defence mechanisms) and pathophysiological (inflammation, infection, neoplastic diseases, liver cirrhosis, diabetes) conditions (Lechner et al. 2005). According to our findings, treatment with ethyl acetate extract reduced *iNOS* expression (Senthilkumar and Kim 2013). The authors found that the alkaloid group hindered the appearance of *iNOS* and *COX-2* in marine organisms. Moreover, (Surh et al. 2001) investigated that some phenolics have anti-inflammatory properties and found that they can suppress the utterance of *iNOS*, *COX-2*, and pro-inflammatory cytokines by decreasing the expression of their gene transcription factor Nuclear factor kappa B (NF-kappa B).

5. Conclusion

According to this study, the ethyl acetate extract of *H. atra* contained phenolic and flavonoid compounds. GC-MS revealed the presence of fatty acids and fatty acid esters. Moreover, the crude extract demonstrated moderate antioxidant activity in DPPH radical scavenging and reducing power assays. Interestingly, it exhibited anti-inflammatory activity by inhibiting the expression of key inflammatory mediators such as *iNOS/NO* and *COX-2/PGE2*. The high polyphenol content of *H. atra* extract could be contributed to this anti-inflammatory effect. As a result, *H. atra* may possess anti-inflammation therapeutic potential.

Author contributions

All authors contributed to the conception and realization of the work. All the authors have contributed to the paper redaction and approved the final version of the manuscript.

Conflict of interest: There are no conflicts of interest stated by the authors.

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