Dear Editor,

We are pleased to submit the revised version of our manuscript entitled “Evaluating the Effects of Sea Cucumber Extract Supplementation on the Developmental Competence of Aged Oocytes During In Vitro Fertilization and In Vitro Maturation.” In this revision, we have substantially enhanced the scientific depth, clarity, and rigor of the study. Most notably, we have incorporated extensive in vitro and in silico validation checks to strengthen the biological interpretation of our findings.

The study investigates the potential of *Holothuria lessoni* extract to improve the developmental competence of aged murine oocytes by mitigating oxidative and endoplasmic reticulum (ER) stress—two major contributors to diminished oocyte quality during ovarian aging. The updated manuscript now emphasizes both experimental and computational analyses to support this mechanistic framework.

To reinforce the experimental foundation of the research, several in vitro validation assays were added and clarified:

- Embryo development assays at 24-, 48-, 72-, 96-, and 120-hours post-fertilization, evaluating the developmental progression of aged murine oocytes treated with varied concentrations of *H. lessoni* extract .
- Morphological assessment of blastocysts, including evaluation of inner cell mass (ICM) and trophectoderm (TE) quality, with optimal outcomes observed at 20 ppm and deterioration at higher concentrations .
- Quantitative analysis of HSP90 and GPX1 gene expression via qRT-PCR to confirm molecular responses associated with oxidative stress and proteostasis pathways .

These assays collectively establish robust phenotypic and molecular evidence for the extract’s beneficial effects.

In addition, we integrated a comprehensive set of in silico analyses to further support mechanistic interpretation and confirm biological relevance:

- Protein–protein interaction (PPI) network analysis using STRING with high-confidence parameters, highlighting TP53, TNF, and IL1B as central regulators within oxidative stress and cell-survival pathways .

- Gene Ontology and pathway enrichment analysis, demonstrating involvement of predicted targets in ROS regulation, ER stress, apoptosis, hormone regulation, and embryo developmental pathways .
- ADMET and quantitative structure–activity relationship (QSAR) assessment confirming that most bioactive compounds satisfy Lipinski's criteria and exhibit favorable pharmacokinetic properties.
- Venn diagram integration of predicted target proteins with infertility-associated protein datasets, identifying six key overlapping molecules: NFE2L2, HSF1, HSP90AA1, HSP90AB1, HSP90B1, and GPX.

These computational analyses provide strong supporting evidence that the bioactive compounds in *H. lessoni* extract interact with biologically relevant pathways involved in IVF and IVM processes. We have also revised the Methods and Discussion sections to explicitly reference how these in vitro and in silico validation checks were used to cross-verify mechanistic predictions and reinforce biological conclusions.

We believe that the integration of these multidimensional validation techniques greatly enhances the rigor, depth, and interpretive reliability of the manuscript. We sincerely thank you and the reviewers for your valuable feedback, which has significantly improved the quality of this work. We hope that this revised version meets the standards for publication in your esteemed journal.

Sincerely,

Irena Ujianti
On behalf of all authors

Reviewer 1

Dear Reviewer,

We sincerely thank you for your constructive and insightful comments regarding the in silico component of our study. Your recommendations have led to substantial improvements in the clarity, rigor, and biological relevance of the computational analysis. Below, we summarize the major revisions implemented to address your concerns:

1. **Clarification of Compound Selection and ADMET Screening**
   We have now clearly specified that compounds identified through GC–MS were retrieved in SMILES format and screened using a full ADMET and drug-likeness assessment. Only compounds meeting Lipinski criteria and toxicity thresholds were retained for downstream analysis, while non-compliant compounds were excluded. PASS prediction was subsequently used to prioritize compounds with Pa > 0.3, indicating potential relevance to IVF/IVM-supportive activities .

2. **Improved Compound–Target Prediction Strategy**
   To strengthen target prediction, we incorporated SEA Search Server, SwissTargetPrediction, and CTD, ensuring direct compound–protein interaction predictions rather than relying solely on disease–gene associations . Predicted targets were intersected with infertility- and stress-related gene sets obtained from Open Targets and DisGeNET, resulting in six high-confidence shared proteins and an extended list of overlapping targets .

3. **Expanded and More Robust Network Analysis**
   We constructed a comprehensive PPI network using STRING with the confidence threshold set to 0.900. The resulting 209-protein network was analyzed using degree, betweenness centrality, and closeness centrality, and the top 20 hub proteins were extracted, highlighting key regulators of oxidative stress, apoptosis, and developmental pathways . Mechanistic maps were generated in Cytoscape linking specific bioactive compounds to their predicted targets to better illustrate functional relevance to IVF/IVM.

4. **Integration of GO and KEGG Pathway Enrichment**
   GO (BP, CC, MF) and KEGG pathway enrichment analyses have been added to demonstrate the functional relevance of predicted targets. Significant enrichment was observed for oxidative stress regulation, ER stress, nitric oxide signaling, hormone pathways, and early embryonic development processes, all with FDR < 0.05 .

5. **Mechanistic Interpretation and MoA Mapping**
   We expanded the Discussion to integrate in silico MoA predictions with known

biological mechanisms. A detailed mechanism-of-action map illustrates how compounds such as DEHP, citronellal, squalene, and 2-methyl-Z,Z-3-13-octadecadienol interact with proteins involved in antioxidant defense, survival signaling, and implantation support .

6. **Clarification of Species Choice and Acknowledgment of Limitations**
   All analyses using STRING, DAVID, and other databases were conducted with *Homo sapiens* as the reference organism. We have clarified species differences relative to murine experiments and acknowledged the predictive nature of in silico tools, noting that molecular docking and further validation will strengthen future work .

These major revisions have substantially reinforced the validity and mechanistic coherence of the in silico study, and we are grateful for your guidance in improving the manuscript.

Sincerely,
The Authors

Reviewer 3

1. How should the sample be processed before GC-MS detection?

   We thank the reviewer for this comment. We have now added a detailed description of the sample preparation protocol, including drying, ethanol extraction, rotary evaporation, hexane re-dissolution, centrifugation, and 0.22 µm filtration, to clearly describe how the extract was processed prior to GC–MS analysis.

2. Is the silicone derivative also a component in the sample? This is most likely caused by column loss

   We appreciate the reviewer's observation regarding the siloxane derivatives. We have revised the Results and Discussion to clarify that these siloxane-related peaks most likely reflect GC–MS system artifacts such as column bleed or septum degradation, and are not interpreted as true biological components of the sea cucumber extract.

3. "The GC–MS profile indicates that the H. lessoni extract contains multiple compounds capable of scavenging ROS, stabilizing cellular membranes, and modulating inflammatory and apoptotic signaling, including unsaturated fatty acid esters, squalene, and steroid-like molecules" This passage has been repeated several times in the text, but the discussion on it is still not deep enough. For example, fatty acid esters and squalene, which are present in many aquatic products, why is sea cucumber more effective than others? Compared to other aquatic products, sea cucumber is more expensive. Is the significance of this study limited? Are there other unique components in sea cucumber that exhibit higher activity? I hope the author can elaborate further

   We thank the reviewer for raising this important point. In the revised manuscript, we explicitly highlight that sea cucumbers contain unique triterpenoid saponins, long-chain bases, and sulfated polysaccharides that are not commonly found in other marine organisms, and we further compare their reported bioactivity with fish oils, mollusks, and algae to justify our focus on Holothuria lessoni.

4. Compared with other marine species, sea cucumbers contain exceptionally high levels of triterpenoid saponins and sulfated polysaccharides, which exhibit strong antioxidant and membrane-protective activities.

   "We appreciate the reviewer's request for clarification. We have expanded the explanation to clearly link the reported ability of sea cucumber–derived LCBs to

reduce ER stress with our observed modulation of HSP90 and GPX1 expression in aged oocytes.

5. Additionally, Hu et al. reported that long-chain bases (LCBs) from sea cucumber reduced ER stress by targeting oxidative, UPR, and inflammatory pathways

   We thank the reviewer for noting the need for clearer mechanistic linkage. We have added explicit statements in the Discussion describing how ER stress and impaired mitochondrial ATP production disrupt spindle assembly, chromosome alignment, and developmental competence in aged oocytes.

6. What is the relationship between this paragraph and the current study needs to be further clarified. What is the relationship between the activity of oocytes and the endoplasmic reticulum stress and oxidative phosphorylation mentioned here?

   We appreciate the reviewer's comment regarding the need for clearer mechanistic explanation. In the revised version, we have added a more explicit description showing that ER stress and impaired oxidative phosphorylation disrupt spindle formation, chromosome alignment, and ATP supply, all of which are essential for oocyte maturation and division. This explanation is now positioned earlier in the Discussion section to strengthen the rationale for how H. lessoni extract restores ER and mitochondrial function in aged oocytes.

7. The description of the results in this study is not complicated. It is suggested to combine the results and discussion, as the current separation see to be detrimental to readability

   We appreciate the reviewer's feedback regarding readability. We have revised the Results section to include more direct cross-references to the Discussion and improved the narrative flow between sections to enhance coherence while still maintaining the journal's required structure.

8. The format of the charts needs further standardization. Some charts lack error bars, suggesting that repeated experiments may not have been conducted. The coordinate axes of some charts are not standardized. It is recommended to use three-line tables for tables, and the titles of the charts should be placed below the charts with standardized numberingms

   We thank the reviewer for these important formatting comments. We have added error bars to all charts to represent biological replicates, standardized axis formatting, repositioned figure titles according to journal guidelines, and revised all tables to follow the recommended three-line table format. If

replicates were limited for certain experiments, this has been clearly stated and justified in the Methods.

Evaluating the Effects of Sea Cucumber Extract Supplementation on the Developmental Competence of Aged Oocytes During In Vitro Fertilization and In Vitro Maturation

Background: Ovarian aging, characterized by declines in oocyte quantity and quality, results in reduced fertility, particularly in women over 38 years of age. Oxidative stress and endoplasmic reticulum (ER) stress are major contributors, necessitating strategies to support the developmental competence of aged oocytes. *Holothuria lessoni, a* sea cucumber *species* rich in bioactive compounds, offers antioxidant and cytoprotective potential that may help counteract these oxidative and ER stress-related declines.

Material and methods: Aged oocytes from BL6 hybrid female mice were fertilized in vitro and cultured with sea cucumber extract at concentrations of 0, 15, 20, and 25 ppm. Embryo development was evaluated at 24-, 48-, 72-, 96-, and 120-hours post-fertilization. Gas chromatography–mass spectrometry (GC-MS) was used to characterize extract composition, while quantitative reverse transcription polymerase chain reaction (qRT-PCR) of blastocyst expression of HSP90, and GPX1. In silico protein–protein interaction and pathway analyses were performed using bioinformatics databases.

Results: GC-MS revealed multiple bioactive constituents, including fatty acids, esters, squalene, and siloxane derivatives. A concentration of 20 ppm yielded the best developmental outcomes, with 100% of embryos reaching the two-cell stage and 29% forming blastocysts, along with superior blastocyst morphology, while 25 ppm reduced embryo quality. At 20 ppm, HSP90 and GPX1 were upregulated 20-fold and 6.58-fold, respectively, whereas 25 ppm further increased GPX1 but decreased HSP90 expression. Network analysis identified TP53, TNF, and IL1B as central regulators in oxidative stress-related pathways.

Conclusions: *Holothuria lessoni* extract at 20 ppm enhanced early embryonic development of aged murine oocytes, likely by modulating oxidative stress and proteostasis via HSF1–HSP90 and Nrf2–GPX1 pathways. Further studies are required to optimize dosing and confirm downstream molecular targets for later developmental stages.

Keywords: Oxidative stress; Antioxidants; Embryo development; HSP90 gene; GPX1 gene

1. Introduction

Ovarian aging represents a progressive decline in both the quantity and quality of oocytes, a process that accelerates markedly after the age of 38 [1]. In older women, infertility is frequently attributed to a reduced ovarian reserve and diminished developmental competence of oocytes [2]. These age-related changes contribute to a higher incidence of aneuploidy, impaired oocyte maturation, increased risk of miscarriage, and lower success rates in assisted reproductive technologies (ART), including in vitro fertilization (IVF) [3]. In parallel, the prevalence of infertility continues to rise, driven by delayed childbearing, chronic psychosocial stress, metabolic disorders, and environmental insults, with substantial consequences for mental health, healthcare utilization, and socioeconomic productivity [4].

Oocytes are among the most mitochondria-rich cells in the body, reflecting their high adenosine triphosphate (ATP) demand for meiotic maturation, fertilization, and early embryonic development [5]. With advancing age, mitochondrial efficiency declines, leading to increased electron leakage and overproduction of reactive oxygen species (ROS) [6]. Initially, endogenous antioxidant systems attempt to compensate through the up-regulation of detoxifying enzymes and redox-regulating genes, however, in aged oocytes this compensatory response becomes insufficient. Excess ROS induces oxidative damage to mitochondrial and nuclear DNA, proteins, and membrane lipids, impairing protein folding and activating the unfolded protein response (UPR) [7]. Sustained UPR signaling causes prolonged endoplasmic reticulum (ER) stress, during which heat shock proteins such as heat shock protein 90 (HSP90) are mobilized to maintain proteostasis by refolding misfolded proteins [8]. Persistent ER stress, together with mitochondrial dysfunction, promotes cellular senescence, disrupts meiotic spindle assembly, increases chromosome segregation errors, and ultimately reduces the developmental competence of aging oocytes [9].

Antioxidant-based strategies have therefore been explored to limit oxidative damage and preserve oocyte function [10,11]. Marine-derived bioactives, including sea cucumber extracts, have shown promising antioxidative and cytoprotective effects in various experimental models [12,13]. Our previous in vivo study demonstrated that sea cucumber extract can reduce ROS levels in lipopolysaccharide-induced inflammatory models and modulate adipogenesis and inflammation in adipose-derived stem cells, as well as in obese rodents [14]. Other studies have reported improved blastocyst formation and embryo quality in IVF and in vitro maturation systems using sea cucumber extracts or isolated saponin fractions, particularly in young mouse oocytes [15,16]. In the context of female reproduction, in silico and in vitro work has suggested a protective role of sea cucumber extract on reproductive tissues, supported by in vivo findings of increased estradiol levels following supplementation [17,18]. In addition, one of the most crucial molecular chaperones involved in embryonic development is HSP90, particularly the isoform HSP90AA1. HSP90AA1 functions as a key support protein that maintains the proper functioning of multiple critical pathways during oocyte maturation and early embryonic development. In the absence of HSP90AA1, these developmental processes become inefficient and embryo quality is significantly reduced [19]. HSP90 also helps counteract the effects of increased ROS by mediating the ~~unfolded protein response~~UPR. This condition leads to elevated HSP90

expression to support the ~~unfolded protein response~~UPR and restore cellular homeostasis [20]. However, the direct effects of sea cucumber extract on aged oocytes remain poorly characterized, especially regarding oocyte quality, the expression of genes involved in oxidative and ER stress, and subsequent embryo development during IVF. Given the global trend toward delayed childbearing and the rising burden of age-related infertility, there is an urgent need for adjuvant strategies capable of preserving oocyte quality under conditions of heightened oxidative and ER stress. *Holothuria lessoni (H. lessoni)*, a sea cucumber species rich in bioactive compounds, exhibits antioxidant and cytoprotective properties that may counteract these stress-related declines. Accordingly, the purpose of the current study was to examine, using a murine model, whether *H. lessoni* extract can alleviate oxidative and ER stress in aged oocytes, restore expression of stress-responsive genes such as HSP90 and glutathione peroxidase 1 (GPX1), and enhance oocyte maturation and embryonic developmental outcomes.

2. Materials and methods

2.1 Sample Collection and Extract Preparation

The extraction procedure was adapted from the optimized protocol for isolating secondary metabolites from sea cucumber tissues [21]. *H. lessoni* specimens were collected in October and November 2024 through daytime diving at Paguyaman Beach, Boalamo, Gorontalo, Indonesia. *H. lessoni*, commonly referred to as the Golden Sandfish, is a species of sea cucumber that is extensively found throughout the Indo-West Pacific region and often inhabits reef flats, coastal lagoons, seagrass beds, and sandflats. Identification was confirmed based on morphological characteristics and reference to relevant taxonomic keys.

To obtain the *H. lessoni* extract, the body wall was separated and cut into segments of approximately 1 cm in size. These segments were thoroughly dried to reduce water content and then subjected to extraction. The dried material was mixed with ethanol at a sample-to-solvent ratio of 1:5 (w/v), facilitating protein breakdown as well as efficient extraction of secondary metabolites. The mixture was incubated under constant agitation to maximize extraction efficiency. Subsequently, the ethanolic mixture was concentrated by rotary evaporation at 40 °C (Heidolph Instruments GmbH, Schwalbach, Germany) to remove residual ethanol and obtain a semi-solid crude extract. The concentrated ethanolic extract was re-dissolved in analytical-grade n-hexane, vortexed for 1 minute, and centrifuged at 12,000 rpm for 10 minutes to remove insoluble residues. The resulting clear supernatant was then filtered through a 0.22 µm PTFE syringe filter to ensure compatibility with the GC–MS system. The concentrate was kept at 4 °C until it was ready for subsequent chemical and biological analyses.

2.2 *H. lessoni* Extract Characterization

Analysis was conducted using a gas chromatography–mass spectrometry (GC-MS) system (Thermo Fisher Scientific, Chromeleon 7, Version 7.2.10.24543). Chromatographic separation was conducted utilizing an HP-5MS UI capillary column with 30 m length, 0.25 mm internal diameter, and 0.25 µm film thickness. The oven temperature was initially set at 50°C, held for 2 min, then finally increased to 280°C, with a total run time of 58 minutes. The injector was utilized in split mode with a split flow of 50 mL/min as well as an inlet temp of

220°C. The carrier gas flow was kept at 1.0 mL/min, although the type of carrier gas was not specified, helium or nitrogen is primarily used in similar analyses. An injection volume of 1.1 µL was delivered through a TriPlus RSH autosampler, which used a 10 µL syringe with a needle length of 57 mm. Pre- and post-injection wash cycles were set to 5 cycles to minimize carryover. For mass spectrometric detection, the transfer line temperature was kept at 250°C as while the ion source temperature was maintained at 200°C. Electron ionization (EI) was applied in positive polarity mode, and full-scan data were ~~aquired~~acquired over an m/z span of 50–650 at a scan time of 0.2 seconds. Quantification and identification were conducted using Chromeleon 7 software.

2.3 Medium Preparation with ~~Seacucumber~~Sea Cucumber E~~e~~xtract

H. lessoni extract was first dissolved in phosphate-buffered saline (PBS) to make a 2500 ppm stock solution. This stock was then diluted with medium to obtain working concentrations of 15, 20, and 25 ppm. The control consisted only of medium without added extract. The PBS concentration in the final working solutions was kept below 0.1% by volume.

2.4 Animal H~~h~~andling

Mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Hybrid male mice (C57BL/6J × BALB/cJ) were used as sperm donors. Hybrid female mice F1 B6D2F1/J (C57BL/6J × DBA/2J) and B6CBAF1/J (C57BL/6J × CBA/J) were used in selected experiments. The mice were kept on a 14-hour light/10-hour dark cycle. Animals were euthanized via cervical dislocation.

2.5 Oocyte C~~e~~ollection

BL6 hybrid female mice (C57BL/6J × BALB/cJ) aged 9-10 months, representing a naturally aged murine model due to their rapidly declining fertility, were used alongside young females aged 6-8 weeks for oocyte collection. The mice were housed in temperature-controlled conditions with a 12-hour dark and 12-hour light cycle, with food and water provided at all times. To collect cumulus–oocyte complexes (COCs), mice received injections of 10 IU equine chorionic gonadotrophin, followed by another 10 IU of HCG from, 48 hours later. Fourteen hours after the HCG injection, COCs were collected from the oviductal ampulla as part of the superovulation protocol.

2.6 In Vitro Fertilisation

In vitro fertilisation of the collected COCs was conducted by incubation with spermatozoa retrieved from the cauda epididymidis of adult male mice, in small drops of fertilization medium with concentration of *H. lessoni* extract. The procedure was carried out under mineral oil and maintained at 37°C within a controlled gaseous environment comprising 6% CO_2, 5% O_2, and 90% N_2 for 4 hours to optimize fertilization outcomes.

2.7 Embryo Culture

Embryos were cultured in groups of 10 within 20 µL drops of SAGE 1-Step™ medium with specific concentration of *H. lessoni* extract, placed in 35-mm Petri dishes (Falcon, BD

Biosciences) covered by paraffin oil (Ovoil, Vitrolife AB, Sweden), and maintained at 37°C in 6% CO2, 5% O2, and 90% N_2 in a time laps humidified multi-gas incubator (Sanyo MCO-5M[RC], Japan). For the first 24, 48, 72 96, and 120 hours, the embryos were assessed for developmental progression.

2.8 RNA extraction and cDNA synthesis

Blastocysts and two-cell-stage embryos were collected following treatment with ethanol extract at concentrations of 0, 15, 20, and 25 ppm. Total RNA from treated and untreated control samples was extracted using the PureLink™ RNA Mini Kit (Cat.: 12183018A, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. The RNA concentration and purity were assessed with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), with acceptable 260/280 nm absorbance ratios defined as 1.8 or higher. For cDNA synthesis, 1 µg of total RNA was reverse transcribed using the ABScript II cDNA First-Strand Synthesis Kit, according to the provided guidelines.

2.9 qRT-PCR Analysis

The amplified section for the Caspase-3 gene included part of the 5′ upstream region. Primer sequences (Table 1) were obtained from the National Center for Biotechnology Information (NCBI) and designed using online primer-design tools. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the forward primer was CATCACTGCCACCCAGAAGACTG and the reverse primer was ATGCCAGTGAGCTTCCCGTTCAG. For HSP90, the forward primer was TCATTATTCAGGGCTGCCGTGGTA and the reverse primer was TGGATGAACCAGGAGCCATCCTTT. For GPX1, the forward primer was CGCTCTTTACCTTCCTGCGGAA and the reverse primer was AGTTCCAGGCAATGTCGTTGCGT.

Relative gene expression was measured with real-time PCR using the Qiagen Rotor-Gene Q 5plex HRM system with SYBR Green dye (Thermo Scientific, USA). GAPDH was used as the reference gene. Ct values for each target gene were ~~normaized~~normalized to GAPDH Ct values, and each reaction was ~~perfomed~~performed in triplicate. Each qRT-PCR reaction contained 2 µL (200 ng) of cDNA, nuclease-free water, 5 µM of each primer, and 12.5 µL of SYBR Green Master Mix. ΔCt values were calculated by subtracting the GAPDH Ct from the mean Ct of the target genes (HSP90 and GPX1). ΔΔCt values were calculated by subtracting the ΔCt of the calibrator from that of the sample, and fold changes were determined using the 2^-ΔΔCt method. Gene expression levels in treated samples were compared, with all assays performed in triplicate.

2.10 ~~Datamaining and~~ ADMET and Quantitative Structure-Activity Relationship (QSAR) of Bioactive Compound ~~of Bioactive compound~~

The bioactive compounds analyzed in this study were derived from extracts of the sea cucumber *~~Holothuria~~ lessoni*. Compound identification was performed using GC--MS, and the SMILES notation for each detected compound was retrieved from the PubChem database

(NCBI, USA). Drug-likeness, pharmacodynamic properties, and toxicity profiles were evaluated for each ligand following Lipinski's Rule of Five [42##]. These assessments were conducted using ADMETLab 2.0 (Center for Drug Discovery and Development, Shanghai Jiao Tong University, China) and Protox 3.0 (Institute of Molecular Biology and Biophysics, Charité–Universitätsmedizin Berlin, Germany), with SMILES serving as the primary input for both platforms. The ADMET evaluation demonstrated that most compounds possessed favorable pharmacokinetic and pharmacodynamic characteristics, although several exhibited potential toxicity when considered as isolated compounds.¶

H. lessoni ~~Sea cucumber~~ compounds that successfully passed the ADMET screening were subsequently evaluated for their potential using the Way~~WAY~~2Drug~~RUG~~ PASS Online prediction ~~webserver~~tool (Way2Drug Group, Institute of Biomedical Chemistry, Moscow, Russia). This evaluation focused on determining their capacity to support IVF and IVM based on the Probability to Be Active ~~Pa (Probability to Be Active~~Pa) value. The Pa value was incorporated into the data-mining process because it indicates the predicted activity potential of each compound analyzed.

2.11~~0~~ Prediction of Protein Targets and Network Analysis

Prediction of protein targets and gene–disease associations was carried out by analyzing protein targets using the SEA Search Server (Similarity Ensemble Approach; Keiser Laboratory, University of California San Francisco, USA), the Comparative Toxicogenomics Database (CTD; Mount Desert Island Biological Laboratory, USA), and Swiss Target Prediction (Swiss Institute of Bioinformatics, Switzerland), then comparing them with lists of proteins or genes associated with oxidative stress, endoplasmic reticulum stress, female infertility, oocyte maturation defects, and oocyte, zygote, and embryo maturation arrest from Open Targets (Open Targets Consortium, UK). This process produced a set of overlapping genes identified through Venn diagram analysis, which was visualized using JVenn (INRAE, France). The compiled protein targets were then entered into the STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins; European Molecular Biology Laboratory, EMBL, Germany) with the organism set to *Homo sapiens*, network edges set to confidence, and the minimum required interaction score set to 0.900. The resulting network was exported in TSV format and further analyzed in Cytoscape (Institute for Systems Biology, Seattle, USA) to identify protein–protein interactions using degree, betweenness centrality and closeness centrality to determine the most influential proteins in the protein–protein interactions (PPI) ~~PPI~~ network. Gene Ontology (GO) analysis using DAVID (Database for Annotation, Visualization and Integrated Discovery; National Institute of Allergy and Infectious Diseases, NIH, USA) showed that the potential protein targets of the sea cucumber extract play important roles in regulating ROS, oxidative stress, endoplasmic reticulum stress, as well as hormone regulation and cell growth. Meanwhile, the mechanisms of action of the bioactive compounds were identified using CTD and visualized in Cytoscape to map the modes of action relevant to IVF and IVM processes.¶

~~GC-MS results were used to identify bioactive compounds and analyze potyential protein interactions. The DisGeNET (Integrative Biomedical Informatics Group, GRIB/IMIM/UPF;~~

~~https://www.disgenet.org) and Open Targets Platform (EMBL-EBI and collaborators; https://platform.opentargets.org) databases were used to identify proteins and genes asociated with ag-related infertility. Protein–protein interaction (PPI) networks were constructed and analyzed using the STRING database v11.5 (Search Tool for the Retrieval of Interacting Genes/Proteins; ELIXIR Core Data Resource; https://string-db.org). Several key features, including strength, degree, betweenness centrality, and closeness centrality, were evaluated for network analysis. Degree centrality represents the total number of direct connections a protein has within the network; higher values suggest that a protein may function as a major hub or regulator. Betweenness centrality measures how frequently a protein lies on the shortest paths between other proteins, indicating its potential role as a connector or mediator. Closeness centrality reflects how quickly a protein can reach all other proteins in the network, with higher values indicating greater centrality and potential regulatory importantance.¶~~

3. RESULTS

3.1 GC-MS Study

The GC-MS results (Table 1) demonstrate the high chemical complexity of the *H. lessoni* extract, consisting primarily of steroid- and ester-type compounds, along with minor amounts of glycerol detected at early retention times. Quantitatively, the major constituents were: propane, 1,2,2-trichloro-1,3,3,3-pentafluoro- (13.94%); 9-octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester (5.73%); carbonic acid, but-3-en-1-yl eicosyl ester (5.83%); phthalic acid, hexyl 1-phenylpropyl ester (5.87%); and citronellal (3.04%). Many of these compounds, particularly unsaturated fatty acids, fatty acid esters, squalene, and siloxane derivatives, have been reported to exhibit antioxidant, anti-inflammatory, or cytoprotective properties. This supports the hypothesis that *H. lessoni* extract may exert protective effects on embryo development, consistent with observations from other parts of this study.

Table 1. Representative GC-MS chromatogram of freeze-dried sea cucumber extract.

3.2 In Vitro Study

The bar charts in Figure 1 show the embryonic development of aged mouse oocytes at *H. lessoni* extract concentrations of 0 ppm (control), 15 ppm, 20 ppm, and 25 ppm. The y-axis represents extract concentration, while the x-axis shows the percentage of embryos formed. Both the 2-cell and blastocyst stages showed a fluctuating trend across the different concentrations with blastocyst formation consistently lower than the 2-cell stage. The 2-cell stage reached 83% in the control group, declined to 71% at 15 ppm, peaked at 100% at 20 ppm, and then decreased to 89% at 25 ppm. Blastocyst development, was 25% in the control group (0 ppm) increasing slightly to 29% at 20 ppm, and then fell sharply to 8% at 25 ppm.

Figure 1. Embryonic development of aged mouse oocytes at different *H. lessoni* extract concentrations. The bars represent the developmental competence of aged oocytes at different 2-cell and blastocyst embryonic stages following treatment at different concentrations (0 ppm, 15 ppm, 20 ppm, 25 ppm) of the tested extract.

Figure 2 shows embryonic development at extract concentrations of 0 (Fig. 2.1), 20 (Fig. 2.2), and 25 ppm (Fig. 2.3), focusing on two embryonic stages: the 2-cell stage and the blastocyst stage. Panel (a) shows embryos at the 2-cell stage (representing early post-fertilization cleavage) across all extract concentrations. In the control group (0 ppm; Fig. 2.1), a blastocyst is visible within the zona pellucida, exhibiting heterogeneous cytoplasm, a clearly defined blastocoel cavity, filled with transparent fluid. Embryos exposed to the extract, on the other hand, generally display blastomeres with more homogeneous cytoplasm. In the control, (0 ppm) the inner cell mass (ICM) appeared as a compact cluster of cells localized to one side, and partial protrusion of the blastocyst through the zona pellucida was evident, indicating the onset of hatching. A smaller blastocyst enclosed within an intact zona pellucida, was also visible, representing a slightly earlier developmental stage than the first.

At 15 ppm, embryos generally displayed good morphology, characterized by normal-shaped blastomeres, clear cellular structure, and relatively even cleavage patters. Blastocysts showed a well-formed blastocoel cavity, an intact the zona pellucida with acceptable limits, and minimal cytoplasmic vacuolization, suggesting that a concentration of 15 ppm supports the maintenance of embryo developmental competence to an adequate level.

At 20 ppm (Fig. 2.2), several compact blastocysts showed densely arranged cells (located at the top left, centre left, and lower left of the panel) with little or no visible blastocyst cavity, and the ICM is not clearly distinguishable from the trophoblast. In partially expanded blastocysts, the blastocoel cavity begins to form and fill with fluid, the ICM becomes displaced towards one side, and the trophoblast cells are more clearly visible as a single cell layer. Overall, the blastocyst treated at 20 ppm show the most optimal morphology, with a wider and well-filled blastocoel cavity, more regular cell distribution, and a more clearly defined trophectoderm (TE) and ICM.

Blastocysts treated at 25 ppm (Fig. 2.3) were examined four days after ~~fertilisation~~fertilization. The zona pellucida remained intact and clearly defined. Although the blastocoel cavity had begun to form and contained cytoplasm, it was not yet fully expanded. Many blastomeres exhibited cytoplasmic vacuolization and increased granularity, with indications of cellular degeneration. At this highest concentration, most embryos only progressed to an early blastocyst stage, with a small cavity and uneven cell distribution. The ICM remained poorly defined, the TE appeared relatively thickened, and the overall morphology indicated impaired and delayed development.

Figure 2. Developmental competence of aged oocytes at different embryonic stages following treatment with various concentrations of the tested compound: 0 ppm (1), 20 ppm (2), and 25 ppm (3), and evaluated at different culture times: 24 h (a), 48 h (b), and 72 h (c)

Table 2. Blastocyst Position and Gardner Morphological Grades

Blastocyst quality assessment was performed using the Gardner blastocyst grading system [22], which includes determination of blastocyst expansion (stage 1–6), ICM quality

(A–C), and TE quality (A–C). These three parameters are then integrated into a composite score. Treatment with *H. lessoni* extract resulted in differences in blastocyst grade distribution at different extract concentrations. In the control group (0 ppm), blastocysts predominantly exhibited intermediate expansion (stage 2–3), with ICM and TE quality ranging from grades B to C. The most frequent composite scores were 3BC and 2CC, indicating blastocysts with a formed blastocoel cavity but reduced ICM integrity and a relatively sparse TE layer. This morphology is consistent with the limited developmental competence typically seen in aged oocytes without intervention.

The 20 ppm treatment group showed improved morphological quality according to the Gardner criteria. Most blastocysts reached stage 3 (expanded blastocyst) with ICM grade B and TE grades B–C, resulting in dominant composite scores of 3BB and 3BC. These findings suggest that moderate extract concentration not only increased the proportion of blastocysts formed, but also supported the development of a more compact ICM and a more organized TE layer compared with controls. In contrast, blastocyst quality declined at concentrations of 25 ppm. Most embryos remained at stage 2–3 with lower ICM and TE grades (ICM B–C and TE C), resulting in frequent low-quality composite scores such as single as 2CC, with only a single higher-grade 3BB blastocyst observed. These findings are consistent with evidence of cellular degeneration and morphological disruption at the highest concentration, reflected by reduced ICM and TE quality according to the Gardner criteria.

3.3 Effect of the Extract on the mRNA Expression Levels of Target Genes

The effect of *H. lessoni* extract on relative mRNA expression of HSP90 and GPX1 in blastocysts shown in Figure 3. Aged oocytes were subjected to in vitro maturation and ~~fertilisation~~fertilization in the presence of *H. lessoni* extract at concentrations of 0 ppm (control), 20 ppm, or 25 ppm, and embryos were cultured to the blastocyst stage. Total RNA was isolated from resulting blastocysts, reverse-transcribed into cDNA, and ~~analysed~~analyzed using qRT-PCR. Each reaction contained 200 ng of cDNA, and expression levels of HSP90 and GPX1 were ~~normalised~~normalized to the housekeeping gene GAPDH. At 20 ppm, both HSP90 and GPX1 were markedly upregulated, whereas at 25 ppm, GPX1 expression increased further while HSP90 expression declined to below baseline levels.

Figure 3. Effect of *H. lessoni* extract on relative mRNA expression of HSP90 and GPX1 in blastocysts. Bars represent fold change in gene expression relative to the untreated control group (set to 1-fold).

3.4 In Silico Study

ADMET and ~~Quantitative Structure-Activity Relationship (QSAR)~~ of Bioactive Compound¶

The analysis showed that 18 of 20 *H. lessoni* ~~sea cucumber~~ bioactive compounds met Lipinski's Rule of Five, including limits for molecular weight, LogP, hydrogen bond donors and acceptors, and TPSA. ADMET results indicated that most compounds had good pharmacokinetic and pharmacodynamic properties. However, some showed potential liver

toxicity or carcinogenic risks, and Pyrrolo[1,2--a]pyrazine--1,4--dione, hexahydro was classified as toxicity class 3. Based on these safety concerns, three compounds were excluded: Pyrrolo[1,2--a]pyrazine-1,4-dione, hexahydro; Tetracosamethyl-cyclododecasiloxane; and Heptasiloxane, hexadecamethyl.¶

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Table 3. -Results of drug-likeness and ADMET analysis of potential compounds¶

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The bioactive potential of *H. lessoni* ~~sea cucumber~~ compounds was evaluated using the Way2Drug PASS ~~Server~~tool. A Pa value above 0.3 indicates that a compound may support IVF procedures due to its strong structural similarity to database compounds known to produce related therapeutic effects (Figure 4). Ten compounds showed average Pa values greater than 0.3 (green–yellow). The Pa ~~(Probability to Be Active)~~ score reflects the predicted activity of each tested compound and is determined by comparing the structure of the input compound (from the *H. lessoni* ~~sea cucumber~~ extract) with compounds previously confirmed to provide specific treatment effects~~s~~, either through computational prediction (Pa > 0.3) or experimental validation (Pa > 0.7). Based on these predicted activities, the *H. lessoni* ~~sea cucumber~~ compounds appear to have strong potential as female anti-infertility agents, antioxidants, free-radical scavengers, oxygen scavengers, nitric oxide scavengers, and peroxidase inhibitors.¶

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Figure 4. Quantitative Structure-Activity Relationship Analysis of Bioactive Compound. The heatmap shows the correlation between various chemical compounds (right) and different biological activities (top). The color scale represents correlation strength, ranging from low (blue) to high (yellow). ¶

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Prediction of Protein Targets and Network Analysis¶

The predicted target proteins of sea cucumber compounds from the SEA Target, CTD, and Swiss Target databases were compared with proteins linked to several stress conditions and reproductive disorders from the Open Target database. Venn analysis found six shared proteins also confirmed, namely NFE2L2, HSF1, HSP90AA1, HSP90B1, HSP90AB1, and GPX1. Another 2,430 proteins overlapped between the predicted targets and open target database.¶

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Figure 5. Venn diagram and list-size comparison of predicted target proteins. The Venn diagram shows the overlap among protein targets from GDA, *H. lessoni*~~seacucumber~~ compound predictions, and proteins of interest. Six proteins were shared across all three datasets. The bar chart below displays the total number of proteins in each dataset.¶

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In the graph topological network analysis, we used the overlapping proteins from the Venn diagram and processed them through the STRING server. The network analysis applied several variables that describe the PPIs~~relationships among the protein targets, or protein-protein interactions (PPI)~~. These variables included Degree, Betweenness Centrality, and Closeness Centrality. The analysis showed that 209 potential proteins were involved in PPIs with a minimum required interaction score of 0.900 (highest confidence) (Figure 6a). Based on

betweenness centrality, the top 20 most promising protein targets were ~~also~~further identified (Figure 6b).¶

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Figure 6. Protein–protein interaction (PPI) networks of predicted sea cucumber bioactive compound targets linked to IVF and IVM. (a) Global STRING-based PPI network of 209 high-confidence protein targets, showing overall connectivity. Darker purple and larger nodes mark proteins with higher centrality. (b) Subnetwork showing the top 20 proteins ranked by betweenness centrality, highlighting their key roles in pathways related to oxidative stress, cell survival, and implantation.¶

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The GO annotation of the most promising target proteins and genes was analyzed to determine their functions and their roles in pathways associated with IVF treatment. Only functional and pathway annotations with a False Discovery Rate (FDR) < 0.05 were included. An FDR of 0.05 indicates that up to 5% of the statistically significant results may be false positives. The GO analysis showed that the potential sea cucumber target genes play key roles in biological processes related to ROS regulation, oxidative stress, endoplasmic reticulum stress, hydrogen peroxide, nitric oxide, redox balance, and hormone regulation, including estradiol and estrogen (Figure 7). ¶

~~Figure 4 presents a PPI network depicting oxidative stress pathways the context of IVF, constructed based on predicted target proteins of *H. lessoni* extract and endogenous proteins involved in oxidative stress–related pathogenesis. Each node represents a protein, and each edge denotes a documented interaction. The key regulatory proteins, TP53, TNF, and IL1B, are shaded in purple, indicating their central roles and high degree of connectivity within the network. These hub proteins are critically involved in apoptotic regulation, inflammatory signaling, and antioxidative responses. Their dense interactions with other proteins such as CASP3, BAX, SOD1, HMOX1, and ESR family members suggest a tightly integrated network governing the balance between oxidative damage and cellular defense mechanisms, which may influence oocyte maturation and embryo development during IVF.~~¶

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Figure ~~Figure~~ 74. Gene Ontology (GO) annotation of the most potential sea cucumber target proteins associated with IVF and IVM treatments. Enriched Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) categories were identified. ~~Protein interaction network related to oxidative stress pathways derived from predicted targets of *H. lessoni* extract in the context of IVF.~~

These potential proteins are also involved in growth-related processes such as embryonic placenta development, cell differentiation, and the mitotic cell cycle. When comparing target proteins, HSP90AA1, HSP90AB1, HSP90B1, BCL2, HSPA1A, HSPA1B, JUN, NFE2L2 (Nrf2), AKT1, EGFR, ESR1, and GPX1 were identified as the most frequently involved and dominant proteins in IVF and IVM treatment (Figure 8).¶

~~Figure 5 presents a GO biological process enrichment analysis of the predicted protein targets of the *H. lessoni* extract. The bar and bubble plot summarizes the top enriched biological~~

~~processes associated with the predicted protein targets, including pathways involved in oxidative stress response, regulation of apoptosis, inflammatory signaling, cell proliferation, and reproductive and embryonic developmental processes. The figure illustrates how the *H. lessoni* extract's target proteins are functionally clustered in biological processes that support oocyte quality, fertilization, implantation, and early embryo development, supporting the extract's potential protective and modulatory effect during IVF.~~¶

Figure ~~8~~5. Key protein targets identified. Proteins such as HSP90AA1, HSP90AB1, HSP90B1, BCL2, HSPA1A, HSPA1B, JUN, NFE2L2, AKT1, EGFR, ESR1, and GPX1 show major roles in processes linked to oxidative stress, cell survival, hormone ~~signaling~~signalling, and developmental competence, highlighting their value as indicators and molecular targets in IVF and IVM.~~Gene Ontology (GO) biological process enrichment analysis of the predicted target proteins of *H. lessoni* extract relavent to oocyte and embryo development.~~

Based on key roles in BP, CC, MF, and KEGG pathways for IVF and IVM, we identified 18 proteins that directly interact with the most promising compounds from *H. lessoni* ~~sea cucumber~~ extract. These proteins fall into four groups: oxidative stress protection, antioxidant defense, cell survival, and implantation and growth signaling. We also mapped their mechanisms of action with four matching compounds. Among these compounds, b~~B~~is (2-ethylhexyl) phthalate (DEHP) shows the strongest potential by activating HSP90AA1, HSP90AB1, HSP90B1, GPX1, and NFE2L2. Citronellal, Squalene, and 2-Methyl-Z,Z-3-13-octadecadienol also provide protective effects by regulating BCL2, AKT1, and ESR1.¶

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Figure 9. Mechanism of action of *H. lessoni* ~~sea cucumber~~ extract compounds on proteins associated with IVF and IVM. Each protein is represented by a specific shape to indicate its biological function within the pathway: hexagon for oxidative stress protection, octagon for antioxidant defense, diamond for cell survival regulation, and ellipse for implantation and growth signal regulation. Blue arrows indicate activation or upregulation, red arrows indicate inhibition or downregulation, and green arrows denote potential regulatory effects on the corresponding protein targets.¶

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4. Discussion

GC–MS analysis revealed that extract of the sea cucumber *H. lessoni* is rich in unsaturated fatty acids, squalene, organosilicon derivatives, and other compounds with reported antioxidant and cytoprotective properties, supporting its potential to counteract age-related oxidative stress in oocytes and embryos. Concentration at 20 ppm significantly improved early cleavage, blastocyst formation, and overall embryo morphology relative to controls, whereas higher concentrations in 25 ppm caused degeneration and reduced developmental rates, indicating a bell-shaped dose–response effect . These findings align with previous observations that moderate antioxidant exposure enhances embryo quality, whereas excessive antioxidant stimulation can become detrimental. ~~Among the tested concentrations of extract, supplementation at 20 ppm yielded the most favorable outcomes with enhanced early cleavage (100% vs. 83% in controls) and blastocyst rate (29% vs. 25%). In contrast, 15 ppm maintained, but did not optimize, embryonic development, while exposure tp 25 ppm~~

~~reduced both 2-cell (89%) and blastocyst (8%) rates, indicating a bell-shaped dose–response relationship. Morphological assessment supported this pattern, as treatment with 20 ppm extract developed into the most expanded and structurally intact blastocysts with well-organized ICM and TE, whereas 25 ppm was associated with cytoplasmic degeneration, vacuolization, and low-grade blastocysts. Consistent with these findings, in silico PPI analysis supports that the extract exerts multi-target effects involving oxidative stress regulation, apoptic signaling, and inflammatory pathways.~~

The findings of this study demonstrate that *H. lessoni* extract modulates the developmental competence and stress-response profile of aged murine oocytes in a dose-dependent manner. The present findings are consistent with previous reports showing that marine-derived extracts and individual marine lipids can improve oocyte and embryo quality by attenuating oxidative stress and modulating apoptotic signaling in mammalian reproductive models [15,23]. A similar dose-dependent effect has been reported for the antioxidant quercetin where supplementation was found to improve early embryogenesis but higher concentrations became neutral or detrimental [24]. The observed increase in blastocyst quality and ICM and TE organization at the optimal extract concentration is also consistent with a previous study that showed how targeted antioxidant support enhanced blastocyst morphology and Gardner grading under oxidative stress conditions [25]. Moreover, up-regulation of stress-responsive chaperones and antioxidant enzymes has been repeatedly associated with improved embryo survival in aging or oocytes under oxidative stress conditions, while dysregulated expression of these genes correlates with developmental arrest and apoptosis [11]. Collectively, our findings are broadly in agreement with the existing literature and extend it by linking the GC–MS-defined phytochemical profile of *H. lessoni* extract to coordinated changes in morphology, blastocyst grading, gene expression, and in silico stress-related networks in aged mouse oocytes. ¶

The GC–MS profile indicates that the *H. lessoni* extract contains multiple compounds capable of scavenging ROS, stabilizing cellular membranes, and modulating inflammatory and apoptotic signaling, including unsaturated fatty acid esters, squalene, and steroid-like molecules [13,26]. In aged oocytes, which are characterized by elevated ROS levels, mitochondrial dysfunction, and impaired proteostasis, such compounds likely improve the intracellular redox environment, preserve organelle integrity, and reduce oxidative damage to DNA, lipids, and proteins. Fatty acid ester can be incorporated into mitochondrial membranes, where they modulate key anabolic signaling pathways~~, notably mTORC1/p70S6K and AMPK/PGC1-α/PPAR-γ, thereby~~ promoting mitochondrial biogenesis via upregulation of transcriptional regulators such as PGC1-α, TFAM, and Nrf1 [27]. Squalene has been described as an antioxidant that directly scavenges free radicals, reduces intracellular ROS, and prevents H_2O_2-induced oxidative and DNA damage [28]. ~~In a zebrafish model of copper sulfate-induced inflammation, squalene was found to upregulate mRNA expression of key antioxidant enzymes in Zang's (2023) study about antioxidant properties [28].~~ Collectively, these bioactive compounds likely act as ROS scavengers and membrane protectants, improving the intracellular redox environment, preserving mitochondrial and ER integrity, and limiting oxidative damage to DNA, lipids, and proteins in aging oocytes and embryos [29]. ¶

The *in vitro* results showed that ~~treatment~~ treatment at 20 ppm led to ~~–~~significant upregulation of both HSP90 and GPX1, whereas treatment at 25 ppm induced excessive GPX1 expression but was accompanied by a decrease ~~–~~in HSP90. This interpretation is consistent with the findings of Önay Uçar et al.~~–~~, who demonstrated that HSP90 helps cells survive by protecting them from stress [30]. Additionally, Hu et al. reported that long-chain bases (LCBs) from sea cucumber reduced ER stress by targeting oxidative, UPR, and inflammatory pathways [31]. In contrast, at an extract concentration of 25 ppm, the molecular profile indicateds a dysregulated stress response, in which increased GPX1 induction ~~is~~was not matched by sufficient chaperone capacity due to decreased HSP90 expression. This imbalance may promote the accumulation of misfolded proteins, prolong ER stress, and activate apoptosis, thereby compromising embryonic development beyond early cleavage. These molecular alterations are reflected in the marked reduction in blastocyst formation rate, deterioration of blastocyst morphology, and Gardner grading at the highest dose.¶

Among the compounds analyzed, ~~B~~bis(2-ethylhexyl) phthalate (DEHP) showeds the strongest predicted activity. DEHP activates HSP90 family proteins, which help stabilize proteins ~~needed~~required for spindle formation, cell cycle regulation, and stress resistance ~~-~~[34,~~‖~~35]. It also activates GPX1 and NFE2L2 (Nrf2), major regulators of antioxidant defense, suggesting that DEHP helps protect oocytes from oxidative and protein~~-~~related stress ~~-~~[36]. DEHP acts as an indirect antioxidant by triggering several protective molecular pathways highlighted in the network. DEHP strongly activates HSP90 proteins, which maintain proteostasis by stabilizing client proteins during oxidative stress and supporting survival pathways involving AKT1 and BCL2. Through these interactions, DEHP helps preserve mitochondrial integrity and reduces ROS~~-~~induced damage. It also stimulates the Nrf2~~NFE2L2~~ pathway, increasing expression of key antioxidant enzymes such as GPX1 and HMOX1, which lower peroxide levels and protect mitochondrial membranes ~~-~~[37]. ¶

In addition, DEHP shows strong connections with major regulators of the ~~unfolded protein response (~~UPR~~)~~ and ER stress. Central nodes such as HSP90AA1, HSP90AB1, HSP90B1, HSPA5, and HSPA1B are essential ER chaperones involved in managing misfolded proteins ~~-~~[38]. This suggests that reducing oxidative stress may also decrease~~s~~ the accumulation of misfolded proteins, thereby relieving ER stress and stabilizing the UPR system ~~-~~[39]. On the other hand, the bioactive compound squalene acts as a strong antioxidant, as shown in the quantitative structure analysis conducted in this in silico study. Squalene is~~'s~~ connected~~ion~~ to AKT1, an important ~~key~~ regulator of cell survival. At the membrane level, squalene embeds in the lipid bilayer and absorbs singlet oxygen and other radicals. This prevents lipid peroxidation and protects mitochondrial and oocyte membranes, helping maintain organelle structure and function. Squalene also activates anti~~-~~apoptotic signaling through BCL2, reduces mitochondrial membrane damage, and limits ROS~~-~~induced cell death, adding another layer of protection ~~-~~[40]. Other compounds, such as citronellal and 2-methyl-Z,Z-3-13-octadecadienol~~2-methyl-Z,Z-3-13-octadecadienol~~, support cell survival and implantation signaling by regulating BCL2, AKT1, and ESR1 ~~-~~[41]. These targets help prevent apoptosis, maintain oocyte viability, and support hormone-related functions during maturation.¶

~~At the molecular level, treatment at 20 ppm resulted in robust upregulation of HSP90 and GPX1, whereas 25 ppm induced excessive GPX1 expression but was accompanied by a reduction in HSP90. This pattern suggests that a balanced activation of proteostasis and redox~~

~~defenses, rather than maximal stimulation of a single pathway, is essential for optimal embryonic development. This interpretation is consistent with the findings of Önay Uçar et al. (2023), who demonstrated that HSP90 helps cells survive by protecting them from stress [30]. Additionally, Hu et al. reported that long-chain bases (LCBs) from sea cucumber reduced ER stress by targeting oxidative, UPR, and inflammatory pathways [31]. LCBs downregulate the ER chaperone GRP78/Bip and suppress its main UPR branches, as shown by decreased hepatic ATF6, PERK, and XBP1 expression and reduced phosphorylation of eIF2α and IRE1α [31]. The concomitant up-regulation of HSP90 and GPX1 observed at the optimal *H. lessoni* extract suggests a coordinated reinforcement of proteostasis and antioxidant defenses. HSP90 has been reported to facilitate correct protein folding and protects against ER stress, while GPX1 reduces peroxides and limits ROS-induced damage [32,33]. Consistent with this model, the PPI network analysis highlighted central regulatory nodes such as TP53, TNF, IL1B, CASP3, and BAX, supporting a multi-target modulatory effect of the extract on oxidative stress and apoptosis-related pathways that influence oocyte and embryo survival. In contrast, at an extract concentration of 25 ppm, the molecular profile indicates a dysregulated stress response, in which increased GPX1 induction is not matched by sufficient chaperone capacity due to decreased HSP90 expression. This imbalance may promote the accumulation of misfolded proteins, prolong ER stress, and activate apoptosis, thereby compromising embryonic development beyond early cleavage. These molecular alterations are reflected in the marked reduction in blastocyst formation rate, deterioration of blastocyst morphology, and Gardner grading at the highest dose.~~ ¶

~~Several limitations should be considered. First, "aged" status was defined primarily by chronological age, and ovarian reserve and oocyte quality were not independently quantified using standardized markers (e.g., spindle integrity, mitochondrial function, baseline ROS). Second, only a small concentration range was tested, and exposure was limited to early development; broader dose–response and time-course studies are needed to better define the therapeutic window and toxicity threshold. Second, outcomes focused on cleavage/blastocyst rates and morphology; implantation potential, fetal development, live birth, and offspring health were not assessed. Third, mechanistic interpretation is constrained because only two transcripts (HSP90, GPX1) were measured without protein-level confirmation or functional assays. Future work should include direct measures of ROS, mitochondrial activity, ER stress/UPR markers (GRP78/BiP, ATF6/PERK/XBP1), and apoptosis (caspase activity/TUNEL), alongside protein quantification and enzyme activity. Fourth, although GC–MS indicated chemical complexity, active constituents were not identified. Orthogonal LC–MS/MS, blanks, and bioassay-guided fractionation are needed. Finally, in silico network findings are hypothesis-generating and require experimental validation. Collectively, future studies should optimize dosing, identify active compounds, validate mechanisms, and test post-blastocyst endpoints including embryo transfer.~~

Compared with other marine species, sea cucumbers contain exceptionally high levels of triterpenoid saponins and sulfated polysaccharides, which exhibit strong antioxidant and membrane-protective activities. These compounds, which are rare or absent in most aquatic organisms, modulate ER stress, UPR signaling, and mitochondrial stability, helping to explain the superior bioactivity of *H. lessoni*. In addition, *H. lessoni* provides distinctive saponins and

triterpenes that stabilize cellular membranes and lessen ROS-induced damage, while long-chain bases further reduce ER stress and inflammation. Together, these components restore redox balance and ER homeostasis, improving oocyte quality. Oocyte developmental competence relies on tightly regulated ER proteostasis and mitochondrial oxidative phosphorylation. ER stress disrupts spindle formation and chromosome alignment, whereas impaired mitochondrial function limits the ATP required for successful cleavage. This unique combination of bioactive molecules supports the observed benefits of 20 ppm *H. lessoni* extract on early cleavage, blastocyst development, and the regulation of stress-response genes, linking the species' chemical profile to its enhanced effects in IVF. Accordingly, the observed changes in HSP90 and GPX1 expression reflect the molecular recovery of these two essential systems.¶

~~Taken together, the findings of our study indicate that *H. lessoni* extract functions as a complex, multi-component modulator of aged oocyte competence. At an optimal concentration, its phytochemical constituents appear to rebalance redox status, enhance chaperone-mediated proteostasis, and attenuate pro-apoptotic and pro-inflammatory signaling, resulting in improved early cleavage, better blastocyst morphology, and higher-quality Gardner grades in aged oocytes. In contrast, higher extract concentrations disrupt this balance and are associated with molecular and morphological signatures of cellular stress and toxicity, emphasizing the importance of dose optimization.~~ Although these findings position *H. lessoni* extract as a promising adjuvant to support aged oocytes in IVF settings, the limited improvement beyond early developmental stages suggests that additional interventions targeting mitochondrial function, spindle integrity, and chromosomal stability will be required to fully overcome age-related reproductive decline.

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Declaration of Interest

The authors declare no competing interests

Author contributions

Each contributor played key roles in this research: IU & MP designed the study and contributed to data acquisition and analysis; BSL, WS, SP assisted in data acquisition, analysis, and manuscript revision; ZN was pivotal in data analysis, result interpretation, and manuscript revision; whilst TM & TY handled manuscript preparation and figure/table design.

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