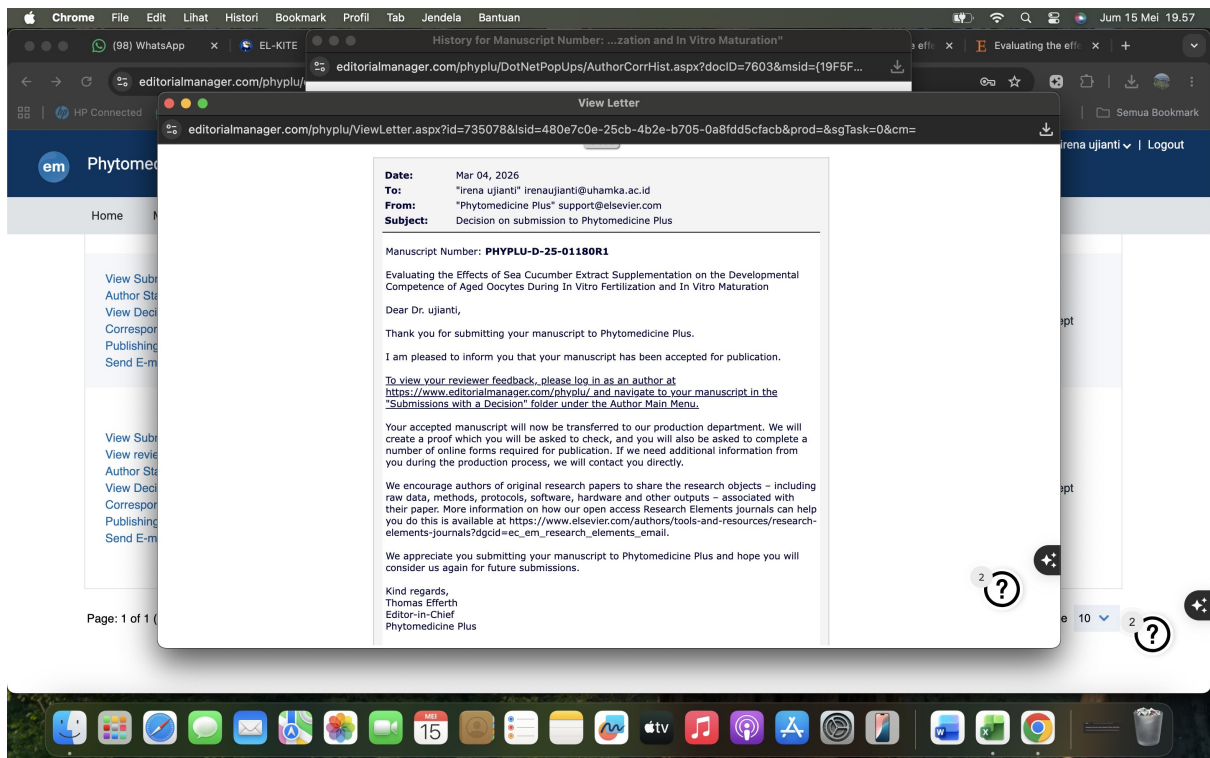
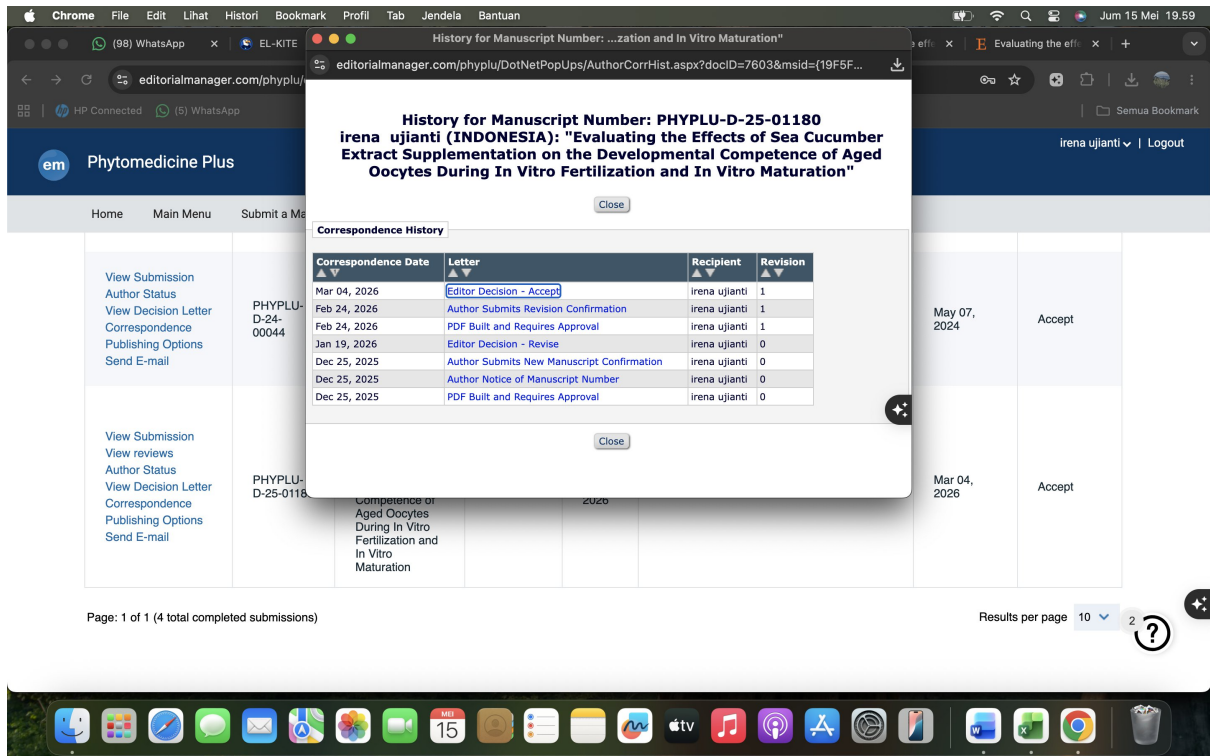


## Accapted Letter



## Corespondence



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 Ujjanti

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  $P_a > 0.3$ , 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  $\mu\text{m}$  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 seem 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.

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

4  
5 **Background:** Ovarian aging, characterized by declines in oocyte quantity and quality, results in  
6 reduced fertility, particularly in women over 38 years of age. Oxidative stress and endoplasmic  
7 reticulum (ER) stress are major contributors, necessitating strategies to support the developmental  
8 competence of aged oocytes. *Holothuria lessoni*, a sea cucumber *species* rich in bioactive compounds,  
9 offers antioxidant and cytoprotective potential that may help counteract these oxidative and ER stress-  
10 related declines.

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

17 **Results:** GC-MS revealed multiple bioactive constituents, including fatty acids, esters, squalene, and  
18 siloxane derivatives. A concentration of 20 ppm yielded the best developmental outcomes, with 100%  
19 of embryos reaching the two-cell stage and 29% forming blastocysts, along with superior blastocyst  
20 morphology, while 25 ppm reduced embryo quality. At 20 ppm, HSP90 and GPX1 were upregulated  
21 20-fold and 6.58-fold, respectively, whereas 25 ppm further increased GPX1 but decreased HSP90  
22 expression. Network analysis identified TP53, TNF, and IL1B as central regulators in oxidative stress-  
23 related pathways.

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

28  
29 **Keywords:** Oxidative stress; Antioxidants; Embryo development; HSP90 gene; GPX1 gene  
30  
31  
32  
33  
34

## 35 1. Introduction

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

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

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

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

90

## 91 **2. Materials and methods**

### 92 **2.1 Sample Collection and Extract Preparation**

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

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

112

### 113 **2.2 *H. lessoni* Extract Characterization**

114 Analysis was conducted using a gas chromatography–mass spectrometry (GC-MS)  
115 system (Thermo Fisher Scientific, Chromeleon 7, Version 7.2.10.24543). Chromatographic  
116 separation was conducted utilizing an HP-5MS UI capillary column with 30 m length, 0.25  
117 mm internal diameter, and 0.25 µm film thickness. The oven temperature was initially set at  
118 50°C, held for 2 min, then finally increased to 280°C, with a total run time of 58 minutes. The  
119 injector was utilized in split mode with a split flow of 50 mL/min as well as an inlet temp of

120 220°C. The carrier gas flow was kept at 1.0 mL/min, although the type of carrier gas was not  
121 specified, helium or nitrogen is primarily used in similar analyses. An injection volume of 1.1  
122 µL was delivered through a TriPlus RSH autosampler, which used a 10 µL syringe with a  
123 needle length of 57 mm. Pre- and post-injection wash cycles were set to 5 cycles to minimize  
124 carryover. For mass spectrometric detection, the transfer line temperature was kept at 250°C  
125 as while the ion source temperature was maintained at 200°C. Electron ionization (EI) was  
126 applied in positive polarity mode, and full-scan data were ~~acquired~~ acquired over an m/z span of  
127 50–650 at a scan time of 0.2 seconds. Quantification and identification were conducted using  
128 Chromeleon 7 software.

129

### 130 **2.3 Medium Preparation with ~~Sea cucumber~~ Sea Cucumber Extract**

131 *H. lessoni* extract was first dissolved in phosphate-buffered saline (PBS) to make a  
132 2500 ppm stock solution. This stock was then diluted with medium to obtain working  
133 concentrations of 15, 20, and 25 ppm. The control consisted only of medium without added  
134 extract. The PBS concentration in the final working solutions was kept below 0.1% by volume.

135

### 136 **2.4 Animal Handling**

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

142

143

### 144 **2.5 Oocyte Collection**

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

153

### 154 **2.6 In Vitro Fertilisation**

155 In vitro fertilisation of the collected COCs was conducted by incubation with  
156 spermatozoa retrieved from the cauda epididymidis of adult male mice, in small drops of  
157 fertilization medium with concentration of *H. lessoni* extract. The procedure was carried out  
158 under mineral oil and maintained at 37°C within a controlled gaseous environment comprising  
159 6% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> for 4 hours to optimize fertilization outcomes.

160

### 161 **2.7 Embryo Culture**

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

164 Biosciences) covered by paraffin oil (Ovoil, Vitrolife AB, Sweden), and maintained at 37°C  
165 in 6% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> in a time laps humidified multi-gas incubator (Sanyo MCO-  
166 5M[RC], Japan). For the first 24, 48, 72 96, and 120 hours, the embryos were assessed for  
167 developmental progression.

168

## 169 **2.8 RNA extraction and cDNA synthesis**

170 Blastocysts and two-cell-stage embryos were collected following treatment with  
171 ethanol extract at concentrations of 0, 15, 20, and 25 ppm. Total RNA from treated and  
172 untreated control samples was extracted using the PureLink™ RNA Mini Kit (Cat.:  
173 12183018A, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's  
174 instructions. The RNA concentration and purity were assessed with a NanoDrop™ 2000  
175 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), with acceptable 260/280  
176 nm absorbance ratios defined as 1.8 or higher. For cDNA synthesis, 1 µg of total RNA was  
177 reverse transcribed using the ABScript II cDNA First-Strand Synthesis Kit, according to the  
178 provided guidelines.

179

## 180 **2.9 qRT-PCR Analysis**

181 The amplified section for the Caspase-3 gene included part of the 5' upstream region.  
182 Primer sequences (Table 1) were obtained from the National Center for Biotechnology  
183 Information (NCBI) and designed using online primer-design tools. For glyceraldehyde-3-  
184 phosphate dehydrogenase (GAPDH), the forward primer was  
185 CATCACTGCCACCCAGAAGACTG and the reverse primer was  
186 ATGCCAGTGAGCTTCCCGTTCAG. For HSP90, the forward primer was  
187 TCATTATTCAGGGCTGCCGTGGTA and the reverse primer was  
188 TGGATGAACCAGGAGCCATCCTTT. For GPX1, the forward primer was  
189 CGCTCTTTACCTTCCTGCGGAA and the reverse primer was  
190 AGTTCAGGCAATGTCGTTGCGT.

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

201

## 202 **2.10 Datamaining and ADMET and Quantitative Structure-Activity Relationship** 203 **(QSAR) of Bioactive Compound of Bioactive compound**

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

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

215 [H. lessoni Sea cucumber](#) compounds that successfully passed the ADMET screening  
216 [were subsequently evaluated for their potential using the WayWAY2DrugRUG PASS Online](#)  
217 [prediction ~~webservertool~~ \(Way2Drug Group, Institute of Biomedical Chemistry, Moscow,](#)  
218 [Russia\)](#). This evaluation focused on determining their capacity to support IVF and IVM based  
219 [on the Probability to Be Active Pa \(~~Probability to Be Active~~Pa\) value](#). The Pa value was  
220 [incorporated into the data-mining process because it indicates the predicted activity potential](#)  
221 [of each compound analyzed](#).

222

## 223 **2.110 Prediction of Protein Targets and Network Analysis**

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

247 [GC-MS results were used to identify bioactive compounds and analyze potential protein](#)  
248 [interactions](#). The DisGeNET ([Integrative Biomedical Informatics Group, GRIB/IMIM/UPF;](#)

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

261

### 262 3. RESULTS

#### 263 3.1 GC-MS Study

264 The GC-MS results (Table 1) demonstrate the high chemical complexity of the *H. lessoni*  
265 extract, consisting primarily of steroid- and ester-type compounds, along with minor amounts of  
266 glycerol detected at early retention times. Quantitatively, the major constituents were: propane, 1,2,2-  
267 trichloro-1,3,3,3-pentafluoro- (13.94%); 9-octadecenoic acid (*Z*)-, 2,3-dihydroxypropyl ester  
268 (5.73%); carbonic acid, but-3-en-1-yl eicosyl ester (5.83%); phthalic acid, hexyl 1-phenylpropyl ester  
269 (5.87%); and citronellal (3.04%). Many of these compounds, particularly unsaturated fatty acids, fatty  
270 acid esters, squalene, and siloxane derivatives, have been reported to exhibit antioxidant, anti-  
271 inflammatory, or cytoprotective properties. This supports the hypothesis that *H. lessoni* extract may  
272 exert protective effects on embryo development, consistent with observations from other parts of this  
273 study.

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

275

#### 276 3.2 In Vitro Study

277 The bar charts in Figure 1 show the embryonic development of aged mouse oocytes at  
278 *H. lessoni* extract concentrations of 0 ppm (control), 15 ppm, 20 ppm, and 25 ppm. The y-axis  
279 represents extract concentration, while the x-axis shows the percentage of embryos formed.  
280 Both the 2-cell and blastocyst stages showed a fluctuating trend across the different  
281 concentrations with blastocyst formation consistently lower than the 2-cell stage. The 2-cell  
282 stage reached 83% in the control group, declined to 71% at 15 ppm, peaked at 100% at 20 ppm,  
283 and then decreased to 89% at 25 ppm. Blastocyst development, was 25% in the control group  
284 (0 ppm) increasing slightly to 29% at 20 ppm, and then fell sharply to 8% at 25 ppm.

285

286

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

291

292 Figure 2 shows embryonic development at extract concentrations of 0 (Fig. 2.1), 20  
293 (Fig. 2.2), and 25 ppm (Fig. 2.3), focusing on two embryonic stages: the 2-cell stage and the  
294 blastocyst stage. Panel (a) shows embryos at the 2-cell stage (representing early post-  
295 fertilization cleavage) across all extract concentrations. In the control group (0 ppm; Fig. 2.1),  
296 a blastocyst is visible within the zona pellucida, exhibiting heterogeneous cytoplasm, a clearly  
297 defined blastocoel cavity, filled with transparent fluid. Embryos exposed to the extract, on the  
298 other hand, generally display blastomeres with more homogeneous cytoplasm. In the control,  
299 (0 ppm) the inner cell mass (ICM) appeared as a compact cluster of cells localized to one side,  
300 and partial protrusion of the blastocyst through the zona pellucida was evident, indicating the  
301 onset of hatching. A smaller blastocyst enclosed within an intact zona pellucida, was also  
302 visible, representing a slightly earlier developmental stage than the first.

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

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

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

324  
325 Figure 2. Developmental competence of aged oocytes at different embryonic stages following  
326 treatment with various concentrations of the tested compound: 0 ppm (1), 20 ppm (2), and 25 ppm (3),  
327 and evaluated at different culture times: 24 h (a), 48 h (b), and 72 h (c)

328  
329  
330  
331  
332 Table 2. Blastocyst Position and Gardner Morphological Grades  
333

334  
335 Blastocyst quality assessment was performed using the Gardner blastocyst grading  
336 system [22], which includes determination of blastocyst expansion (stage 1–6), ICM quality

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

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

356

### 357 **3.3 Effect of the Extract on the mRNA Expression Levels of Target Genes**

358

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

368

369

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

373

374

### 375 **3.4 In Silico Study**

#### 376 ADMET and Quantitative Structure-Activity Relationship (QSAR) of Bioactive 377 Compound¶

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

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

386 ¶

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

388 ¶

389 The bioactive potential of *H. lessoni sea cucumber* compounds was evaluated using the  
390 Way2Drug PASS [Server](#)tool. A Pa value above 0.3 indicates that a compound may support  
391 IVF procedures due to its strong structural similarity to database compounds known to produce  
392 related therapeutic effects (Figure 4). Ten compounds showed average Pa values greater than  
393 0.3 (green–yellow). The Pa ([Probability to Be Active](#)) score reflects the predicted activity of  
394 each tested compound and is determined by comparing the structure of the input compound  
395 (from the *H. lessoni sea cucumber* extract) with compounds previously confirmed to provide  
396 specific treatment [effectss](#), either through computational prediction (Pa > 0.3) or experimental  
397 validation (Pa > 0.7). Based on these predicted activities, the *H. lessoni sea cucumber*  
398 compounds appear to have strong potential as female anti-infertility agents, antioxidants,  
399 free-radical scavengers, oxygen scavengers, nitric oxide scavengers, and peroxidase inhibitors.¶

400 ¶

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

404 ¶

405 ¶

#### 406 **Prediction of Protein Targets and Network Analysis¶**

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

413 ¶

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

418 ¶

419 ¶

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

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

428 ¶  
429 Figure 6. Protein–protein interaction (PPI) networks of predicted sea cucumber bioactive compound  
430 targets linked to IVF and IVM. (a) Global STRING-based PPI network of 209 high-confidence  
431 protein targets, showing overall connectivity. Darker purple and larger nodes mark proteins with  
432 higher centrality. (b) Subnetwork showing the top 20 proteins ranked by betweenness centrality,  
433 highlighting their key roles in pathways related to oxidative stress, cell survival, and implantation.

434 ¶  
435 ¶  
436 The GO annotation of the most promising target proteins and genes was analyzed to  
437 determine their functions and their roles in pathways associated with IVF treatment. Only  
438 functional and pathway annotations with a False Discovery Rate (FDR) < 0.05 were included.  
439 An FDR of 0.05 indicates that up to 5% of the statistically significant results may be false  
440 positives. The GO analysis showed that the potential sea cucumber target genes play key roles  
441 in biological processes related to ROS regulation, oxidative stress, endoplasmic reticulum  
442 stress, hydrogen peroxide, nitric oxide, redox balance, and hormone regulation, including  
443 estradiol and estrogen (Figure 7).

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

454 ¶

455 ¶

456 ¶

457 ¶

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

462

463

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

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

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

477  
478

479 **Figure 85.** Key protein targets identified. Proteins such as HSP90AA1, HSP90AB1, HSP90B1,  
480 BCL2, HSPA1A, HSPA1B, JUN, NFE2L2, AKT1, EGFR, ESR1, and GPX1 show major roles in  
481 processes linked to oxidative stress, cell survival, hormone ~~signalingsignalling~~, and developmental  
482 competence, highlighting their value as indicators and molecular targets in IVF and IVM. Gene  
483 Ontology (GO) biological process enrichment analysis of the predicted target proteins of *H.*  
484 *lessoni* extract relevant to oocyte and embryo development.

485

486 Based on key roles in BP, CC, MF, and KEGG pathways for IVF and IVM, we  
487 identified 18 proteins that directly interact with the most promising compounds from *H. lessoni*  
488 ~~sea cucumber~~ extract. These proteins fall into four groups: oxidative stress protection,  
489 antioxidant defense, cell survival, and implantation and growth signaling. We also mapped  
490 their mechanisms of action with four matching compounds. Among these compounds, ~~bBis~~  
491 (2-ethylhexyl) phthalate (DEHP) shows the strongest potential by activating HSP90AA1,  
492 HSP90AB1, HSP90B1, GPX1, and NFE2L2. Citronellal, Squalene, and 2-Methyl-Z,Z-3-13-  
493 octadecadienol also provide protective effects by regulating BCL2, AKT1, and ESR1.¶

494 ¶

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

501 ¶

502 ¶

503

#### 504 4. Discussion

505

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

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

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

543 The GC-MS profile indicates that the *H. lessoni* extract contains multiple compounds  
544 capable of scavenging ROS, stabilizing cellular membranes, and modulating inflammatory  
545 and apoptotic signaling, including unsaturated fatty acid esters, squalene, and steroid-like  
546 molecules [13,26]. In aged oocytes, which are characterized by elevated ROS levels,  
547 mitochondrial dysfunction, and impaired proteostasis, such compounds likely improve the  
548 intracellular redox environment, preserve organelle integrity, and reduce oxidative damage to  
549 DNA, lipids, and proteins. Fatty acid ester can be incorporated into mitochondrial membranes,  
550 where they modulate key anabolic signaling pathways, ~~notably mTORC1/p70S6K and~~  
551 ~~AMPK/PGC1- $\alpha$ /PPAR- $\gamma$ , thereby~~ promoting mitochondrial biogenesis via upregulation of  
552 transcriptional regulators such as PGC1- $\alpha$ , TFAM, and Nrf1 [27]. Squalene has been described  
553 as an antioxidant that directly scavenges free radicals, reduces intracellular ROS, and prevents  
554 H<sub>2</sub>O<sub>2</sub>-induced oxidative and DNA damage [28]. ~~In a zebrafish model of copper sulfate-~~  
555 ~~induced inflammation, squalene was found to upregulate mRNA expression of key antioxidant~~  
556 ~~enzymes in Zang's (2023) study about antioxidant properties [28].~~ Collectively, these bioactive  
557 compounds likely act as ROS scavengers and membrane protectants, improving the  
558 intracellular redox environment, preserving mitochondrial and ER integrity, and limiting  
559 oxidative damage to DNA, lipids, and proteins in aging oocytes and embryos [29]. ¶

560 The *in vitro* results showed that ~~treatment~~ treatment at 20 ppm led to ~~significant~~ significant  
561 upregulation of both HSP90 and GPX1, whereas treatment at 25 ppm induced excessive GPX1  
562 expression but was accompanied by a decrease ~~in~~ HSP90. This interpretation is consistent  
563 with the findings of Önay Uçar et al., who demonstrated that HSP90 helps cells survive by  
564 protecting them from stress [30]. Additionally, Hu et al. reported that long-chain bases (LCBs)  
565 from sea cucumber reduced ER stress by targeting oxidative, UPR, and inflammatory pathways  
566 [31]. In contrast, at an extract concentration of 25 ppm, the molecular profile indicateds a  
567 dysregulated stress response, in which increased GPX1 induction ~~iswas~~ was not matched by  
568 sufficient chaperone capacity due to decreased HSP90 expression. This imbalance may  
569 promote the accumulation of misfolded proteins, prolong ER stress, and activate apoptosis,  
570 thereby compromising embryonic development beyond early cleavage. These molecular  
571 alterations are reflected in the marked reduction in blastocyst formation rate, deterioration of  
572 blastocyst morphology, and Gardner grading at the highest dose.¶

573 Among the compounds analyzed, ~~B~~bis(2-ethylhexyl) phthalate (DEHP) showed the  
574 strongest predicted activity. DEHP activates HSP90 family proteins, which help stabilize  
575 proteins ~~needed~~required for spindle formation, cell cycle regulation, and stress  
576 resistance ~~:[34,¶35]~~. It also activates GPX1 and NFE2L2 (Nrf2), major regulators of  
577 antioxidant defense, suggesting that DEHP helps protect oocytes from oxidative and protein-  
578 ~~related stress~~ ~~:[36]~~. DEHP acts as an indirect antioxidant by triggering several protective  
579 molecular pathways highlighted in the network. DEHP strongly activates HSP90 proteins,  
580 which maintain proteostasis by stabilizing client proteins during oxidative stress and  
581 supporting survival pathways involving AKT1 and BCL2. Through these interactions, DEHP  
582 helps preserve mitochondrial integrity and reduces ROS--induced damage. It also stimulates  
583 the Nrf2NFE2L2 pathway, increasing expression of key antioxidant enzymes such as GPX1  
584 and HMOX1, which lower peroxide levels and protect mitochondrial membranes ~~:[37]~~. ¶

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

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

604 defenses, rather than maximal stimulation of a single pathway, is essential for optimal  
605 embryonic development. This interpretation is consistent with the findings of Önay Uçar et  
606 al. (2023), who demonstrated that HSP90 helps cells survive by protecting them from stress  
607 [30]. Additionally, Hu et al. reported that long-chain bases (LCBs) from sea cucumber reduced  
608 ER stress by targeting oxidative, UPR, and inflammatory pathways [31]. LCBs downregulate  
609 the ER chaperone GRP78/Bip and suppress its main UPR branches, as shown by decreased  
610 hepatic ATF6, PERK, and XBP1 expression and reduced phosphorylation of eIF2 $\alpha$  and IRE1 $\alpha$   
611 [31]. The concomitant up-regulation of HSP90 and GPX1 observed at the optimal *H. lessoni*  
612 extract suggests a coordinated reinforcement of proteostasis and antioxidant defenses. HSP90  
613 has been reported to facilitate correct protein folding and protects against ER stress, while  
614 GPX1 reduces peroxides and limits ROS-induced damage [32,33]. Consistent with this model,  
615 the PPI network analysis highlighted central regulatory nodes such as TP53, TNF, IL1B,  
616 CASP3, and BAX, supporting a multi-target modulatory effect of the extract on oxidative  
617 stress and apoptosis-related pathways that influence oocyte and embryo survival. In contrast,  
618 at an extract concentration of 25 ppm, the molecular profile indicates a dysregulated stress  
619 response, in which increased GPX1 induction is not matched by sufficient chaperone capacity  
620 due to decreased HSP90 expression. This imbalance may promote the accumulation of  
621 misfolded proteins, prolong ER stress, and activate apoptosis, thereby compromising  
622 embryonic development beyond early cleavage. These molecular alterations are reflected in  
623 the marked reduction in blastocyst formation rate, deterioration of blastocyst morphology, and  
624 Gardner grading at the highest dose. ¶

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

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

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

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

669

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676

## 677 **Declaration of Interest**

678 The authors declare no competing interests

679

## 680 **Author contributions**

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

685

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