Proliferative Effect of Aqueous Extract of Sea Cucumber (Holothuria Parva) Body Wall on Human Umbilical Cord Mesenchymal Stromal/stem Cells

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Abstract

Background: The sea cucumber potentials for stem cell proliferation induction and their mechanisms of bioactive compounds in its extract have been studied. Human umbilical cord mesenchymal stromal/stem cells (hUC-MSCs) were exposed to aqueous extract of *Holothuria parva* body wall.

Methods: Using GC-MS analysis on aqueous extract of *H. parva*, proliferative molecules were detected. The extract concentrations of 5, 10, 20, 40, and 80 µg/mL and 10 and 20 ng/mL of human epidermal growth factor (EGF) as positive controls were used. MTT proliferation, cell count, viability, and cell cycle assays were performed. Using Western blot analysis, effects of aqueous extract of *H. parva* and EGF on cell proliferation markers were detected. Computational modeling done to detect effective proliferative compounds in aqueous extract of *H. parva*.

Results: MTT assay showed that the 10, 20, and 40 µg/mL aqueous extract of *H. parva* had proliferative effects on hUC-MSCs. Count of the cells treated with the 20 µg/mL of the extract was increased faster and higher than the control group ($P<0.05$). This concentration of extract did not have significant effects on hUC-MSCs viability. The cell cycle assay of hUC-MSCs showed that percent of cells in the G2 stage of the extract was biologically higher than the control group ($P>0.05$). Expression of the cyclin D1, cyclin D3, cyclin E, HIF-1α, and TERT were increased comparing with the control group. Moreover, expression of the p21 and PCNA decreased after treating hUC-MSCs with the extract. However, the CDC-2/cdk-1 and ERK1/2 had almost the same expression as the control group. The expression of the cdk-4 and cdk-6 was decreased after treatment with the extract. Between the detected compounds, 1-methyl-4-(1-methylethenyl)-benzene showed better affinity to cdk-4 and p21 than tetradecanoic acid.

Conclusions: The *H. parva* aqueous extract showed proliferative potential on the hUC-MSCs.

1. Introduction

Human umbilical cord mesenchymal stromal/stem cells (hUC-MSCs) as an allogeneic source of stem cells have been clinically studied for skin repair (1). The hUC-MSCs can play roles in skin wound healing phases including hemostasis, inflammation, proliferation and maturation (2). Skin injuries are categorized into superficial, partial-thickness, or full-thickness in terms of the damage caused to the skin structure (3). These injuries can be treated by wound excision, skin grafting, skin substitutes, or wound dressings (3). Introducing proliferative agents into skin wound dressings has the potential to improve tissue regeneration (4). Combination of stem cell therapy and proliferative agents in complementary and alternative medicine can be promising tissue regeneration (5, 6).

Sea cucumbers have the ability of whole organ regeneration because they direct their wound healing abilities towards restoring their organs (7). The bioactive compounds producing during wound healing and organ regeneration in sea cucumber may give us the opportunity to repair human wounds. Sea cucumber extracts and their bioactive compounds have been studied for their beneficial therapeutic properties, including anti-cancer, anti-bacterial, anti-viral, anti-diabetic, and anticoagulant properties (8). Recently, different type extract from various species of sea cucumber, have been shown to promote cell proliferation

in cell lines and/or differentiation of pluripotent MSCs under standard *in vitro* or *in vivo* conditions (9-12). Sea cucumber therapeutic effects have been gaining interest in complementary medicine because of their advantages over other semi-biological and synthetic proliferative inducers, such as availability, low toxicity, and comparatively low cost.

We presume that bioactive molecules derived from sea cucumber, could be utilized to enhance the proliferation of stem cells. In this project, we aimed to evaluate the impact of *Holothuria parva* aqueous extract on the viability and proliferation capacity of human umbilical cord mesenchymal stem cells (hUC-MSCs).

2. Materials And Methods

2.1. Sampling and identification of sea cucumber

Sea cucumbers were harvested alive from coastal waters of Dayyer, the Persian Gulf, Iran (28°57'44. 6"N 50°48'42. 5"E). They were sent to the laboratory in containers filled with fresh seawater for extraction and further processing. They were rinsed with distilled water to remove debris and transferred to the aquarium with 29 ppt salinity and 26℃ water temperature. The samples were identified according to identification keys (13, 14).

2.2. Preparation of sea cucumber aqueous extract

The sea cucumbers were washed with tap water at the laboratory to eliminate all particles from their body. The sea cucumber body was cut longitudinally and taking out all the internal organs. The body wall was washed by distilled water properly then was cut into small pieces. The samples were homogenized using a blender and mixed with distilled water. The prepared mixture was filtered using Whatman filter paper. Following filtration, we freeze-dried the liquid and stored the final powder at − 80°C (15).

2.3. Gas chromatography–mass spectrometry (GC-MS) assay

The GC-MS analysis was used to evaluate the chemical composition of the sea cucumber extract (16). The lyophilized fractions, were subjected to the 7890B Agilent Gas Chromatography–Mass Spectroscopy. Electronionization (EI) mass spectra (scan range, m/z 50–500) were obtained using electrons with energy of 70 eV and filament emission of 0.5 mA. The GC separations were conducted using an HP-5MS UI column (30 m × 0.25 mm i.d., film thickness 0.5 µm). Helium was used as the carrier gas (flow: 0.8 mL/min) for EI. The GC oven temperature was programmed at 5°C/min from 80℃ after 3 min since the sample injection and held at 250℃ for 10 min. The injection port of the gas chromatograph, transfer line, and ion source of 5977MSD were maintained at 240℃, 250℃, and 220°C, respectively. The separated compounds were identified by comparing them with the compound data from the National Institute of Standards and Technology (NIST MS database, 2014) library. The relative percent amount of each component was measured by comparing its average peak area to the total areas.

2.4. hUC-MSCs culture and characterization

The hUC-MSCs were isolated from infants umbilical cords, and cultured (17). The cells were cultured in 75 cm^2. Tissue culture flask (NEST, Cat. No. 708003, China) and incubated (Memmert, INB200, Germany) at 37°C with 5% CO_2 in a 95% humidity cultured in a Dulbecco's Modified Eagle's medium (DMEM) (Gibco™, Cat. No. 12-800-082, UK) medium containing 10% FBS (Kiazist, Cat. No. KFBS100, Iran), 1% penicillin streptomycin (Gibco™, Cat. No. 15-140-122, UK) and 1% gentamicin (Sigma-Aldrich, CAS. No1405-41-0, USA) (18).

Flasks of hUC-MSCs were incubated at 4℃ in dark with either phycoerythrin (PE) or fluorescein isothiocyanate (FITC)-conjugated antibodies specific for CD44, CD90, CD73, CD34, and CD45. Cells were analyzed by flow cytometry using a BD FACS Calibur (BD biosciences, San Jose, CA, USA) (17).

Adherent cells were subjected to adipogenic, osteogenic, and chondrogenic differentiations *in vitro*, according to established protocols (19). In a 24-well cell culture plate, 5×10^4 cells were seeded and 2 mL culture medium was added. Then the cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. After 24 h, the culture medium was replaced with differentiation media (Kiazist, Iran). Cells were treated with differentiation media for three weeks with medium changes every 3 d. To document the adipogenic differentiation, the culture was rinsed three times with 1X phosphate buffer saline (PBS, Sigma-Aldrich, P4417-100TAB, USA), fixed with 4% formalin (merck, Cat. No. 1040022500, Germany) for 20 min. Then formalin was removed and washed with sterile distilled water and stained for 20 min with Oil Red O staining to stain lipid droplets or Alizarin Red staining to observe the calcium-rich extracellular matrix and observed under the microscope (Optika, Cat. No. IM-3FL4, Italy). For validating chondrogenic differentiation, after removing formalin, the cells were washed with sterile distilled water, and 40 µL HCl 0.1M (Merck, Germany) was added. HCl was removed and 0.5 mL Alcian Blue was added. After 20 min, the stain was removed and washed twice with PBS 1X. The cells were observed under light a microscope for glycosaminoglycans.

2.5. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) proliferation assay

For the MTT assay4×10^4 hUC-MSCs were seeded in each well of a 96-well tissue culture plate (Sorfa, Cat. No. 220400, China) and added 200µl culture medium then incubate for 24h to let cells attached to the bottom of the plate (20). After that, replaced the medium with different doses of aqueous extract (5, 10, 20, 40, and 80 µg/mL) after some modification of previous findings (9), epidermal growth factor (EGF) (10 and 20 ng/mL) as positive control based on previous findings (9) or culture media as negative control. After 72 h treatment, the cells medium was removed and washed twice with PBS 1X. Then, 100 µL MTT solution (bio-idea, BI-2004, Iran) was added per well and incubate for 4 h. After that, 150 µL Dimethyl sulfoxide (DMSO, bio-idea, BI-2004, Iran) was added. The plate was put in an incubator for 20 min. Quantification was then carried out using a microplate reader at 570 nm.

2.6. Cell count and cell viability assays

For these assays, 1×10^4 hUC-MSCs were seeded in each well of a 24-well tissue culture plate and added 2 mL culture medium. Then it was incubated for 24 h to let cells be attached to the bottom of the plate (21).

Then, the medium was replaced with culture media, 20 µg/mL aqueous extract and 10 ng/mL EGF and replaced it every 3 d. This doses were selected based on the MTT results. Every 24 h, the medium was removed from wells and washed it with PBS 1X. Adding 0.5 ml trypsin-EDTA (Gibco™, Cat. No. 25300054, UK) and 4 min incubation and then enzyme neutralization with 1.5 mL by adding culture medium, cell suspension was collected. The suspension was centrifuged, the supernatant was removed and 1 mL culture medium was added to cells and shaken well to a homogeneous mixture. To count the cells, 10 µL of this mixture was pipetted and mixed with same volume of trypan blue and count with a hemocytometer under light microscope. Based on the point that viable cells with intact membranes do not absorb trypan blue stain, dead cells were distinguished from viable ones by their blue color while live cells appeared white.

2.7. Cell cycle assay

Treated hUC-MSCs with 20 µg/mL aqueous extract or 10 ng/mL EGF for 72 h were harvested at 80–90% confluence for cell cycle analysis. The cell concentration was adjusted to 5×10^5 cell/mL. Cells were washed with PBS 1X and were fixed with 70% ethanol at 4°C for 2 h. Fixed cells were then centrifuged and the supernatant was discarded. Fixed cells were washed and incubated in 1 mL propidiumlodide (PI) master mix included 40 µL PI, 10 µL RNase (DNase free), and 950 µL PBS for 30 min. Cell cycles were assessed by flow cytometry and analysis was performed using FlowJo software (22).

2.8. Western blot

Western blot analysis was done based on the standard procedures with slight modifications (23). After 72 h treatment of hUC-MSCs with 20 µg/mL aqueous extract or 10 ng/mL EGF, they were used for western blot analysis. In details, cells were lysed by lysis buffer including 500 µL tris-HCL pH=8, 0.003 gr EDTA, 0.08 gr NaCl, 0.025 gr sodium deoxycholate, 0.01 gr sodium dodecyl sulfate, 1 tablet protease inhibitor cocktail, and 10µl NP40 (1%) triton at 4°C for 20 min. The lysates were centrifuged at 12000 ×g for 10 min at 4°C, and the protein concentration was measured by a Bradford protein assay. Then, proteins were transferred to microporous polyvinylidene difluoride membrane (Millipore, France). Membranes were incubated in a blocking buffer for 1 h at room temperature. After blocking, the membranes were incubated with the corresponding primary antibodies separately overnight at 4°C.

Immunoblotting was performed with β-actin (c4): sc-47778 (Santa Cruz Biotechnology), cdc2 p34 (17): sc-54 (Santa Cruz Biotechnology), cdk-4 (dcs-35): sc-23896 (Santa Cruz Biotechnology), cdk6 (b-10): sc-7961 (Santa Cruz Biotechnology), cyclin D1 (a-12): sc-8396 (Santa Cruz Biotechnology), cyclin D3 (1): sc-135875 (Santa Cruz Biotechnology), cyclin E (he12): sc-247 (Santa Cruz Biotechnology), ERK 1/2 (h-72): sc-292838 (Santa Cruz Biotechnology), HIF-1α (28b): sc-13515 (Santa Cruz Biotechnology), p21 (f-5): sc-6246 (Santa Cruz Biotechnology), PCNA (pc11): sc-53407 (Santa Cruz Biotechnology), and TERT polyclonal antibody e-ab-33070 (Elabscience Biotechnology). Membranes were washed 3 times (10 min each) in Tris-buffered saline before incubating with m-IgGκBP-HRP: sc-516102 (Santa Cruz Biotechnology) or mouse anti-rabbit IgG-HRP: sc-2357 (Santa Cruz Biotechnology) secondary antibodies. One of the most accurate and sensitive techniques for detecting the desired protein band (identified by its specific antibody) is the use of chemoluminescence kits. ECL advanced reagents kit and its protocol was used.

2.9. Computational details

2.9.1. Preparation of ligands and receptors

Fifty-eight compounds detected through the GC analysis of the aqueous extract of sea cucumber (*H. parva*) body wall. Accordingly, these compounds were selected as ligands to study whose interactions with nine target proteins through the docking process. Their three-dimensional (3D) structure downloaded from the PubChem database. Nine proteins of CDK4, CDK6, Cyclin D1, Cyclin D3, Cyclin E, HIF-1α, p21, PCNA and TERT were downloaded from the Protein Database Bank (PDB) with PDB code of 2w96, 1blx, 2w96, 3g33, 7kjs, 4h6j, 5e0u, 5e0u, and 5ugw. The HyperChem software version 8.0.10 was used to optimize the ligands geometry. All receptors were prepared for docking process using Chimera 1.15.

2.9.2. Generation of grid box

The grid box was manually generated with a space of 0.375 Å at the position of the active sites chosen according to the result of the CASTp calculation.

2.9.3. Study of target proteins-marine derived compounds interactions

In order to perform the docking process, Autodock Vina 1.1.2 software was utilized to conduct to investigate interactions between receptors and ligands.

2.10. Statistical analysis

The data were statistically analyzed using IBM SPSS Statistics 26 software (SPSS for Windows, version 26, SPSS Inc, Chicago, Illinois, USA). Comparison between groups was done using one-way ANOVA and *post hoc* LSD test (for comparing the MTT and cell proliferation and viability assays) or chi-square test (for comparing the cell cycle analysis and flow cytometry). Data were demonstrated as mean ± standard error of mean. The significant difference between groups was statistically considered $p<0.05$. The graphs were drawn using Graph pad prism (v7.0a, GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Sea cucumber was identified as *H. parva*

Identification of sea cucumbers was aided by the examination of the skeletal elements (ossicles) found in various parts of the body (Figure S1). Separated ossicles confirmed that the collected sea cucumbers were *H. parva* based on the identification keys (13, 14).

3.2. GC-MS analysis detected proliferative compounds in the aqueous extract of H. parva

Sixty compounds were detected in the aqueous extract of *H. parva* via GC-MS analysis (Figure S2 and Table 1). It was shown that the molecules in the aqueous extract from *H. parva* had different biological effects. As it was shown in Table 1, there were two molecules including methyl ester octadecanoic acid (24-26) and 1-methyl-4-(1-methylethenyl)-benzene (27-29) which has been proved to have proliferative biological

activity. Other biological activities were also observed in other molecules of aqueous extract of *H. parva* including anti-oxidant, anti-microbial, anti-inflammatory, etc. (Table 1).

3.3. Morphologic and immunophenotypic assays and differentiation to adipocytes, osteocytes, and chondrocytes confirmed hUM-MSCs isolation

Morphologically, the MSCs were thin and long spindle-shaped cells with small cell bodies (Figure 1A). The cells' morphology did not change during the four passages. At the molecular level, MSCs did not express CD34 (0.04%) and CD45 (0.16) (Figures 1B and 1C). At the molecular level, MSCs express surface antigens including CD44 (99.9%), CD73 (98.5%), and CD90 (98.3%) (Figures 1D-1F).

To verify the multipotency of the hUC-MSCs, the cells were assessed for their adipogenic, chondrogenic, and osteogenic differentiation capacities *in vitro*. In the adipogenic differentiated cells, red-stained intracellular vacuoles were observed (Figure 1G). In osteogenic differentiation, adherent monolayers of spindle-shaped cells became multilayered cell clusters surrounded by a matrix-like substance (Figure 1H). In chondrogenic differentiation, at day 21, the cartilaginous elements were numerous and well-differentiated and the cells were observed under the light microscope for sulfated proteoglycan (Figure 1I).

3.4. Aqueous extract of *H. parva* induced proliferation of hUC-MSCs

The findings of MTT assay demonstrated that the 10, 20 and 40 µg/mL aqueous extract of *H. parva* had proliferative effects on hUC-MSCs (p=0.01, p<0.001, and p=0.014, respectively, Figure 2A). Moreover, the EGF-10 and EGF-20, as positive controls, had also proliferative effects on hUC-MSCs (p<0.001, Figure 2A). In addition, the EGF-10 had higher proliferative effects on hUC-MSCs than the 10 and 40 µg/mL aqueous extract of *H. parva* (p<0.05, Figure 2A). However, the EGF-10 had no statistically significant difference in proliferative effect on hUC-MSCs with the 20 µg/mL aqueous extract of *H. parva* (Figure 2A). This evidence showed that the 20 µg/mL concentration of the aqueous extract is the optimum dose of sea cucumber extract with minimum cytotoxicity. This concentration was chosen for further analysis.

The number of cells treated with the 20 µg/mL concentration of the extract and EGF-10 were increased faster and higher than the control group (Figure 2B). In details, the cell number of the extract treatment group was higher than the control group from day 6 (p<0.01). Moreover, this result was observed for the EGF-10 treatment group from day 7 (p<0.01). On the other hand, the 20 µg/mL concentration of aqueous extract of *H. parva* and EGF-10 did not have significant effects on hUC-MSCs viability comparing to the control group during 13 days (Figure 2C).

3.5. Cell cycle assayed did not show differences in proportion of stages

The cell cycle assay of hUC-MSCs in the three groups showed that although percent of cells in the G2 stage of the extract and EGF-10 was mathematically higher than the control group, but proportions of different

stages of all groups were not statistically different (Figure 3).

3.6. Western blot analysis findings

The hUC-MSCs were treated with the 20 µg/mL concentration of aqueous extract of *H. parva* or EGF-10, expression of the cyclin D1, cyclin D3, cyclin E, HIF-1α, and TERT were increased comparing with the control group (Figure 4). Moreover, expression of the p21 and PCNA decreased after treating hUC-MSCs with the 20 µg/mL concentration of aqueous extract of *H. parva* or EGF-10 comparing with the control group. However, the CDC-2/cdk-1 and ERK1/2 had almost the same expression as the control group. The expression of the cdk-4 was decreased and increased after treatment with the extract and EGF-10, respectively. In addition, the cdk-6 expression was decreased and increased after cell treatment with the extract and EGF-10, respectively.

3.7. Six compounds showed better affinity to proteins

The docking process of each ligand-protein complex resulted in 10 conformations with different binding affinity as a docking score. Among them, the conformation with RMSD ≤2 Å and the lowest binding affinity, ΔG [U total in kcal/mol was chosen as the best or the most stable one. Table 2 presents ΔG corresponding to the best conformations. The binding energies are in the range of -2.5 to -7.9 Kcal/mol with mean value of -4.7 Kcal/mol. The highest ΔG was related to the complex of p21 with Decane 4-methyl- and Tridecane. The lowest ΔG was related to the complex of Cyclin D3 with 3,4-Dihydro-1h-isoquinoline-2-carboxamidine hydrochloride.

Figures S3-S11 shows the 3D plot of the active sites and the 2D plot of interactions of these proteins with mentioned compounds. As observed, different interactions, including hydrogen bonds, which are strong bonds, van der Waals and π-π interactions play role in the binding of compounds to proteins. According to the result of docking study, the highest affinity to CDK4 was related to 2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, (1S)-. The highest affinity to CDK6 was related to 1, 2-benzenedicarboxylic acid, diisooctyl ester and 3,4-Dihydro-1h-isoquinoline-2-carboxamidine hydrochloride. The highest affinity to Cyclin D1 was related to 1, 2-benzenedicarboxylic acid, diisooctyl ester and 3,4-Dihydro-1h-isoquinoline-2-carboxamidine hydrochloride and 8-amino-6-methoxyquinoline. The highest affinity to Cyclin D3 was related to 3,4-Dihydro-1h-isoquinoline-2-carboxamidine hydrochloride, Isopinocarveol, E-2, 3-epoxycarane, 2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, (1S)-, and Benzene, 2-(butenyl)-5-(1, 1-dimethylethyl)-1, 3-dimethyl-. The highest affinity to Cyclin E was related to 1, 2-benzenedicarboxylic acid, diisooctyl ester and 3,4-Dihydro-1h-isoquinoline-2-carboxamidine hydrochloride. The highest affinity to HIF-1α was related to (5E,9E)-6,10-Dimethyldodeca-5,9-dien-2-one and 1, 2-benzenedicarboxylic acid, diisooctyl ester. The highest affinity to p21 was related to 3,4-Dihydro-1h-isoquinoline-2-carboxamidine hydrochloride, Azulene and D-glucose, cyclic ethylene mercaptal, pentaacetate and Phenol,2,4-bis(1,1dimethylethyl)-. The highest affinity to PCNA was related to (5E,9E)-6,10-Dimethyldodeca-5,9-dien-2-one and 3,4-Dihydro-1h-isoquinoline-2-carboxamidine hydrochloride, and Cyclooctaneacetic acid, 2-oxo-. The highest affinity to TERT was related to 1, 2-benzenedicarboxylic acid, diisooctyl ester and 3,4-Dihydro-1h-isoquinoline-2-carboxamidine hydrochloride. Therefore, six compounds can be considered as the most effective ones.

4. Discussion

In the present study aqueous extract of *H. parva* induced proliferation of hUC-MSCs same as EGF. Growth factors, including EGF, fibroblast growth factor (FGF), transforming growth factor beta (TGFβ), and bone morphogenetic protein (BMP) induce proliferation in multipotent MSCs cells (30). These cytokines play roles in regeneration and development of sea cucumbers (31). Consistent with our findings protein fractions from PBS and acetic acid crude extracts of *H. scabra* body wall increased the growth kinetics of placenta-derived MSCs (10). In addition, the pepsin-solubilized collagen extract of *Stichopus japonicus* enhanced human keratinocyte cell proliferation (12). Water extract of *S. variegatus* induced proliferation activity of spinal astrocytes cell lines (9). Optimum concentration of *S. chloronotus* aqueous extract enhanced wound healing in rat models (11). Therefore, extract of *H. parva* has bioactive compounds for induction of proliferation in hUC-MSCs.

On the other hand, the proportion of the cells at G2 stage after treating with aqueous extract of *H. parva* and EGF were higher than control group. This result indicated that the cells after treatment with both extract and EGF undergoes proliferation and they are in pre-mitosis stage. In line with the current result, previous studies showed that the extracts which induce cell proliferation, increased S and G2 phases and finally mitosis (32). Moreover, the effects of aqueous extract of *H. parva* on proliferation related peptides in hUC-MSCs were assessed. Aqueous extract of *H. parva* or EGF upregulated expression of the cyclin D1, cyclin D3, and cyclin E in the hUC-MSCs. Cyclin D protein subfamilies cyclin D1 and cyclin D3 plays an important role in cell proliferation (33). They do this role by activating cdk-4 or cdk-6 (33). Similar to our findings, EGF induces hair follicle-derived MSC proliferation through the EGFR/ERK and AKT pathways associated by upregulation of cyclin D1 expression and stimulation of G1/S transition (34). It is shown that cyclin D3 expressed in cells stimulated by EGF for a G1 phase progression (35). Cyclin E form the CDK2-cyclin E complexes which both promote the G1/S phase progression (36). On the other hand, Aqueous extract of *H. parva* or EGF downregulated expression of the p21 in the hUC-MSCs. The p21 protein is a CDK inhibitor and downregulates proliferation by preventing the transcription of cell cycle-regulated pro-proliferative proteins (37). EGF promotes cell growth by suppressing p21 (38). Therefore, aqueous extract of *H. parva* as same as EGF induced proliferation of hUC-MSCs through upregulation of cyclins subfamilies and suppressing cellular senescence-related protein (Figure 5).

Aqueous extract of *H. parva* or EGF upregulated expression of the TERT in the hUC-MSCs. TERT (telomerase reverse transcriptase) maintains telomere length to enable cells to proliferate (39). TERT mRNA expression increased in the hUC-MSCs treated by EGF (40). TERT expression is high in stem cells during proliferation and reduced upon differentiation (39). EGF activates TERT transcription in cancer cells but not in somatic cells (41). Therefore, aqueous extract of *H. parva* as same as EGF induced proliferation of hUC-MSCs through maintaining telomere length.

Aqueous extract of *H. parva* or EGF increased expression of the HIF-1α in the hUC-MSCs. Hypoxia-inducible factor 1 (HIF-1) has been recognized for its key role in transcriptional control of proliferation (42). The EGF is able to stabilize HIF-α under non-hypoxic conditions (43). Growth factors promotes HIF-α binding to DNA to induce gene transcription in cells (44). Similarly, HIF-1α contributes to the proliferative response of cells

to growth factors (45). Therefore, aqueous extract of *H. parva* as same as EGF induced proliferation of hUC-MSCs through maintaining telomere length.

After docking analysis of GC-MS-detected bioactive compounds, 13 compounds with the effective proteins in the stem cell proliferation the following compounds showed the highest affinity. 3,4-Dihydro-1h-isoquinoline-2-carboxamidine hydrochloride other name is debrisoquine which is an antihypertensive drug (46). Debrisoquine hydrochloride showed the highest affinity to CDK4, CDK6, Cyclin D1, Cyclin D3, Cyclin E, PCNA, and TERT. The most affinity of debrisoquine hydrochloride was calculated with Cyclin D3. Increase of Cyclin D3 was observed after sea cucumber extract exposure. Based on best of our knowledge there is no data that show the effects of Debrisoquine hydrochloride on cell proliferation. Cyclooctaneacetic acid, 2-oxo- showed the highest affinity to Cyclin D3, CDK4, and PCNA. The most affinity of debrisoquine hydrochloride was calculated with Cyclin D3. After sea cucumber extract exposure into MSCs, Cyclin D3 increased. Based on best of our knowledge there is no data that show the effects of Cyclooctaneacetic acid, 2-oxo- on cell proliferation. 8-amino-6-methoxyquinoline has antimalarial and antiplasmodial activity effect (47). 8-Quinolinamines analogues did not show any cytotoxicity on cancerous and noncancerous cells (48). However, hematotoxic effect of a metabolite of 8-amino-6-methoxyquinoline on erythrocyte has been reported (49). 8-amino-6-methoxyquinoline showed the highest affinity to Cyclin D1, Cyclin D3, and CDK4. The most affinity of 8-amino-6-methoxyquinoline was calculated with Cyclin D3. There was no data on the effect of this compound on cell growth. Isopinocarveol which has been also known as Pinocarveol has anti-viral effect (50, 51). It has been reported that trans-pinocarveol has antigenotoxic potential (52). Pinocarveol showed the highest affinity to Cyclin D3 in our present study. 1, 2-benzenedicarboxylic acid, diisooctyl ester other name is Diisooctyl phthalate which is shown previously in the sea cucumber (15). 1, 2-benzenedicarboxylic acid, diisooctyl ester has antioxidant activity (53). Diisooctyl phthalate had fungitoxic effect on six fungi and cytotoxic activity on newborn shrimp (54). Diisooctyl phthalate showed the highest affinity to CDK6, Cyclin D1, Cyclin E, HIF-1α, PCNA, and TERT. The most affinity of Diisooctyl phthalate was calculated with CDK6. Decrease of CDK6 was observed after sea cucumber extract exposure. E-2, 3-epoxycarane is a terpenoid. E-2, 3-epoxycarane has anti-aging effects on skin (55, 56). E-2, 3-epoxycarane showed the highest affinity to Cyclin D3 in our present study.

5. Conclusions

The aqueous extracts of sea cucumber, *H. parva*, same as EGF, were able to induce proliferation in hUC-MSCs. The GC-MS detected six effective bioactive compounds in sea cucumber aqueous extract had the property of inducing stem cell proliferation.

Abbreviations

3D, Three-dimensional

BMP, Bone morphogenetic protein

DMEM, Dulbecco's Modified Eagle's medium

DMSO, Dimethyl sulfoxide

EGF, Epidermal growth factor

EI, Electronionization

FGF, Fibroblast growth factor

FITC, Fluorescein isothiocyanate

GC-MS, Gas chromatography–mass spectrometry

H. parva, *Holothuria parva*

H. scabra, *Holothuria scabra*

hUC-MSCs, Human umbilical cord mesenchymal stromal/stem cells

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PBS, Phosphate buffer saline

PDB, Protein Database Bank

PE, Phycoerythrin

PI, propidiumlodide

TGFβ, Transforming growth factor beta

Declarations

Ethics approval and consent to participate

This investigation was performed in accordance with relevant guidelines and regulations of the ethical committee of Bushehr University of Medical Sciences (Permission number: IR.BPUMS.REC.1398.091).

Consent for publication

Not applicable.

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Competing interests

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Authors' contributions

A. T., S. A., R. S. and I. N. conceived and designed the format of the manuscript. P. R., A. K., A. K., N. B., G. M., A. B., H. A., Y. H., A. D., A. B., Z. K. and S. Z. collected the data, and drafted and edited the manuscript. A. T., N. B, G. M., and A. K. computational and statistical analysis. N. B., A. T., A. K., P. R., G. M. and A. K. drew the Figures and Tables. All the authors reviewed the manuscript and all of them contributed to the critical reading and discussion of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Availability of data and material

The dataset supporting the conclusions of this article are included within the article and its additional files.

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Tables

Table 1

GC-MS compounds in aqueous extract of sea cucumber (*Holothuria parva*) based on PubChem database

Compounds	Formula	Effects	MW (g/mol)	References
1, 2-benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	Anti-microbial	390.6	(57)
10-methyl-e-11-tridecen-1-ol propionate	$C_{17}H_{32}O_2$	ND	268.4	ND
1-decanol, 2-hexyl-	$C_{16}H_{34}O$	Proteolytic activity	242.44	(58)
1-decanol, 2-methyl-	$C_{11}H_{24}O$	ND	172.31	ND
1-decen-4-yne, 2-nitro-	$C_{10}H_{15}NO_2$	ND	181.23	ND
1-eicosanol	$C_{20}H_{42}O$	Anti-microbial Anti-cancer	298.5	(59, 60)
1-heptadecanol	$C_{17}H_{36}O$	ND	256.5	ND
1-nonadecanol	$C_{19}H_{40}O$	Anti-microbial	284.5	(61)
1-octanol 2-butyl-	$C_{12}H_{26}O$	ND	186.33	ND
3,4-Dihydro-1h-isoquinoline-2-carboxamidine hydrochloride	$C_{10}H_{13}N_3$	Anti-hypertensive	175.23	(46)
2-(prop-2-enoyloxy)tetradecane	$C_{17}H_{32}O_2$	ND	268.4	ND
2-azido-2,4,4,6,6-pentamethylheptane	$C_{12}H_{25}N_3$	ND	211.35	ND
2-cyclohexane-1-ol,2-methyl-5-(1-methylethenyl)	$C_{10}H_{16}O$	ND	152.23	ND
2-dodecanol	$C_{12}H_{26}O$	Anti-microbial Anti-dotes for bungarotoxin	186.33	(62, 63)
2-hexyl-1-octanol	$C_{14}H_{30}O$	Anti-microbial Anti-oxidant	214.39	(64)
3-hexadecyloxycarbonyl-5-(2-hydroxyethyl)-4-methylimidazolium ion	$C_{24}H_{45}N_2O_3^+$	ND	409.6	ND
5, 9-dodecadien-2-one, 6, 10-dimethyl-, (e, e))-	$C_{14}H_{24}O$	ND	208.34	ND
8-amino-6-methoxyquinoline	$C_{10}H_{10}N_2O$	Hemotoxic	174.2	(49)
Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	Growth stimulator	298.5	(24-26)

		Anti-inflammatory Inhibit the detrimental effects of cerebral ischemia		
Azulene	$C_{10}H_8$	Cytotoxic Tyrosine kinase inhibitors	128.17	(65, 66)
Benzene, 1-methyl-4-(1-methylethenyl)-	$C_{10}H_{14}$	Anti-ulcer Gastric healing activities Anti-viral Anti-cancer	134.22	(27-29)
Benzene, 2-(butenyl)-5-(1, 1-dimethylethyl)-1, 3-dimethyl-	$C_{16}H_{24}$	ND	216.36	ND
Cyclooctaneacetic acid, 2-oxo-	$C_{10}H_{16}O_3$	ND	184.23	ND
Decane	$C_{10}H_{22}$	Anti-cancer	142.28	(67)
2,3,5,8-tetramethyldecane	$C_{14}H_{30}$	ND	198.39	ND
Decane 4-methyl-	$C_{11}H_{24}$	ND	156.31	ND
D-glucose, cyclic ethylene mercaptal, pentaacetate	$C_{18}H_{26}O_{10}S_2$	ND	466.5	ND
Dodecanal	$C_{12}H_{24}O$	ND	184.32	ND
2,6,11-trimethyldodecane	$C_{15}H_{32}$	Analgesic activity	212.41	(68)
E-2, 3-epoxycarane	$C_{10}H_{16}O$	ND	152.23	ND
Eicosane	$C_{20}H_{42}$	ND	282.5	ND
Heptacosane	$C_{27}H_{56}$	Cytotoxic Anti-microbial	380.7	(64, 69)
5-ethyl-2,2,3-trimethylheptane	$C_{12}H_{26}$	ND	170.33	ND
Hexadecane	$C_{16}H_{34}$	Cytotoxic	226.44	(70)
Isopinocarveol	$C_{10}H_{16}O$	Anti-viral Anti-microbial	152.23	(50, 51)
Methoxyacetic acid, 2-tetradecyl ester	$C_{17}H_{34}O_3$	ND	286.4	ND
Methoxyacetic acid, 3-tridecyl ester	$C_{16}H_{32}O_3$	ND	272.42	ND

Methoxyacetic acid, pentadecyl ester	$C_{18}H_{36}O_3$	ND	300.5	ND
Methyl 3-hydroxytetradecanoate	$C_{15}H_{30}O_3$	ND	258.4	ND
N,n'-methylenebisacrylamide	$C_7H_{10}N_2O_2$	Adverse maternal and developmental effects	154.17	(71)
N-hexadecanoic acid	$C_{16}H_{32}O_2$	Insulin resistance Anti-proliferative Inhibitory effect on cells Inhibited activation of the NLR signaling pathway Reduces cell migration Inflammatory responses	256.42	(72-77)
Nonadecane	$C_{19}H_{40}$	Anti-microbial Anti-cancer	268.5	(78)
Octacosane	$C_{28}H_{58}$	Mosquitocidal activities Prolong the survival time of hypoxic mice Cytotoxic	394.8	(79-81)
O-xylene	C_8H_{10}	Poisoning (liver disease) Apoptosis induction	106.16	(82, 83)
Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	ND	270.5	ND
Pentanoic acid, octyl ester	$C_{13}H_{26}O_2$	ND	214.34	ND
Phenol,2,4-bis(1,1dimethylethyl)-	$C_{14}H_{22}O$	Anti-fungal Cytotoxic Anti-oxidant Anti-microbial Anti-viral	206.32	(84, 85)
Sulfurous acid, 2-ethylhexyl nonyl ester	$C_{17}H_{36}O_3S$	ND	320.5	ND
Tetracosane	$C_{24}H_{50}$	Anti-viral Cytotoxic	338.7	(86-88)

Tetradecane	$C_{14}H_{30}$	Erythema Cytotoxic	198.39	(89, 90)
Tetradecanoic acid	$C_{14}H_{28}O_2$	Marker of severe inflammation and sepsis Bacteraemia marker Anti-apoptosis Anti-biofilm activity	228.37	(91-93)
Tridecane	$C_{13}H_{28}$	Systemic toxicity Transepidermal water loss	184.36	(94, 95)
Undecane	$C_{11}H_{24}$	Anti-allergic Anti-inflammatory Inhibited degranulation and the secretion of histamine and tumor necrosis factor α (TNF-α)	156.31	(96)
Z-10-tetradecen-1-ol acetate	$C_{16}H_{30}O_2$	Anti-oxidant	254.41	(97)
ND, no data				

Table 2

The values of binding affinity (Kcal/mol) of ligands to receptors

Compounds	CDK4	CDK6	Cyclin D1	Cyclin D3	Cyclin E	HIF-1α	p21	PCNA	TERT
(5E,9E)-6,10-Dimethyldodeca-5,9-dien-2-one	-6.3	-5.1	-5.4	-4.4	-5.9	-5.8	-3.6	-6	-5.4
1, 2-benzenedicarboxylic acid, diisooctyl ester	-5.2	-7	-6.3	-5.1	-6.6	-5.7	-3.3	-5.4	-6.3
10-methyl-e-11-tridecen-1-ol propionate	-5.2	-4.7	-5.2	-4.1	-4.7	-4.5	-2.9	-4.9	-4.3
1-decanol, 2-hexyl-	-5.2	-4.8	-5.2	-4.4	-4.7	-4.4	-2.9	-5.3	-4.7
1-decanol, 2-methyl-	-5	-4.7	-5	-4.1	-4.4	-3.9	-2.7	-4.8	-4.6
1-decen-4-yne, 2-nitro-	-5.6	-5	-4.9	-4.9	-4.9	-4.3	-3.5	-5.7	-5.2
1-eicosanol	-5.1	-3.9	-4.4	-3.8	-4.2	-3.8	-2.7	-4.6	-4.5
1-heptadecanol	-4.8	-4.4	-4.7	-4	-4.5	-4.2	-3	-4.4	-4.7
1-nonadecanol	-3.9	-4.3	-4.4	-4.1	-4.2	-3.6	-2.7	-5	-4.2
2-(prop-2-enoyloxy)tetradecane	-5.2	-5.5	-5.3	-4.5	-5.2	-3.7	-3.1	-5.6	-4.8
2,3,5,8-tetramethyldecane	-5.1	-4.4	-5.2	-4.2	-4.9	-4	-3	-4.8	-4.7
2,6,11-trimethyldodecane	-5.5	-4.4	-4.9	-4.2	-4.3	-4.2	-3.2	-4.7	-4.4
2-azido-2,4,4,6,6-pentamethylheptane	-5.2	-5.4	-5.4	-5.6	-4.8	-4.1	-3.6	-5.4	-4.9
2-butyl-1-octanol	-5.2	-5	-5.1	-5.6	-4.7	-4	-2.8	-4.7	-3.8
2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, (1S)-	-6	-5.5	-5.6	-6.6	-5.3	-4.1	-3.7	-5.5	-5.2
2-dodecanol	-4.9	-4.6	-4.6	-4.3	-4.6	-3.8	-2.9	-5.1	-4.7
2-hexyl-1-octanol	-5.2	-4.7	-4.9	-6.1	-4.3	-4.7	-2.9	-4.8	-4.6
3,4-Dihydro-1h-isoquinoline-2-carboxamidine hydrochloride	-6.4	-6.6	-6.6	-7.4	-6.3	-3.8	-4.4	-6.4	-6.3
3-hexadecyloxycarbonyl-5-(2-hydroxyethyl)-4-methylimidazolium ion	-4.6	-4.8	-4.5	-4.8	-5.1	-4.3	-2.8	-5.3	-4.5
5-ethyl-2,2,3-trimethylheptane	-5.2	-5.2	-4.9	-4.5	-4.8	-4.7	-3.3	-5.1	-4.3

8-amino-6-methoxyquinoline	-6.3	-5.7	-6.5	-7.1	-5.8	-4	-3.6	-5.5	-5.4
Azulene	-5.8	-5.6	-5.9	-6.8	-5.8	-4.9	-4.2	-5.6	-5.3
Benzene, 1-methyl-4-(1-methylethenyl)-	-5.9	-5.5	-5.4	-5.4	-5	-4.7	-3.8	-5.3	-5.2
Benzene, 2-(butenyl)-5-(1, 1-dimethylethyl)-1, 3-dimethyl-	-5.4	-5.2	-5.2	-6.1	-5.2	-4.5	-3.7	-5.3	-5.3
Cyclooctaneacetic acid, 2-oxo-	-6.4	-5.7	-5.9	-7.3	-5.9	-4.6	-3.7	-6.2	-5.2
Decane	-4.8	-4.4	-4.4	-4.2	-4.4	-3.1	-2.6	-5.1	-4.4
Decane 4-methyl-	-4.6	-4	-4	-3.9	-4.5	-3.6	-2.5	-4.8	-4.4
D-glucose, cyclic ethylene mercaptal, pentaacetate	-5.9	-6.3	-5.6	-4.3	-5.7	-4.2	-4.1	-5.3	-4.8
Dodecanal	-4.7	-4.6	-4.3	-3.8	-4.1	-3.8	-3	-4.9	-4.4
E-2, 3-epoxycarane	-5.9	-5.7	-5.7	-7	-5.5	-4.3	-3.2	-5.5	-4.9
Eicosane	-5.1	-4.5	-4.4	-3.7	-4.5	-3.7	-2.9	-4.4	-4.5
Heptacosane	-3.8	-4.3	-4.7	-3.9	-4.3	-3.5	-2.6	-4.1	-4.5
Hexadecane	-4.7	-4.3	-4.6	-4.5	-4.1	-3.6	-3	-4.6	-4.5
Isopinocarveol	-5.5	-5.4	-5.3	-7.1	-5.5	-4.3	-3.3	-5.2	-5
Methoxyacetic acid, 2-tetradecyl ester	-5.3	-4.8	-4.9	-4.3	-4.8	-4.1	-3.2	-4.8	-4.6
Methoxyacetic acid, 3-tridecyl ester	-5.2	-4.6	-4.7	-3.9	-4.8	-4.1	-3	-4.8	-4.6
Methoxyacetic acid, pentadecyl ester	-4.4	-4.2	-4.7	-3.7	-4.7	-3.9	-2.9	-4.8	-4.2
Methyl 3-hydroxytetradecanoate	-5.4	-4.6	-4.8	-4.4	-4.6	-4.4	-2.8	-4.8	-4.5
N,n'-methylenebisacrylamide	-5.1	-4.4	-4.6	-4.6	-4.7	-3.6	-3.1	-4.5	-4.2
N-hexadecanoic acid	-4.5	-4.6	-4.8	-3.9	-4.5	-3.8	-3.2	-4.8	-4.9
Nonadecane	-4.6	-4.1	-4.2	-4	-4.1	-3.6	-2.6	-4.5	-4.9
Octacosane	-4.5	-3.8	-4.5	-3.9	-4	-3.8	-2.8	-4.6	-4.8
Octadecanoic acid, methyl ester	-5.5	-4.3	-4.6	-5.8	-4.4	-3.8	-2.9	-4.8	-5

O-xylene	-5	-5	-5	-4.1	-5	-4.2	-3.6	-4.7	-4.5
Pentadecanoic acid, 14-methyl-, methyl ester	-5.2	-4.1	-4.6	-4	-4.6	-3.9	-2.9	-5	-4.8
Pentanoic acid, octyl ester	-4.9	-4.3	-4.5	-5.2	-4.7	-3.6	-2.9	-4.6	-4.5
Phenol,2,4-bis(1,1dimethylethyl)-	-6.3	-5.8	-6	-5.9	-5.6	-5	-4.1	-6.3	-5.5
Sulfurous acid, 2-ethylhexyl nonyl ester	-4.6	-4.7	-5	-3.8	-4.6	-4.3	-2.6	-4.6	-4.6
Tetracosane	-3.5	-4.9	-4.4	-3.9	-4.5	-3.7	-2.7	-4.9	-5.1
Tetradecane	-4.7	-4.1	-4.3	-4.2	-4.5	-3.6	-2.8	-4.5	-4.5
Tetradecanoic acid	-5.2	-4.4	-4.8	-5.5	-4.3	-3.7	-3.3	-5.1	-4.4
Tridecane	-4.7	-4.1	-4.1	-3.9	-4.2	-3.4	-2.5	-4.9	-4.4
Undecane	-4.8	-4.3	-4.2	-4.1	-4.3	-3.6	-2.6	-5	-4.1
Z-10-tetradecen-1-ol acetate	-5.3	-4.5	-4.8	-4.4	-4.5	-3.9	-3.2	-5	-5

Figures

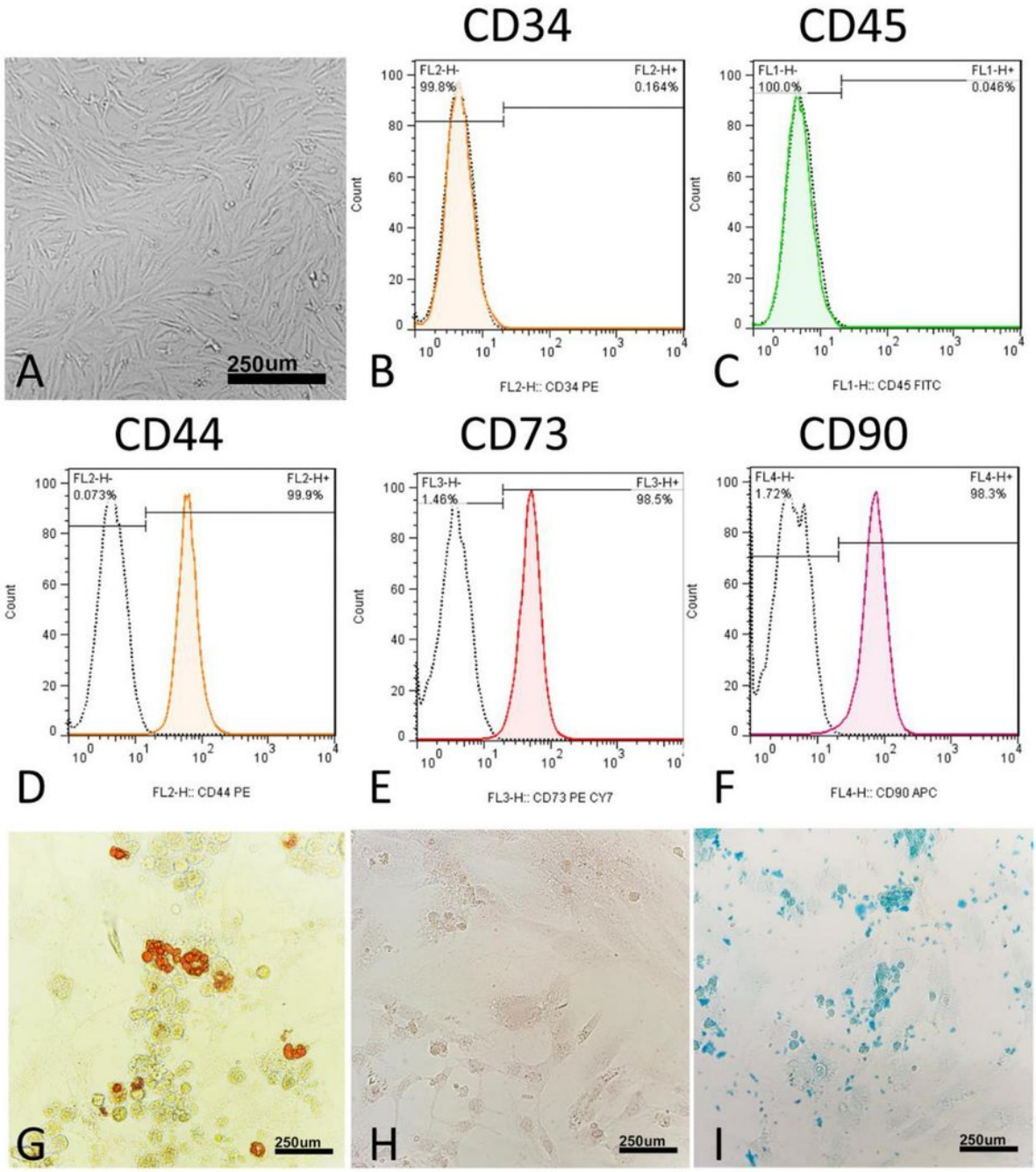

Figure 1.

Figure 1

Characterization of human umbilical cord mesenchymal stem cells (hUC-MSCs). A) hUC-MSCs morphology. Flow cytometric histograms showing the immunophenotype of hUC-MSCs. B and C) The hUC-MSCs are negative for the hematopoietic line markers CD34 and CD45. D-F) Analyzed hUC-MSCs are positive for CD44, CD73, and CD90, which are considered to be markers of MSCs. FITC, fluorescein isothiocyanate; PE, phycoerythrin. G-I) Differentiation of hUC-MSCs to adipogenic differentiation in which the cells were stained

with Oil Red O, osteogenic differentiation in which the cells were stained with Alizarin red, and chondrogenic differentiation that the cells were stained with Alcian blue.

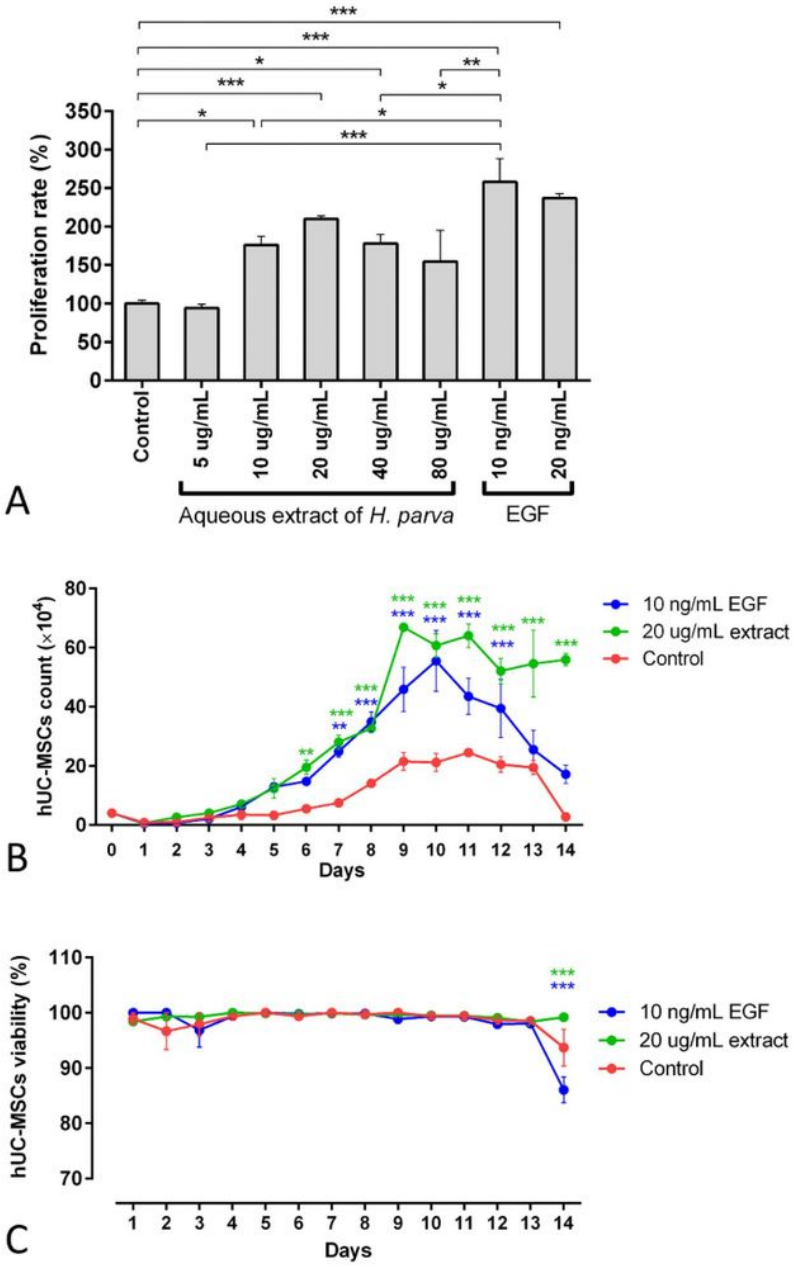

Figure 2.

Figure 2

Mean and standard errors of cell proliferation rate (A), cell count (B), and cell viability (C) of human umbilical cord mesenchymal stem cells (hUC-MSCs) after exposure to aqueous extract of sea cucumber (Holothuria parva) or epidermal growth factor (EGF). *P <0.05; **P <0.01; ***P <0.001.

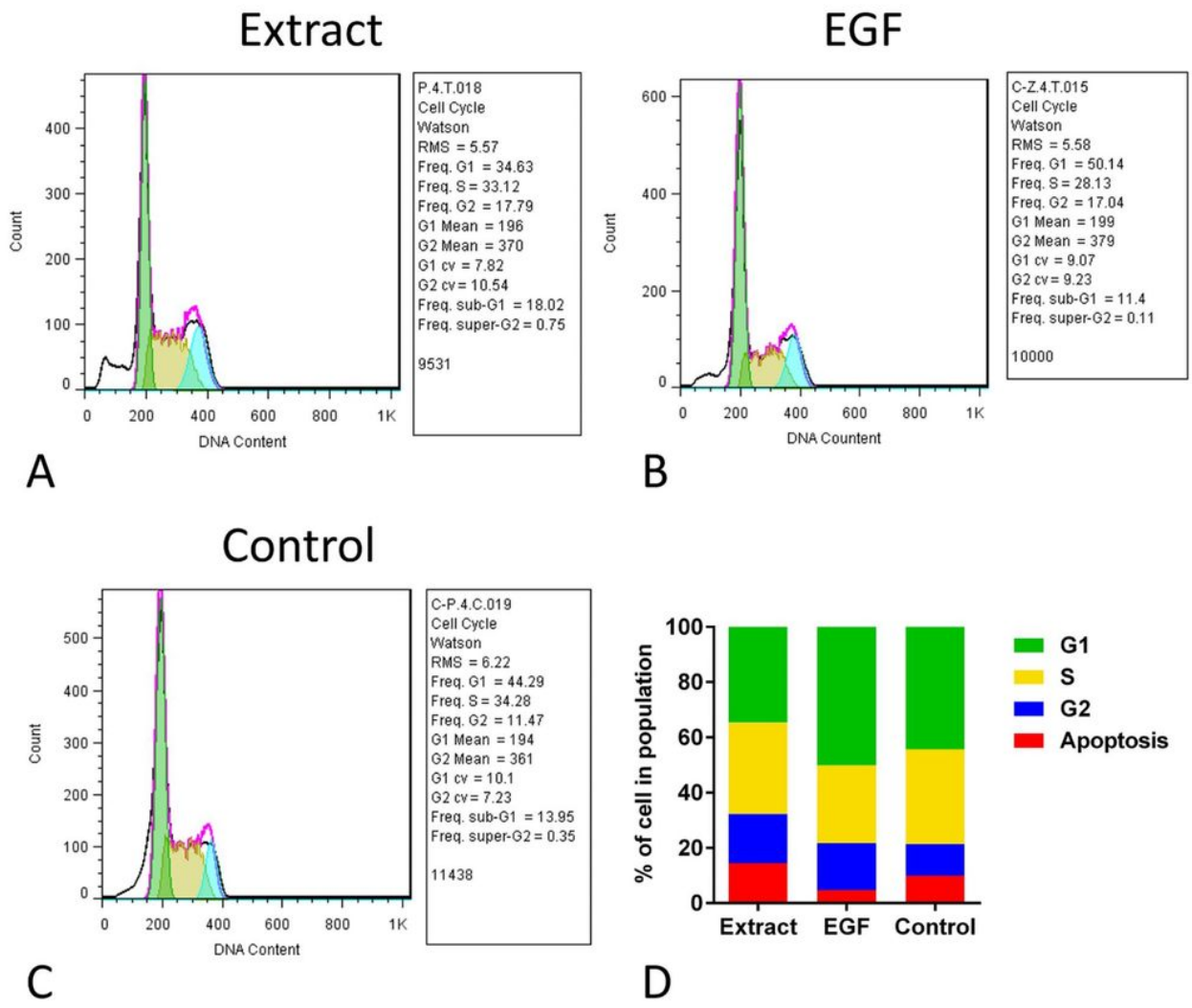

Figure 3.

Figure 3

Cell cycle assay of human umbilical cord mesenchymal stem cells (hUC-MSCs) after exposure to aqueous extract of sea cucumber (Holothuria parva) or epidermal growth factor (EGF).

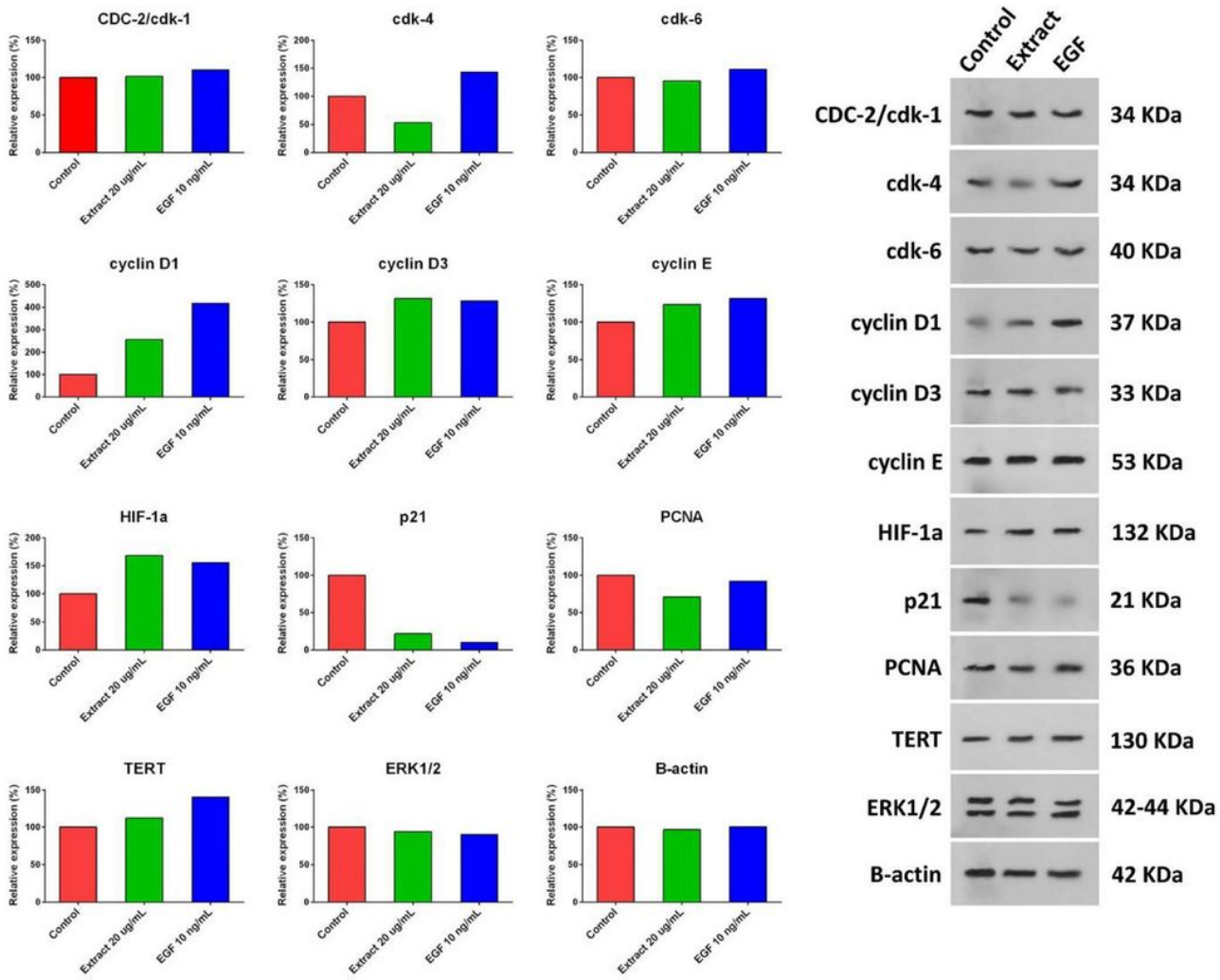

Figure 4.

Figure 4

Expression of proliferation-related proteins after treating of human umbilical cord mesenchymal stem cells (hUC-MSCs) with aqueous extract of sea cucumber (Holothuria parva) or epidermal growth factor (EGF).

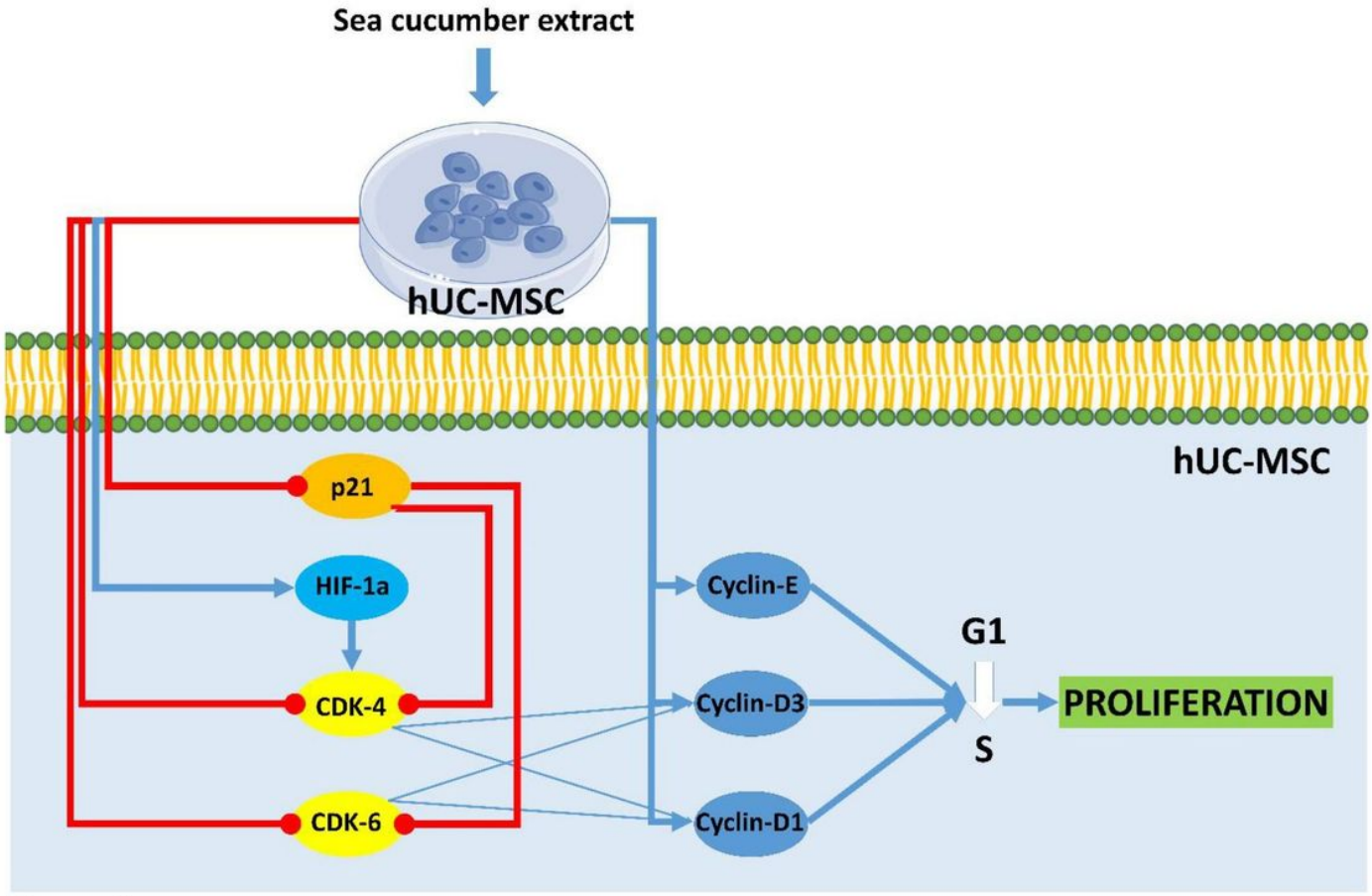

Figure 5.

Figure 5

Aqueous extract of sea cucumber (Holothuria parva) induces proliferation in human umbilical cord mesenchymal stem cells (hUC-MSCs). Small colorful dots, red lighting, blue arrows and red hammer-headlines represent the extract bioactive molecules were detected by docking technique, the extract-induced stimulus, direct reactions and each inhibiting reaction, respectively.

Supplementary Files

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- Supplementaryfile.docx