Research in Pharmaceutical Sciences, April 2016; 11(2): 130-137
Received: July 2015
Accepted: Sept 2015

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Effect of *Holothuria leucospilota* extracted saponin on maturation of mice oocyte and granulosa cells

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Abstract

Sea cucumbers saponins are triterpenoid glycosides which exert beneficial biomedical effects. This study was performed to assess the effect of saponin extracted from sea cucumber *Holothuria leucospilota* (*H. leucospilota*) on maturation of mice oocytes and granulosa cells. The germinal vesicles oocytes were collected from 6–8 weeks old Naval Medical Research Institute (NMRI) mice ovaries, randomly divided into untreated and four experimental groups and cultured *In vitro.* Maturation medium was supplemented with 0, 1, 2, 4 and 8 µg/ml saponin for 12 days. The rates of maturation were recorded through morphological observation by measurement of follicle diameter during treatment. After 4 days, the effects of saponin on granulosa cells were investigated by reactive oxygen species (ROS) measurement, super oxide dismutase (SOD) activity, caspase assay and tumor necrosis factor-alpha (TNF-α) expression. The oocyte maturation rate was significantly higher in treated groups (1 µg/ml). The ROS and SOD assays demonstrated the antioxidant potential of saponin. The caspase assay exhibited that optimum concentrations of saponin (1, 2 µg/ml) reduced caspase activity in granulosa cells. Flow cytometry showed that optimum concentration of saponin promoted oocyte maturation via down regulation of TNF-α as follicular degenerative factor in nursing cells. These results proposed that maturation rate were obtained after the incorporation of 1 µg/ml sea cucumber saponin. Moreover, the extracted saponin at concentrations of 1, 2 µg/ml enhanced follicle growth which is accompanied by attenuating ROS formation, elevating SOD activity and reducing TNF-α expression in granulosa cells. But, further examinations are required to understand precise mechanisms of saponin action on oocyte and granulosa cells.

Keywords: Oocyte maturation; Granulosa; Sea cucumber; *Holothuria leucospilota*; Saponin; Apoptosis

INTRODUCTION

In vitro maturation (IVM) is considered as one of the important tools of assisted reproduction technology (ART) (1). It is estimated that about 8–12% of couples around the world are infertile (2). In addition, the rate of infertility has been considered as a main public health issue by the World Health Organization (WHO) (3).

The two main subjects including fertility preservation and treatment are at the forefront of reproductive health issues, therefore, it is vital to introduce the concept of maturing of immature oocytes *in vitro* (4). Ovarian follicles represent the crucial microenvironment for oocyte maturation and are considered as chief structural and functional units of female reproductive system. Pre-antral follicles provides a prospective source of oocytes to evaluate *in vitro* maturation and fertilization (5,6).

Oxidative stress has been linked to follicle growth inhibition and apoptosis. The enhanced generation of ROS can lead to changes of cellular molecules such as lipids, proteins and nucleic acids, because ROS exert a detrimental effect on early *in vitro* embryo development.

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Therefore, application of antioxidants can prevent harmful effects of oxidative stress associated to maturation of oocytes (7,8).

In vivo, maturation of oocyte is a complicated process which include the function of regulatory factors (gonadotropin and secreted molecules) and interaction of the oocyte and cumulus cells (9). During recent years, it has been shown that natural products have played important role in promoting health and preventing various diseases (10).

The marine environment has been considered as a source of useful entities in the development of new medicinal agents and diverse bioactive natural products which have shown a great potential in the drug discovery (11).

During the past decade, growing attention has been centered towards discovery of novel drugs from marine organisms due to containing pharmacologically bioactive compounds (12,13). Therapeutic and medicinal potentials of sea cucumbers are associated with the presence of an extensive array of naturally occurring substances such as glycosaminoglycan, chondroitin sulfates, sulfated polysaccharides, glycoprotein, lectins, essential fatty acids, peptides and triterpene glycosides (saponin) (14). Saponins are proposed as valuable bioactive compounds in sea cucumber, which are composed of triterpene, or steroid aglycones (sapogenins) and sugar side chains with various medicinal activities (15-17).

Many of surveys, have expressed the hemolytic, antitumor and anti-inflammatory effects of saponins (18-20) and investigated the cytotoxic effect of saponin extracted from sea cucumbers on different cell lines and established its strong cytotoxic effect against cancer cells. However, some of evidences demonstrated that saponin significantly affect reproduction in animals (21). Nevertheless, little is known regarding the role of sea cucumber saponin in maturation of oocyte and granulosa cells. Therefore the aim of this study was to investigate the effect of Persian Gulf sea cucumber (*Holothuria leucospilota*) saponin on *in vitro* growth and the maturation of immature mouse oocyte and its effect on granulosa cells.

MATERIALS AND METHODS

Minimum Essential Medium (α-MEM), fetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA), insulin transferrin selenium (ITS) and antibiotic (Penicillin-streptomycin), follicle stimulating hormone (FSH) and human chorionic gonadotropin (HCG) were obtained from Gibco (USA). Specimens of the sea cucumber (*H. leucospilota*) were obtained from the rocky intertidal flats of the Persian Gulf. All solvents were purchased from Merck (Germany). Caspase-3 colorimetric assay kit and tumor necrosis factor-alpha (TNF-α) primary antibody were purchased from Abcam (England). SOD kit and DCF-DA were purchased from Sigma, USA. All the animal procedures (care, handling, and all of the experiments performed) were approved by the ethical committee of the Kharazmi Institute, Mashhad Islamic Azad University.

Preparation of the sea cucumber saponin extract

Extraction and identification of sea cucumber crude saponin were conducted according to the previous study (22). Briefly, Persian Gulf sea cucumber *Holothuria leucospilota* (*H. leucospilota*) were collected from the Coast of Qeshm, Iran and were transferred to Kharazmi research laboratory. After washing, the sea cucumber body walls were air-dried, grounded, and stored in 70% ethanol, at room temperature for 2 days to release the temperature-sensitive mixtures. Then supernatant were refluxed in ethanol, filtered by Whatman paper 1 μm and evaporated on a rotary evaporator (Heidolph, Germany). The filtrate was diluted in dichloromethane/water for 3 h. The water phase was extracted using *n*-butanol. In the next step, the organic layer was evaporated, dissolved in water, and loaded into a Diaion HP-20 resin column. The column was rinsed with deionized water and 80% or 100% ethanol. The 80% ethanol fraction were air-dried and lyophilized to obtain crude saponin (20).

Collection of pre-antral follicles

In this *in vitro* experimental study, oocytes were obtained from 6–8 week old NMRI female mice (Razi institute, Mashhad, Iran).

Animals were kept under controlled conditions (12 h light/12 h dark) and fed with water *ad libitum*. The animals were slaughtered by cervical dislocation and their ovaries transferred into dissecting medium which contained α-MEM supplemented with 5% FBS, 1% antibiotic, FSH and 10 IU/ml HCG (1IU/L = 2.93 pmol/L). Germinal vesicle-stage oocytes (GV) of the ovarian follicles were dissected with a 31-gauge sterile needle under a stereomicroscope and acquired pre-antral follicles (120-150 µm) were used for the IVM procedure (9).

In vitro maturation

IVM process was performed on the basis of Rajabi-Toustani and coworkers protocol with some modifications (7).

Maturation medium consisted of α-MEM supplemented with, 5% FBS, 7.5 IU/ml recombinant human rhFSH (Organon, Holand), 100 IU/ml HCG (Organon, Holand) and 5 ng/ml ITS (Gibco, USA). The preantral follicles were cultured in α-MEM supplemented with different concentrations of sea cucumber saponin (0, 1, 2, 4, and 8 µg/ml) and were covered with mineral oil for 12 days. Then, the effect of sea cucumber saponin on follicular growth and maturation was analyzed. Follicular viability was evaluated by Trypan blue staining. At various intervals from the onset of incubation, oocytes were observed by inverted microscope (Nikon, Japan), and nucleus morphological changes of GV and germinal vesicle break down were evaluated, or the extrusion of first polar body (Metaphase II: MII) using inverted microscope observed quantitatively.

Evaluation of oxidative stress using ROS measurement

Intracellular ROS levels were determined in granulosa cells after 96 h using the method modified previously (23). Briefly, following cell exposure to 24 mM H_2O_2 at 37 °C for 30 min, 2', 7'-dichlorofluorescein diacetate (5 µm) was added to untreated and treated granulosa cells for 30 min at 37 ºC. Cells were washed with phosphate buffer saline (PBS). Fluorescence was then measured (excitation 492 nm; emission 540 nm) using a plate reader (Epoch, USA).

Antioxidant enzyme activity assays

Granulosa cells were collected after 96 h exposure to sea cucumber saponin and subjected to SOD enzyme activity assay according to the manufacturer's instructions (23). All samples were run in duplicate from at least three separate experiments.

Caspase-3 activity assay

The level of apoptosis in granulosa cells under exposure with different concentrations of sea cucumber extracted saponin was performed using colorimetric Caspase-3 Assay kit following the manufacturer's instructions (24). Untreated and treated granolusa cells were incubated with different concentrations of saponin for 96 h. Caspase activity was measured using a micro plate reader (Epoch, USA) at wavelength 405 nm.

TNF- alpha expression by flow cytometry

To study the expression of TNF-α in follicular granulosa cells flow cytometry was performed (24). Four days after incubation, the granulosa cells were trypsinized, and then transported to eppendorfs and centrifuged at 2,000 rpm for 10 min at 4 °C. After rinsing with PBS and centrifugation, the primary antibody TNF-α (Ab1793 Abcam) at concentration of 1:100 was added to supernatant and placed in the refrigerator overnight. Later, after washing and centrifugation, fluorescein isothiocyanate (FITC) conjugated secondary antibody was added at a concentration of 1:20 and incubated in the refrigerator for 45 min. Then supernatant washed and centrifuged again and 300 µl of formalin 0.1 % added to supernatant for evaluating TNF-α expression using flow cytometry (Becton Dickinson, USA).

Statistical analysis

The data are reported as mean ± standard deviation (SD). Statistical analysis was performed using SPSS software. One-way analysis of variance (ANOVA) followed by Duncan test was used to perform statistical

analysis. $P<0.05$ was considered statistically significant.

RESULTS

Effects of sea cucumber saponin on maturation parameters

In this study, follicles were cultured for 12 days in IVM medium supplemented with various concentrations of sea cucumber saponin (Fig. 1). The effect of saponin concentrations on granulosa cells and oocyte maturation is presented in Table 1. The assessment of follicle viability showed a significant improvement in follicles treated with 1 µg/ml concentration of saponin (P = 0.035) compared to control, 2, 4 and 8 µg/ml saponin treated groups; however, no significant difference observed between control and experimental groups other than 1 µg/ml.

The rate of arrested oocytes at GV stage improved significantly in 1 µg/ml saponin treated group (P = 0.030) compared with control; however, no significant difference observed at percent GV stage between control and other experimental groups. Percent antral-like cavity formation in treated group (1 µg/ml saponin) was significantly higher (P = 0.025) than control and other treatment groups.

Our results showed that the percent oocyte maturation increased at 1 µg/ml of sea cucumber saponin, (***P = 0.001). Further, the incubation of pre-antral follicles with 4, 8 µg/ml sea cucumber saponin decreased the oocyte growth and maturation as compared with the control.

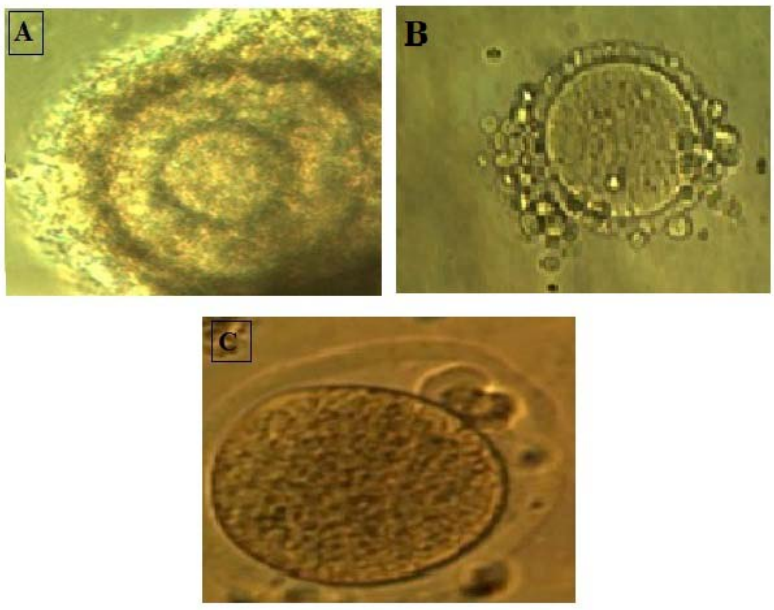

Fig. 1. A; Prenatal follicle, B; Germinal vesicle (GV) stage, C; Oocytes maturation (polar body) (× 400).

Table 1. The survival and growth of preantral follicles at various concentrations of sea cucumber saponin. The data were analyzed using one way ANOVA, Duncan test and represented as mean ± SD.

Saponin concentrations	Total	Viability (%)	GV (%)	Antrum (%)	Maturation (%)
0	125	62 ± 4.58	46 ± 4.00	42 ± 4.35	28 ± 3.66
1	73	77 ± 5.00*	36 ± 5.57*	61 ± 6.00*	43 ± 5.57***
2	82	68 ± 5.57	42 ± 5.57	53 ± 5.57	35 ± 8.45
4	71	39 ± 7.22	57 ± 2.51	49 ± 2.34	23 ± 34.0
8	81	17 ± 6.21	70 ± 4.35	25 ± 4.35	11 ± 5.29

Significant improvement was observed in follicles (P = 0.035)*, the rate of arrested oocytes at GV stage (P = 0.030)*, antral-like cavity formation (P = 0.025)*, and oocyte maturation (P = 0.001)*** treated with 1 µg/ml concentration of saponin compared to control, 2, 4 and 8 µg/ml saponin treated groups.

Effects of sea cucumber saponin on ROS formation

Effect of sea cucumber extracted saponin on oxidative stress levels of granulosa cells was evaluated by measurement of ROS production. The results indicated that higher concentrations of examined saponin (4, 8 µg/ml with $P<0.01$, $P<0.001$, respectively) significantly increased the level of ROS in granulosa cells as compared with untreated cells (Fig. 2) suggesting that higher concentrations of sea cucumber saponin induces oxidative stress and therefore inhibits the growth of pre-antral follicles. Meanwhile sea cucumber saponin (1 µg/ml with $P<0.05$) that promoted maturation of oocytes remarkably reduced the levels of ROS.

Effect of sea cucumber saponin on SOD antioxidant enzyme level

Since sea cucumber saponin inhibited oxidative stress, we then evaluated whether extracted saponin affects the activities of SOD as endogenous antioxidant enzyme in granulosa cells. Our results showed that amongst concentrations of sea cucumber saponin which promoted maturation (1 µg/ml) significantly enhanced the activity of SOD, but the higher concentrations of saponin (4, 8 µg/ml) significantly suppressed the SOD activity ($P<0.05$ (Fig. 3).

Effects of sea cucumber saponin on caspase activation

To assess whether apoptosis has any function on the inhibitory activity of extracted saponin on the granulosa cells, caspases-3 activity was measured in the absence or in the presence of sea cucumber saponin (1–8 µg/ml) for 96 h. As shown in Fig. 4, extracted saponin at lower concentrations (promoting oocyte maturation: 1, 2 µg/ml) highly protected granulosa cells from caspase-3 activity after 96 h. But, the cytotoxic concentrations of extracted saponin (4, 8 µg/ml) increased caspase activity demonstrating apoptosis is correlated with cytotoxic concentration of examined saponin.

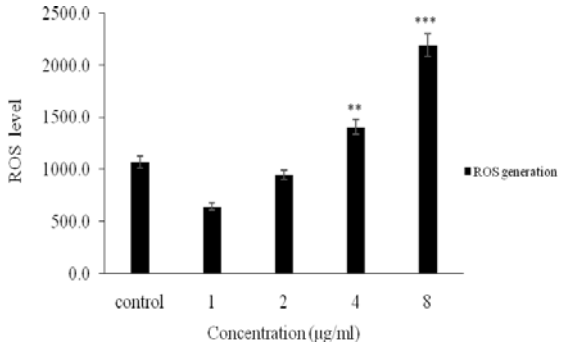

Fig. 2. Effect of sea cucumber saponin on intracellular ROS level in granulosa cells. Cells were treated with 1, 2, 4, 8 µg/ml sea cucumber saponin. Results are represented relative to the control. $^{**}P<0.01$, $^{***}P<0.001$.

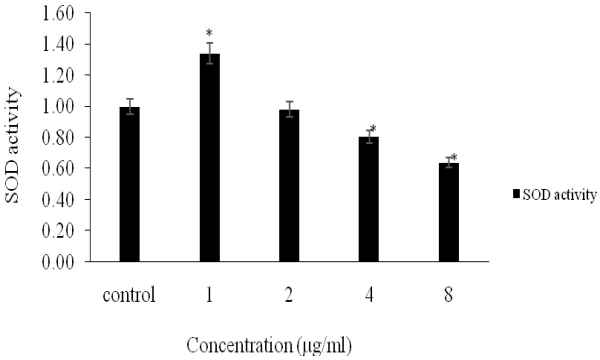

Fig. 3. Effect of sea cucumber saponin treatment on SOD activity in granulosa cells. The granulosa cells were exposed with extracted saponin (1, 2, 4, 8 µg/ml) for 96 h and subjected to SOD activity. Data are presented as means ± SD. Asterisk (*) shows a significant differences with control group.

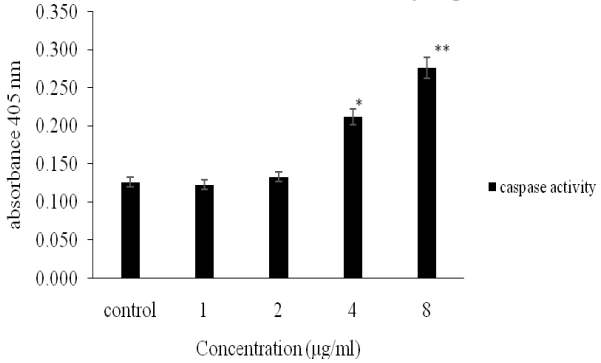

Fig. 4. Effect of sea cucumber saponin treatment on caspase-3 activity in granulosa cells at 1, 2, 4 and 8 µg/ml of saponin. $^{*}P<0.05$, $^{**}P<0.01$ compared to control group.

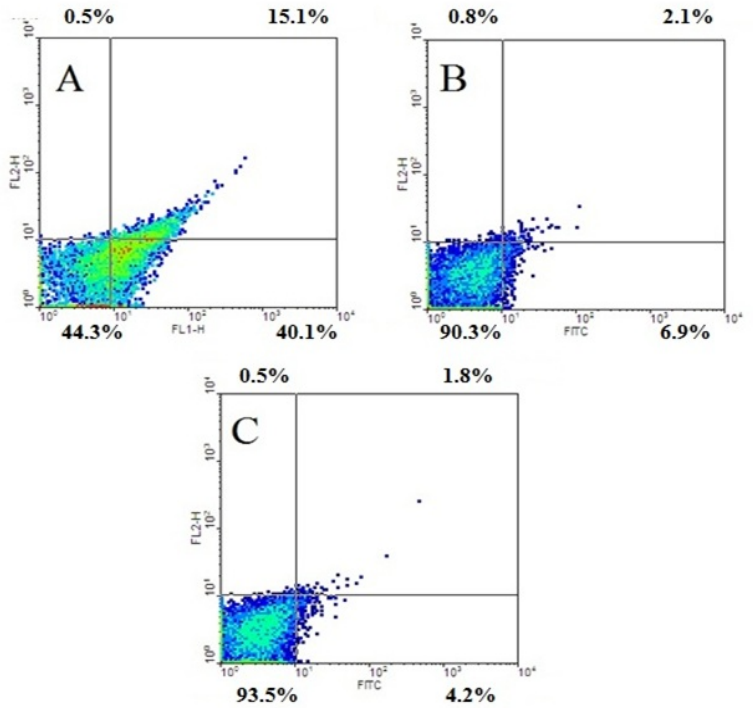

Fig. 5. The measurement of TNF-α expression in untreated and saponin treated groups (1, 2 µg/ml) using flow cytometry: A; The expression of TNF-α in control (untreated group) was 55.7%. B; the expression of TNF-α in treated groups at concentration of 1 µg/ml of sea cucumber saponin was 9.6 %. C; the expression of TNF-α in treated groups at concentration of 2 µg/ml sea cucumber saponin was 6.5%.

Effects of sea cucumber saponin on TNF-α expression

Flow cytometry diagrams exhibited that saponin significantly down regulated TNF-α expression level in a dose-dependent manner in granulosa cells. The expression of TNF-α in the control sample was found to be 55.7 %, while in saponin treated groups (1 and 2 µg/ml) were 9.8 % and 6.5 %, respectively (Fig. 5).

DISCUSSION

Conflicts are presented in the literature with regard to the effect of saponins on reproduction systems. Nevertheless, most of surveys have been reported about terrestrial saponin compounds. The negative effects of terrestrial saponins on animal reproduction have been introduced these bioactive compounds as abortifacient metabolites (21).

To the best of our knowledge, there are no reports on the effects of sea cucumber saponin on efficacy of maturation medium of oocytes. An appropriate *in vitro* maturation system was considered as a chief phase of *in vitro* embryo production process because of its role in subsequent embryonic development (25).

The results of this study indicated that the sea cucumber saponin at concentrations 1, 2 µg/ml positively affected *in vitro* maturation of oocytes, while lower concentrations of sea cucumber saponin 1 µg/ml induced higher rates of oocyte maturation. In the contrary, sea cucumber saponin at higher concentrations (4, 8 µg/ml) significantly reduced *in vitro* maturation as described earlier with terrestrial saponins.

It is reported that the terrestrial steroid saponin directly suppress the proliferation of FSH-modulated granulosa cells in the ovarian follicle. Their mechanism of action appear to exhibit more than a simple permeabilising effect on secretary cell membranes and could probably be attributed to interactions between steroidal saponins and steroid receptors (21).

Further, the present study revealed that sea cucumber saponin at concentration of 1 µg/ml (oocyte maturation promoting concentration), increased growth of mouse antral follicles,

reduced caspase activity, ROS formation, TNF-α protein levels and induced the activity of SOD. Meanwhile, the sea cucumber saponin at concentration of 4, 8 µg/ml (cytotoxic concentrations) significantly augmented ROS levels, increased activation of caspase and attenuated SOD activity *in vitro*.

Previous literature has demonstrated that TNF-α significantly inhibited FSH-induced follicular development and steroidogenesis (26). Therefore the low expression of TNF-α exposed with sea cucumber saponin (concentration of 1 µg/ml) has confirmed promoting the effect of this compound in maturation of mice preantral follicles.

Recent reports indicated that antioxidants, such as, β-mercaptoethanol and cysteamine enhance the maturation of oocyte and has positive effect on embryo development in mice (27,28). Several investigations have been revealed that the addition of natural antioxidants with minimum side effects can be of potentials in *in vitro* maturation outcomes (29). Hence, the search for novel natural bioactive compounds with antioxidant activity is of importance.

In the previous study, we demonstrated the antioxidant capacity of saponins *in vitro* (22). Several investigations have evaluated pharmacological properties of saponin isolated from different spices of sea cucumber, but information about bioactive compounds isolated from Persian gulf sea cucumbers are limited and scarce (15). The positive effects of antioxidants on the developmental competence of embryos exposed to exogenous oxidative stress has been reported (23). Earlier reports have indicated that promotion of oocyte maturation in pre-antral follicles can be attributed to the antioxidant treatment and up regulation levels of endogenous antioxidant such as glutathione *in vitro* (29, 30).

Our results showed that ROS scavenging effect of sea cucumber saponins may be effective in promotion of *in vitro* maturation. Wang and colleagues have shown that the appropriate concentrations of green tea polyphenols that is considered as an antioxidants in green tea enhances the bovine oocytes maturation and the formation of blastocyst (31). Golkar and coworkers showed that the optimum concentration of *Papaver rhoseas* L., caused the improvement of oocytes maturation, and also the development of subsequent embryo (32). The results of present study support the positive potential of marine triterpenoid saponins in defined concentration on oocyte maturation.

CONCLUSION

Sea cucumber saponin in a given concentrations can positively influence maturation of pre-antral follicles by reducing oxidative stress, TNF-α expression and inducing antioxidant enzyme activity. The detailed mechanism of action in granulosa cells remains to be clarified but might be through a similar mechanism of saponins on cancer cells

ACKNOWLEDGMENTS

Financial support of Kharazmi institute, Mashhad, Iran is greatly appreciated.

REFERENCES

1. Smitz JE, Thompson JG, Gilchrist RB. The promise of *in vitro* maturation in assisted reproduction and fertility preservation. Semin Reprod Med. 2011;29:24-37.
2. Boivin J, Bunting L, Collins J, Nygren K. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. Hum Reprod. 2007;22:1506-1512.
3. Covington S, Burns L. Infertility counseling: A comprehensive handbook for clinicians. 2nd ed. Cambridge: Cambridge University Press; 2006. p. 24-28.
4. Chian RC, Uzelac PS, Nargund G. *In vitro* maturation of human immature oocytes for fertility preservation. Fertil Steril. 2013;99:1173-1181.
5. Shackelford TK, Besser A, Goetz AT. Personality, marital satisfaction, and probability of marital infidelity. Individ Differ Res 2008;6:13-25.
6. Jurema M, Nogueira D. *In vitro* maturation of human oocytes for assisted reproduction. Fertil Steril 2006;86:1277-1291.
7. Rajabi-Toustani R, Motamedi-Mojdehi R, Roostaei-Ali Mehr M, Motamedi-Mojdehi R. Effect of Papaver rhoeas L. extract on *in vitro* maturation of sheep oocytes. Small Ruminant Res. 2013;114:146-151.
8. Ghaffari Sh, Hatami H, Dehghan Gh. Saffron ethanolic extract attenuates oxidative stress, spatial

learning, and memory impairments induced by local injection of ethidiumbromide. Res Pharm Sci. 2015;10:222-232.
9. Ali A, Benkhalifa M, Miron P. *In vitro* maturation of oocytes: biological aspects. Reprod Biomed Online. 2006;13:437-446.
10. Handali S, Moghimipour E, Kooshapour H, Rezaee S, Khalili S. *In vitro* cholestrol binding affinity of total saponin extracted from Glycyrrhiza glabra. Asian J Pharm Clin Res. 2014;7:170-173.
11. Gilchrist R, Thompson J. Oocyte maturation: emerging concepts and technologies to improve developmental potential *in vitro*. Theriogenology. 2007;67:6-15.
12. Butler MS. The role of natural product chemistry in drug discovery. J Nat Prod. 2004;67:2141-2153.
13. Hussain S, Fareed S, Ansari S, Sajid M. Marine natural products: A lead for Anti-cancer. Indian J Geo-Marine Sci. 2012;41:27-39.
14. Bordbar S, Anwar F, Saari N. High-value components and bioactives from sea cucumbers for functional foods--a review. Mar Drugs. 2011;9:1761-1805.
15. Soltani M, Baharara J. Antioxidant and antiprolifereative capacity of dichloromethane extract of *Holoturia leucospilota* sea cucumber. Int J Cell Mol Biotechnol. 2014;2014:ID ijcmb-00013.
16. Moghimipour E, Sadaghi-Nejad B, Handali S, Ameri A, Ramezani Z, Azemi Me. *In Vitro* Screening of anti-candida activity of saponins extracted from Glycyrrhiza glabra and *Quillaja Saponaria*. Asian J Pharm Clin Res. 2014;7: 160-162.
17. Inalegwu B, Sodipo O. Phytochemical screening and haemolytic activities of crude and purified saponins of aqueous and methanolic extracts of leaves of *Tephrosia vogelii* Hook. F. Asian J Plant Sci Res. 2013;3:7-11.
18. Tian F, Zhang X, Tong Y, Yi Y, Zhang S, Li L, *et al*. PE, a new sulfated saponin from sea cucumber, exhibits anti-angiogenic and anti-tumor activities *in vitro* and *in vivo*. Cancer Biol Ther. 2005;4:874-882.
19. Li YX, Himaya SW a, Kim SK. Triterpenoids of marine origin as anti-cancer agents. Molecules. 2013;18:78867909.
20. Hu X, Wang Y, Wang J, Xue Y, Li Z, Nagao K, *et al*. Dietary saponins of sea cucumber alleviate orotic acid-induced fatty liver in rats via PPARa and SREBP-1c signaling. Lipids Heal Dis. 2010;9:1-9.
21. FrancisG, Kerem Z, Makkar HPS, BeckerK. The biological action of saponins in animal systems: a review. Br J Nutr. 2002;88:587-605.
22. Soltani M, Parivar K, Baharara J, Kerachian MA, Asili j. Putative mechanism for apoptosis-inducing properties of crude saponin isolated from sea cucumber (*Holothuria leucospilota*) as an antioxidant compound. Iran J Basic Med Sci. 2015;18:180-187.
23. Kitagawa Y, Suzuki K, Yoneda A, Watanabe T. Effects of oxygen concentration and antioxidants on the *in vitro* developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. Theriogenology. 2004;62:1186-1197.
24. Su X, Xu C, Li Y, Gao X, Y L, *et al*. Antitumor activity of polysaccharides and saponin extracted from sea cucumber. J Clin Cell Immunol. 2011;2: 1-5.
25. Barakat IA, Al-Himaidi AR. Effects of fenugreek seed extract on *in vitro* maturation and subsequent development of sheep oocytes. Pakistan J. Zool. 2013;45:1679-1686.
26. Abdo M, Hisheh S, Arfuso F, Dharmarajan A. The expression of tumor necrosis factor-alpha, its receptors and steroidogenic acute regulatory protein during corpus luteum regression. Reprod Biol Endocrinol. 2008;6:50-55.
27. Eimani H, Hassani F, Haeri Rouhani Sa, Nasr Esfahani MH, Dalman A, Rezazadeh Valojerdi M, *et al*. Effect of B-Mercaptoethanol With and without Bso (Dl-Buthionine Sulfoximine) On resumption of meiosis, *in vitro* maturation and Embryo development of immature mouse oocytes. yakhteh. 2006;7:234-241.
28. Eimani H, Hasani F, Haeri Rohani S, Nasr Esfahani M, Rezazadeh M, Dalman A, *et al*. Effect of Cysteamine on *in vitro* maturation, resumption of meiosis and embryo development of immature mouse Oocytes. Yakhteh. 2005;7:1-55.
29. Eimani H, Hassani F, Haeri Rohani S, Nasr Esfahani M, Rezazadeh M, Dalman A. Effect of glutathione on resumption of meiosis *in vitro* maturation and development of immature mouse oocytes. Lournal of Mazandaran University of Medical Sciences. 2005;15:1-10.
30. Lv L, Yue W, Liu W, Ren Y, Lee K-B, Smith GW. Effect of oocyte selection, estradiol and antioxidant treatment on *in vitro* maturation of oocytes collected from prepubertal Boer goats. Ital J Anim Sci. 2010;9:50-54.
31. Wang ZJ, Yu SD, Xu ZR. Effect of supplementation of green tea polyphenols on the developmental competence of bovine oocytes *in vitro*. Braz J Med Biol Res. 2007;40:1079-1085.
32. Golkar-Narenji A, Eimani H, Samadi F, Hasani S, Shahverdi AH, Eftekhari-Yazdi P, *et al*. Effect of Papaver rhoeas extract on *in vitro* maturation and developmental competence of immature mouse oocytes. Reprod Med Biol. 2010;9:211-215.