

# Effects of *Matricaria chamomilla* Extract on Growth and Maturation of Isolated Mouse Ovarian Follicles in a Three-dimensional Culture System

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## Abstract

**Background:** The aim of this study was to design and assess the effects of hydroalcoholic extract of *Matricaria chamomilla* (MC) on preantral follicle culture of mouse ovaries in a three-dimensional culture system.

**Methods:** Isolated preantral follicles were randomly divided into three main groups: the control group containing 10% fetal bovine serum without MC extract (G1), the first experimental group supplemented with 25 µg/ml hydroalcoholic extract of chamomile (G2), and the second experimental group supplemented with 50 µg/ml hydroalcoholic extract of chamomile (G3).

**Results:** After 12 days of culture, the survival rate ( $P < 0.05$ ), antrum formation ( $P < 0.01$ ), metaphase two oocytes ( $P < 0.01$ ), and the expression of *PCNA* ( $P < 0.05$ ) and *FSHR* ( $P < 0.05$ ) genes significantly decreased in G3 as compared with G1. On the other hand, at the last day of culture (day 12), the mean diameter of follicles cultured in the medium which was supplemented with 50 µg/ml hydroalcoholic extract of chamomile significantly decreased as compared with the G1 ( $P < 0.05$ ). In addition, the levels of progesterone and dehydroepiandrosterone hormones significantly increased in the medium of G3 relative to G1 ( $P < 0.01$ ), while in the medium of G1, the level of 17β-estradiol was significantly higher than that of other groups ( $P < 0.01$ ). Reactive oxygen species levels of metaphase II oocytes were significantly decreased in G2 as compared with G1 ( $P < 0.01$ ).

**Conclusion:** Adding chamomile extract to culture media appeared to decrease follicular function and development.

**Key words:** Follicular Development; Follicular Maturation; *FSHR* Gene; *PCNA* Gene; Steroid Hormones; Toxicology

## INTRODUCTION

For many centuries, herbal medicine, which also called botanical medicine or phytomedicine, has been one of the most important solutions for medical therapy in ancient nations.<sup>[1]</sup> Ancient Chinese, Egyptians, Iranians, and Indians have used plants' seeds, roots, leaves, and/or flowers for medicinal purposes.<sup>[2]</sup> There are several species of herbal plants of which several different combinations exist. Nowadays, many chemical analyses have been carried out on plant extracts to identify various compounds of extract. Many combinations such as sterols, flavonoids,

organic sugars, and phenolic compounds were found in the extracts of herbal plants.<sup>[3]</sup> Furthermore, several studies have shown that medicinal plants could play

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an important role in treating many conditions such as allergies, asthma, eczema, and diabetes mellitus.<sup>[4,5]</sup> On the other hand, the World Health Organization estimates that 80% of people worldwide are using herbal medicines for their health care.<sup>[6]</sup> One of the herbal plants is *Matricaria chamomilla* (MC). MC, which is known as chamomile, is a limb of the composite family *Asteraceae* that in the traditional medicine has been used as a drug for treating flatulence, colic, hysteria, wounds, and intermittent fever in many countries from Europe to Asia.<sup>[7-9]</sup> Studies have shown that both lipophilic and hydrophilic forms of chamomile extract are effective for therapeutic activities.<sup>[1,10,11]</sup> The most characteristic constituents of chamomile are unstable oil, sesquiterpene lactones, ascorbic acid, and phenol compounds, primarily the flavonoids, apigenin, quercetin, patulin, luteolin, and glycosides.<sup>[11]</sup> Flavonoids are chemical phenyl benzopyrones which are usually observed in all vascular plants. The benzopyran ring system is a molecular scaffold which can be seen in flavonoid-inherent products and has weak aromatase inhibitory activity.<sup>[11]</sup> One of the Chamomilla compounds is flavonoid antioxidants that neutralize reactive oxygen metabolites.<sup>[12-14]</sup> Antioxidants are human made or natural substances that prevent the formation of free radicals and lipid peroxidation. Antioxidants by binding to free radicals neutralize their destructive properties, such as breakdown of body cells and tissue, DNA fragmentation, and membrane lipid peroxidation. On the other hand, in two- or three-dimensional mouse follicle culture systems, follicles are kept under higher concentrations of O<sub>2</sub> in an incubator.<sup>[15]</sup> During *in vitro* follicle culture, free radicals are continuously produced in aerobic cells; thus antioxidants can lead to removal of free radicals.<sup>[16]</sup> Phytoestrogens are one of the other important compounds of Chamomilla.<sup>[17]</sup> It is a naturally occurring plant compound which is considered as an estrogen-like compound that has similar effects to estrogen and progesterone hormones.<sup>[18]</sup> Experimental and clinical studies which were performed on chamomile concluded that the majority of their pharmacological functions are pertaining to its antioxidant activity which is mostly due to its capability to control the free radicals and/or inhibit lipid peroxidation.<sup>[19]</sup> In one study, Johari *et al.*<sup>[20]</sup> investigated the effects of hydroalcoholic extract of MC in several doses (10, 20, and 40 mg/kg) on the level of production of follicle-stimulating hormone (FSH), luteinizing hormone, estrogen, and progesterone hormones, and also on the changes in ovarian tissue. Moreover, in another study, Johari *et al.*<sup>[21]</sup> showed that in all of the doses of MC extract, the levels of estrogen decreased and the levels of progesterone increased; also, the number of ovarian follicles decreased. Farideh *et al.*<sup>[11]</sup> reported that treatment with chamomile alcoholic extract decreases the signs of polycystic ovarian syndrome (PCOS) in the ovarian tissue and levels of estradiol hormone. MC extracts due to antioxidant activities have positive effects in the treatments of different diseases, such as PCOS, but

repeated use of MC extract or use of high concentration of it may be harmful. Thus, the aim of this study was to assess the effects of MC hydroalcoholic extract on three-dimensional follicle culture because there were no scientific reports of Chamomilla effects in *in vitro* mouse ovarian follicle culture.

## METHODS

### Preparation of hydroalcoholic extract

In order to prepare the whole-plant chamomile extract, a half kilogram of chamomile flower was dried at 25°C and was protected from direct sunlight. For extraction, after grinding the dried plants, they were dissolved in 2 L of alcohol 96% and then kept at room temperature for 48 h. Over this period, after shaking frequently, the solution was filtered. Then, the solution was centrifuged at 3000 r/min for 5 min. At the end of the process, the resulting solution was poured into an open-top container, and the solvent was evaporated. Some 90 g of a semi-solid extract was obtained from 500 g of chamomile powder. To achieve appropriate concentration, the extract was dissolved in normal saline.<sup>[20,21]</sup>

### Animals and collection of ovarian follicles

In this study, 12- to 14-day-old female National Medical Research Institute mice ( $n = 40$ ) were used. They were maintained in the Laboratory Animals of the University at standard conditions (temperature 25°C, 12 h/12 h light/dark cycle, and 55% humidity). Mice were sacrificed by cervical dislocation, and then immediately their bilateral ovaries were dissected free of fat and mesentery using 29G insulin needles and transferred to dissection medium containing  $\alpha$ -minimum essential medium ( $\alpha$ -MEM, WelGENE) supplemented with 10% fetal bovine serum (FBS) (Gibco, Paisley, UK), 100 IU/ml penicillin, and 100 mg/ml streptomycin. All experimental procedures were conducted according to the Guide for the Care and use of laboratory animals of the Tabriz University of Medical Sciences, Tabriz, Iran.

### Study design

In this stage, 150–170  $\mu$ m intact preantral follicles were mechanically isolated from immature mouse ovaries. Then, they were divided into three main groups: G1, the control group containing 10% FBS without MC extract; G2, the first experimental group supplemented with 25  $\mu$ g/ml hydroalcoholic extract of chamomile; and G3, the second experimental group supplemented with 50  $\mu$ g/ml hydroalcoholic extract of chamomile. Moreover, the base of the culture medium of follicles for three groups was composed of  $\alpha$ -MeM supplemented with 0.33 mmol/L sodium pyruvate, 100 mIU/ml recombinant FSH (or Gonadotropin-releasing hormone, Serono, Switzerland), 1% insulin (Gibco, Paisley, UK), transferrin (Gibco, Paisley, UK), 100  $\mu$ g/ml penicillin, 50  $\mu$ g/ml streptomycin, and FBS 5%. The isolated follicles were cultured for 12 days at 37°C in 5% CO<sub>2</sub>. Furthermore, every other day, half of the medium was replaced by fresh medium.<sup>[22]</sup> At the end of culture period, analysis of developmental genes (*PCNA* and *FSHR*)

and steroid hormones (17- $\beta$  estradiol, progesterone, and dehydroepiandrosterone (DHEA)) was performed and also the released metaphase II (MII) oocytes in three groups of culture were collected and randomly considered for reactive oxygen species (ROS) assay.

### Three-dimensional *in vitro* culture of isolated preantral follicles

The preantral follicle was encapsulated in sodium alginate.<sup>[22]</sup> Briefly, alginate was dissolved in deionized water at a concentration of 1% (w/v) and was then mixed with activated charcoal (0.5 g of charcoal per gram of sodium alginate). Following charcoal treatment, the prepared sodium alginate solution was sterile filtered through 0.22- $\mu$ M filters. Then, aliquots of charcoal-stripped and sterilized sodium alginate were diluted with sterile phosphate-buffered saline (PBS) at a concentration of 0.5% (w/v) at room temperature for experimental study.<sup>[23]</sup> Each isolated follicle was individually transferred to the 8  $\mu$ l of alginate solution and then they were slowly immersed in 140 mmol/L NaCl and 50 mmol/L CaCl<sub>2</sub> for 2 min.<sup>[23]</sup> At the end of the process, all alginate droplets were removed and washed in  $\alpha$ -MeM media. Then, they were transferred into 30-mm Petri dishes (SPL Life Science Co., Seoul, South Korea) which were previously filled with 30  $\mu$ l of culture medium (each plate was filled with 25 droplets of 30  $\mu$ l of culture medium) overlaid with mineral oil (Sigma-Aldrich, Munich, Germany). The isolated follicles were cultured for 12 days at 37°C in 5% CO<sub>2</sub>.

### Assessment of follicular morphology and diameter

During the culturing period, the morphology of follicles was checked under an inverted microscope every 48 h. In addition, after the follicular photographs were taken at  $\times 100$  magnification under an inverted microscope with transmitted light and phase objectives (Leica, Bannockburn, IL, USA), measurement of follicular diameter was carried out by ImageJ software (ImageJ, NIH, USA) ( $n = 30$  for each group).

### *In vitro* ovulation induction

On day 12 of culture, by adding 1.5 IU/ml human chorionic gonadotropin (hCG; Organon, Netherlands) to the culture media, oocyte maturation and ovulation were induced. The oocytes are considered as germinal vesicle (GV) stage if the GV is visible, but if not, they are recorded as GV breakdown. On the other hand, if a polar body is present in the perivitelline space, the oocytes are classified as MII. They were assessed 18–24 h after adding hCG to the culture media.

### Hormonal assays

At the end of the culture period, day 12, the levels of 17- $\beta$  estradiol (E2), progesterone (P4), and DHEA hormones were measured in collected media derived from follicle culture. The levels of E2 were measured by a Microplate Enzyme Immunoassay kit (monobind, sensitivity = 6.5 pg/ml), of P4 by an enzyme-linked immunosorbent assay kit (DIAPLUS, sensitivity = 0.1 ng/ml), and of DHEA by a Microplate Enzyme Immunoassay kit (monobind, sensitivity = 0.04 mg/ml). These experiments were minimally done in triplicates.

### RNA extraction and cDNA synthesis for molecular assessment

In order to evaluate gene expression, some follicles in all of the study groups were collected in three replicates (15 follicles in each replicate) at day 12 of culture. Total RNA was extracted from each group using a TRIzol reagent extraction method (Invitrogen, Paisley, UK). The RNA concentration was determined by spectrophotometry and adjusted to a concentration of 400 ng/ml. Using oligo dT, RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase. Sequences for gene-specific *PCNA*, *FSHR*, and *GAPDH* primers were as follows: *PCNA* forward: AGGAGGCGGTAACCATAG, *PCNA* reverse: ACTCTACAACAAGGGGCACATC; *FSHR* forward: CCAGGCTGAGTCGTAGCATC, *FSHR* reverse: GGCGGCAAACCTCTGAACT; and *GAPDH* forward: GGAAAAGAGCCTAGGGCAT, *GAPDH* reverse: CTGCCTGACGGCCAGG.<sup>[24]</sup> *GAPDH* gene was used as an internal control.

### Real-time reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed on Applied Biosystems (UK) by SYBR Green quantitative RT-PCR Kit (Sigma-Aldrich). The real-time thermal condition included holding step at 1 cycle at 95°C for 10 min (as an initial denaturation), cycling step, 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s, and a final extension step, the melt curve step, at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Then, quantitative analysis of the genes in two groups was done by Pfaffl method ( $2^{-\Delta\Delta C_t}$ ,  $\Delta\Delta C_t = \Delta C_{t_{\text{Sample}}} - \Delta C_{t_{\text{Control}}}$ ).<sup>[25]</sup>

### Reactive oxygen species assay

ROS levels were measured in the MII oocytes obtained from all *in vitro* culture groups ( $n = 24$  for each group for three repeats). Eight MII oocytes were pooled in the RNase-free microtube and were washed three times with PBS. In step one that was done in a dark room, pooled MII oocytes were incubated in 40 mmol/L of Tris-HCl buffer at pH = 7.0 containing 5 mmol/L 2',7'-dichlorodihydrofluorescein diacetate (Sigma-Aldrich, Germany) at 37°C for 30 min. In step two, they were washed with PBS and then sonicated at 50 W for 3 min, and immediately centrifuged at 3000  $\times g$  for 10 min at 4°C. In the last step, the supernatants were collected and monitored using a spectrofluorometer at 488 nm excitation and at 525 nm emissions.<sup>[26]</sup>

### Statistical analysis

Statistical analysis was carried out by IBM SPSS Statistics 22.0 Software (IBM Corp., Armonk, NY, USA). Data were tested for normality analysis of the parameters with Kolmogorov-Smirnov test. The results were expressed as mean  $\pm$  standard deviation (SD) and were assessed by one-way analysis of variance. Tukey's honestly significant difference was used for *post hoc* tests. Differences with  $P < 0.05$  were considered statistically significant.



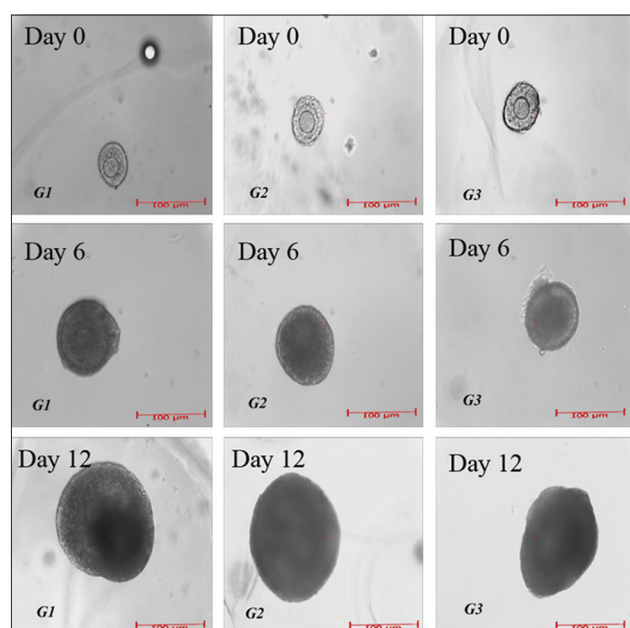
## RESULTS

### The diameter of cultured isolated preantral follicles

The inverted microscope images of the cultured isolated follicles in the three-dimensional culture system are demonstrated in Figure 1. For all groups of the culture, the mean diameter of follicles at the beginning of culture was  $160 \pm 5 \mu\text{m}$ . On day 12 of culture, the mean diameter of cultured follicles was significantly decreased in G3 as compared with G1 ( $P < 0.05$ ). Moreover, on day 6 of culture, the mean diameter of cultured follicles in the G2 and G3 decreased in comparison to G1, although it was not significant ( $P > 0.05$ ). Furthermore, in all groups of the study, the size of preantral follicles increased during the culture period [Figure 2].

### Follicular developmental rate

The developmental rate of cultured follicles in all groups of the study is summarized in Table 1. At the end of



**Figure 1:** The inverted microscopic images of three-dimensional *in vitro* follicular development in alginate on day 0 (first row), day 6 (second row), and day 12 (third row) in G1 (the control group containing 10% FBS without MC extract), G2 (the first experimental group supplemented with 25  $\mu\text{g/ml}$  hydroalcoholic extract of Chamomile), and G3 (the second experimental group supplemented with 50  $\mu\text{g/ml}$  hydroalcoholic extract of Chamomile). FBS: Fetal bovine serum; MC: *Matricaria chamomilla*.

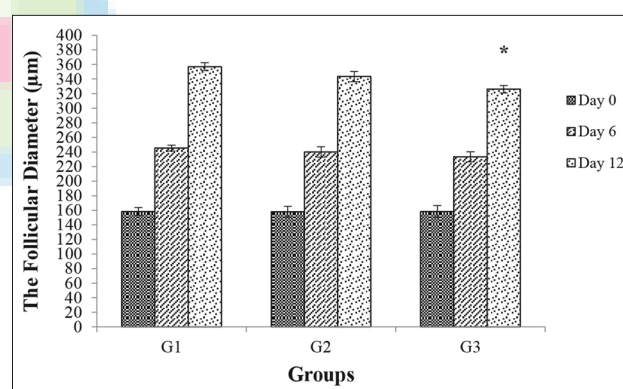
the culture period, day 12, the survival rate of follicles in G3 as compared with other groups was significantly decreased ( $P < 0.05$ ); therefore a higher percentage of survival rate was observed in G1. Moreover, our results showed that the survival rate between both experimental groups has a significant difference. In addition, the rate of antrum formation of follicles cultured in the medium supplemented with 50  $\mu\text{g/ml}$  and 25  $\mu\text{g/ml}$  hydroalcoholic extract of chamomile was  $38.1 \pm 1.5\%$  and  $42.6 \pm 1.5\%$ , respectively. In addition, it was  $45.6 \pm 2.2\%$  for follicles cultured in G1. Thus, there was a significant difference between G3 and two other groups ( $P < 0.01$ ). Moreover, the MII rate was significantly decreased in G3 as compared with two other groups ( $P < 0.01$ ).

### Real-time reverse transcription-polymerase chain reaction analysis

In all of the study groups, the relative expression of *PCNA* and *FSHR* genes as compared with the housekeeping gene (*GAPDH*) is shown in Figures 3 and 4, respectively. Real-time RT-PCR results showed that the levels of mRNA for *FSHR* and *PCNA* were significantly lower in G3 than that of G1 ( $P < 0.05$ ).

### Hormonal assays

At the end of follicular culture period, half of the medium was collected to investigate the concentrations of E2, P4, and

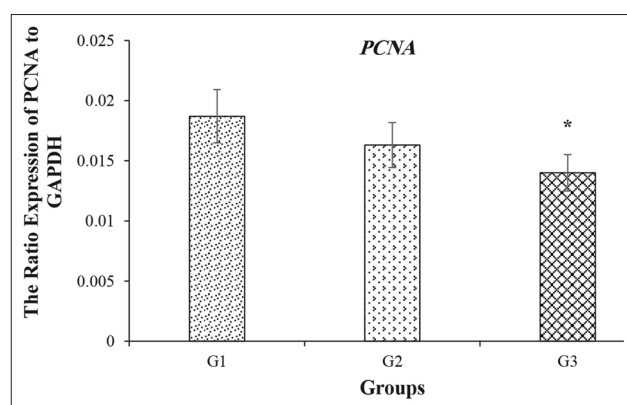


**Figure 2:** The diameter of preantral follicles ( $\mu\text{m}$ ) in different groups during three-dimensional culture system. G1, the control group containing 10% FBS without MC extract; G2, the first experimental group supplemented with 25  $\mu\text{g/ml}$  hydroalcoholic extract of Chamomile; G3, the second experimental group supplemented with 50  $\mu\text{g/ml}$  hydroalcoholic extract of Chamomile. \*Significant difference with the control group ( $P < 0.05$ ). Values are mean  $\pm$  standard deviation. FBS: Fetal bovine serum; MC: *Matricaria chamomilla*.

**Table 1: Developmental rates of *in vitro* culture of isolated preantral follicles**

Groups	Number of follicles	Number of survived (% $\pm$ SD)	Number of antrum formation (% $\pm$ SD)	Number of MII (% $\pm$ SD)
G1	130	90 (69.0 $\pm$ 2.5)	41 (45.6 $\pm$ 2.2)	26 (29.0 $\pm$ 1.7)
G2	175	116 (67.1 $\pm$ 1.5)	49 (42.6 $\pm$ 1.5)	28 (24.8 $\pm$ 2.3)
G3	181	105 (58.0 $\pm$ 1.7)*,†	40 (38.1 $\pm$ 1.5)*,†	23 (21.9 $\pm$ 1.5)*

\*Significant difference with the control group; †The significant difference with the G2. The significant difference for columns 3 and 4 ( $P < 0.05$ ), the significant difference for column 5 ( $P < 0.01$ ). G1: The control group containing 10% FBS without MC extract; G2: The first experimental group supplemented with 25  $\mu\text{g/ml}$  hydroalcoholic extract of Chamomile; G3: The second experimental group supplemented with 50  $\mu\text{g/ml}$  hydroalcoholic extract of Chamomile. SD: Standard deviation; MII: Metaphase II; FBS: Fetal bovine serum; MC: *Matricaria chamomilla*.



**Figure 3:** Expression of *PCNA* gene in different groups during three-dimensional culture system. G1, the control group containing 10% FBS without MC extract; G2, the first experimental group supplemented with 25 µg/ml hydroalcoholic extract of Chamomile; G3, the second experimental group supplemented with 50 µg/ml hydroalcoholic extract of Chamomile. \*Significant difference with the control group ( $P < 0.05$ ). Values are mean  $\pm$  standard deviation. FBS: Fetal bovine serum; MC: *Matricaria chamomilla*.

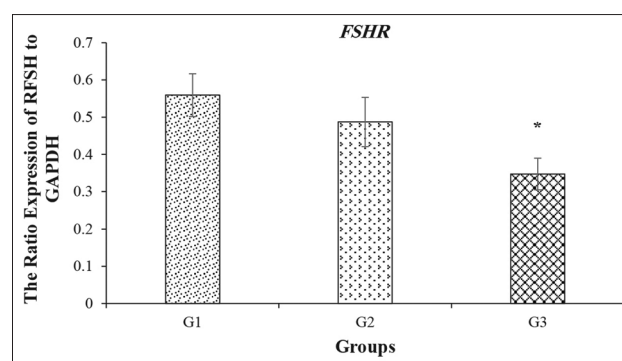
DHEA. Their concentrations are summarized in Table 2. Our observations showed that the E2 levels in two experimental groups were significantly decreased as compared with the control groups ( $P < 0.01$ ). Moreover, the P4 and DHEA levels in G3 and G2 were significantly higher than that of G1 ( $P < 0.01$ ).

### Reactive oxygen species level

There was a significant decrease in the ROS levels of MII oocytes collected from both experimental groups as compared with the control group ( $P < 0.01$ ). On the other hand, there was no significant difference between G3 and G2 and also between G3 and G1 [Figure 5]. Data of ROS levels are shown as mM  $H_2O_2$ .

## DISCUSSION

Folliculogenesis is a complex and dynamic process in which a number of small primordial follicles which contain an immature oocyte surrounded with somatic cells grow to form preovulatory follicles that enter the menstrual cycle.<sup>[27]</sup> In the growth and development of follicles, various para- and autocrine factors are involved.<sup>[28]</sup> On the other hand, for *in vitro* studying of folliculogenesis and effective factors on it, oogenesis, and/or oocyte–somatic cell interactions, three-dimensional culture system has been developed.<sup>[23]</sup> Moreover, in this system, factors such as hormones, antioxidant supplements, and herbal extracts can be added to culture media for investigating their effects on folliculogenesis and oogenesis. However, our results showed that the size and diameter of follicles were increased in all of the study groups, while isolated follicles which were cultured in supplemented media containing 25 and 50 µg/ml hydroalcoholic extract of chamomile have shown smaller diameter than that of the control group on days 6 and 12 of culture. Furthermore, only on day 12 of culture, the mean diameter of follicles which were cultured in the medium supplemented with 50 µg/ml



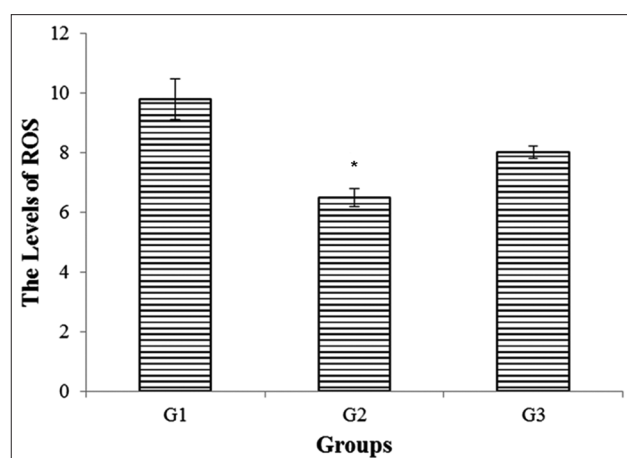
**Figure 4:** Expression of *FSHR* gene in different groups during three-dimensional culture system. G1, the control group containing 10% FBS without MC extract; G2, the first experimental group supplemented with 25 µg/ml hydroalcoholic extract of Chamomile; G3, the second experimental group supplemented with 50 µg/ml hydroalcoholic extract of Chamomile. \*Significant difference with the control and the first experimental group ( $P < 0.05$ ). Values are mean  $\pm$  standard deviation. FBS: Fetal bovine serum; MC: *Matricaria chamomilla*.

**Table 2: The levels of 17-β estradiol, progesterone, and DHEA in collected media on day 12 of culture (Mean  $\pm$  SD)**

Groups	17-β estradiol (pg/ml)	Progesterone (ng/ml)	DHEA (µg/ml)
G1 (n = 130)	2041.66 $\pm$ 60.06	19.41 $\pm$ 2.64	12.55 $\pm$ 2.06
G2 (n = 175)	1841.05 $\pm$ 55.97*	31.53 $\pm$ 1.59*	19.37 $\pm$ 1.06*
G3 (n = 181)	1130.01 $\pm$ 80.01* <sup>†</sup>	39.94 $\pm$ 1.95* <sup>†</sup>	29.29 $\pm$ 0.66* <sup>†</sup>

\*Significant difference with the control group; <sup>†</sup>The significant difference with the G2 ( $P < 0.01$ ). G1: The control group containing 10% FBS without MC extract; G2: The first experimental group supplemented with 25 µg/ml hydroalcoholic extract of Chamomile; G3: The second experimental group supplemented with 50 µg/ml hydroalcoholic extract of Chamomile. SD: Standard deviation; DHEA: Dehydroepiandrosterone; MC: *Matricaria chamomilla*; FBS: Fetal bovine serum.

MC extract significantly decreased relative to the control group. Capcarova *et al.*<sup>[29]</sup> acclaimed that quercetin, one of the compounds of chamomile, had no effect on granulosa cell proliferation. Furthermore, Murray *et al.*<sup>[30]</sup> demonstrated that adding ascorbic acid with different concentrations to culture media had no effect on the growth of the follicles. Therefore, it seems that adding hydroalcoholic extract of chamomile to culture media of follicles has no dramatic effects on follicle growth and size. Moreover, data from the present study showed that the survival rate of follicles cultured in the experimental group 2 as compared with the control group had a significant decrease, and also the rates of MII and antrum formation in the experimental group 2 were significantly decreased. On the other hand, the expression levels of *FSHR* and *PCNA* were carried out by real-time RT-PCR to investigate the follicular development. *FSHR* is one of the most important genes which involved in the normal folliculogenesis process.<sup>[31]</sup> Structurally, it is a G protein-coupled and seven-transmembrane receptor which links to the several pathways, such as adenylate cyclase.<sup>[32]</sup> It has been reported that the *FSHR* is expressed in growing follicles.<sup>[32]</sup> *PCNA* is a protein with a weight of 36,000 and it has been reported in fetal and



**Figure 5:** ROS levels of MII oocyte derived from *in vitro* culture of isolated preantral follicles. G1, the control group containing 10% FBS without MC extract; G2, the first experimental group supplemented with 25 µg/ml hydroalcoholic extract of Chamomile; G3, the second experimental group supplemented with 50 µg/ml hydroalcoholic extract of Chamomile. \*Significant difference with the control group ( $P < 0.01$ ). Values are mean  $\pm$  standard deviation. ROS: Reactive oxygen species; MII: Metaphase II; FBS: Fetal bovine serum; MC: *Matricaria chamomilla*.

adult ovaries of several mammals.<sup>[33]</sup> The expression of *PCNA* increased in the initiation of follicle growth, while there was no expression in granulosa cells of primordial follicles.<sup>[33]</sup> Our results indicated a significant decrease in the *PCNA* gene expression in follicles of the second experimental group in comparison with the control group ( $P < 0.05$ ). Also, the *FSHR* gene expression significantly decreased in the experimental group 2 as compared with the control group ( $P < 0.05$ ). Additionally, data of the present study indicate that MC extract alters the levels of steroid hormones. Especially, in both experimental groups, we observed that the levels of E2 significantly decreased as compared with the control group, but DHEA and P4 levels in the media of experimental groups 2 and 1 significantly increased relative to the control group. Several studies have indicated that estradiol is absolutely necessary for normal follicle growth.<sup>[34-36]</sup> In the estradiol biosynthesis pathway, cholesterol transports by STAR from the outer membrane to the inner membrane of the mitochondria, and then by CYP11A1, it converts to pregnenolone.<sup>[37,38]</sup> The pregnenolone via two pathways converts into estradiol.<sup>[39]</sup> In the first pathway, it converts into the DHEA by CYP17A1 and subsequently by HSD3B1, DHEA converts into androstenedione.<sup>[39]</sup> The androstenedione not only converts into the estrone by CYP19A1, but also converts into the testosterone by HSD17B1.<sup>[38]</sup> Then, at the end of the process, the testosterone converts into the estradiol by CYP19A1.<sup>[38]</sup> On the other hand, in the second pathway, the pregnenolone by HSD3B1 converts into the progesterone.<sup>[38,39]</sup> Then, through CYP17A1, the progesterone is converted into the androstenedione, testosterone, and estradiol, successively.<sup>[37,39]</sup> However, it has been reported that phytoestrogens inhibit the transformation of cholesterol to pregnenolone by reducing the activity of cytochrome p450; therefore, the amount of

estrogen decreases.<sup>[40]</sup> It has been reported that other phytoestrogens such as luteolin and apigenin were competitive with cytochrome p450 aromatase.<sup>[41]</sup> Additionally, phytoestrogens can act as agonists or antagonists of estrogen receptors.<sup>[42]</sup> It has been acclaimed that phytoestrogens can inhibit the progesterone-metabolizing enzyme 20- $\alpha$ -hydroxy steroid dehydrogenase. The main role of the mentioned enzyme is considered as deactivating progesterone that also transforms it to 3- $\alpha$  5- $\alpha$  tetrahydroprogesterone. Phytoestrogen compounds such as flavones, 3- and 7-dihydroxyflavone, and 3- $\alpha$ 7-hydroxy flavone can control the function of the mentioned enzyme. These compounds by binding to the active site of the progesterone-metabolizing enzyme prevent the transformation of progesterone to 3- $\alpha$  5- $\alpha$  tetrahydroprogesterone; therefore, it may be the main cause of the progesterone hormone increase.<sup>[43,44]</sup> Several studies have reported that the apigenin inhibits aromatase activity.<sup>[45,46]</sup> Moreover, genistein is one of the phytoestrogen compounds of hydroalcoholic extract of chamomile that can impact estrogen signaling pathways because of binding to estrogen receptors.<sup>[47]</sup> Several studies have shown that genistein can inhibit steroidogenic enzymes and steroidogenesis, induce follicular atresia, and decrease oocyte maturation.<sup>[48,49]</sup> Patel *et al.*<sup>[50]</sup> reported that adding genistein to the culture media inhibited follicle growth, but did not induce follicle atresia and also genistein increased testosterone and DHEA and decreased progesterone levels. Previous studies demonstrated that genistein exposure (50 µM) can inhibit the ability of granulosa cells to produce progesterone,<sup>[40]</sup> and also genistein exposure (1, 18.5, and 185 µM) inhibits estrogen and progesterone levels in cultured porcine granulosa cells.<sup>[42]</sup> In addition, one study acclaimed that neonatal genistein exposure (50 mg·kg<sup>-1</sup>·d<sup>-1</sup>) has no effect on progesterone and testosterone serum levels during pregnancy and before puberty in mice.<sup>[47]</sup> We guess that hydroalcoholic extract of chamomile at the doses of 25 and 50 µg/ml due to altering the steroidogenesis decreased follicular growth and maturation. The increment of P4 level in the culture media can lead to inhibition of granulosa cell division and, consequently, decrease of estrogen secretion.<sup>[51]</sup> Although the mechanism of how genistein alters steroid hormones is unknown, Patel *et al.*<sup>[50]</sup> reported that 72 h after culture, the STAR and Cyp11a1 gene expressions increased. Two mentioned enzymes are involved in increasing progesterone production.<sup>[52]</sup> Kang *et al.*<sup>[53]</sup> evaluated the effects of quercetin on meiotic maturation of mature oocytes and cumulus cell steroidogenesis. Their results showed that the levels of estrogen and progesterone and also the rates of polar body extrusion decreased. Moreover, another study reported that quercetin at the dose of 50 µg/ml did not affect granulosa cell growth while inhibiting progesterone production by granulosa cells and altered estradiol-17 $\beta$  production in a dose-related manner.<sup>[54]</sup> In the culture of preantral follicles, oxidative stress by inducing an apoptotic mechanism can play an important role in follicular atresia. Our results showed that the ROS level of oocytes in the first experimental group as compared with the control group significantly decreased, but no significant



difference between the second experimental and control groups was observed. ROS are spontaneously generated in the normal metabolism of cells and also in the physiological phenomena against severe infections that lead to cell death. Flavonoids that present in the plant-derived beverages, fruits, and/or vegetables of plant nuts are powerful natural antioxidants that can protect the cells during culture from oxidative stress.<sup>[55]</sup> Also, several studies have shown that the flavonoids of chamomile not only have antiviral, antiallergic, and anticancer effects, but also have antioxidative effects.<sup>[5,56]</sup> On the other hand, it has been suggested that high levels of flavonoids could be toxic to the oocyte.<sup>[49]</sup> It has been demonstrated that apigenin decreases oxidative stress-induced damage in osteoblastic cells.<sup>[13]</sup> Also, Kang *et al.*<sup>[53]</sup> reported that quercetin decreased the ROS level in *in vitro* maturation oocytes.

In conclusion, it seems that the high concentration of hydroalcoholic extract of *Matricaria* can inhibit cell proliferation and also decrease the estradiol level. Therefore, these happenings may be related to the high concentration of flavonoids and/or phytoestrogens. Our results demonstrated that adding chamomile extract to the culture media during follicular culture in a three-dimensional culture system appeared to decrease follicular function and development.

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## Conflicts of interest

There are no conflicts of interest.

## REFERENCES

1. Srivastava JK, Shankar E, Gupta S. Chamomile: A herbal medicine of the past with bright future. *Mol Med Rep* 2010;3:895-901. doi: 10.3892/mmr.2010.377.
2. Bent S. Herbal medicine in the United States: Review of efficacy, safety, and regulation: Grand rounds at university of California, San Francisco medical center. *J Gen Intern Med* 2008;23:854-9. doi: 10.1007/s11606-008-0632-y.
3. Gil-Chávez J, Villa JA, Ayala-Zavala JF, Heredia JB, Sepulveda D, Yahia EM, *et al.* Technologies for extraction and production of bioactive compounds to be used as nutraceuticals and food ingredients: An overview. *Compr Rev Food Sci Food Saf* 2013;12:5-23. doi: 10.1111/1541-4337.12005.
4. Aggarwal BB, Prasad S, Reuter S, Kannappan R, Yadev VR, Park B, *et al.* Identification of novel anti-inflammatory agents from ayurvedic medicine for prevention of chronic diseases: "reverse pharmacology" and "bedside to bench" approach. *Curr Drug Targets* 2011;12:1595-653. doi: 10.2174/138945011798109464.
5. Maschi O, Cero ED, Galli GV, Caruso D, Bosisio E, Dell'Agli M, *et al.* Inhibition of human cAMP-phosphodiesterase as a mechanism of the spasmolytic effect of *Matricaria recutita* L. *J Agric Food Chem* 2008;56:5015-20. doi: 10.1021/jf800051n.
6. Wachtel-Galor S, Benzie IFF. Herbal Medicine: An Introduction to Its History, Usage, Regulation, Current Trends, and Research Needs. In: Benzie IFF, Wachtel-Galor S, editors. *Herbal Medicine: Biomolecular and Clinical Aspects*. 2<sup>nd</sup> ed. Boca Raton, FL: CRC Press/Taylor & Francis; 2011.
7. Mason HS, Warzecha H, Mor T, Arntzen CJ. Edible plant vaccines: Applications for prophylactic and therapeutic molecular medicine. *Trends Mol Med* 2002;8:324-9. doi: 10.1016/S1471-4914(02)02360-2.
8. McGuffin M, Hobbs C, Upton R, Goldberg A. American Products Association's Botanical Safety Handbook. Boca Raton, FL: CRC Press; 1997.
9. Szoke E, Máday E, Tyihák E, Kuzovkina IN, Lemberkovics E. New terpenoids in cultivated and wild chamomile (*in vivo* and *in vitro*). *J Chromatogr B Analyt Technol Biomed Life Sci* 2004;800:231-8. doi: 10.1016/j.jchromb.2003.09.038.
10. Soltani M, Moghimian M, Abtahi H, Shokoohi M. The protective effect of *Matricaria chamomilla* extract on histological damage and oxidative stress induced by Torsion/Detorsion in adult rat ovary. *Int J Womens Health Reprod Sci* 2017;5:187-92. doi: 10.15296/ijwhr.2017.34.
11. Farideh ZZ, Bagher M, Ashraf A, Akram A, Kazem M. Effects of chamomile extract on biochemical and clinical parameters in a rat model of polycystic ovary syndrome. *J Reprod Infertil* 2010;11:169-74.
12. Cemek M, Yilmaz E, Büyükkokuroğlu ME. Protective effect of *Matricaria chamomilla* on ethanol-induced acute gastric mucosal injury in rats. *Pharm Biol* 2010;48:757-63. doi: 10.3109/13880200903296147.
13. Jung WW. Protective effect of apigenin against oxidative stress-induced damage in osteoblastic cells. *Int J Mol Med* 2014;33:1327-34. doi: 10.3892/ijmm.2014.1666.
14. Şanlı S, Lunte C. Determination of eleven flavonoids in chamomile and linden extracts by capillary electrophoresis. *Anal Methods* 2014;6:3858-64. doi: 10.1039/C3AY41878B.
15. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev* 2010;4:118-26. doi: 10.4103/0973-7847.70902.
16. Abedelahi A, Salehnia M, Allameh AA. The effects of different concentrations of sodium selenite on the *in vitro* maturation of preantral follicles in serum-free and serum supplemented media. *J Assist Reprod Genet* 2008;25:483-8. doi: 10.1007/s10815-008-9252-z.
17. Piersen CE. Phytoestrogens in botanical dietary supplements: Implications for cancer. *Integr Cancer Ther* 2003;2:120-38. doi: 10.1177/1534735403002002004.
18. Naji T, Hosseinzadeh Sahafi H, Saffari M. The effects of phytoestrogens *Matricaria recutita* on growth, maturation of oocytes in the three spot gourami (*Trichogaster trichopterus*). *ISFJ* 2015; 23:85-94.
19. Sebai H, Jabri MA, Souli A, Rtibi K, Selmi S, Tebourbi O, *et al.* Antidiarrheal and antioxidant activities of chamomile (*Matricaria recutita* L.) decoction extract in rats. *J Ethnopharmacol* 2014;152:327-32. doi: 10.1016/j.jep.2014.01.015.
20. Johari H, Sharifi E, Mardan M, Kafizadeh F, Hemayatkhah V, Kargar H, *et al.* The Effects of hydro alcoholic extract of *Matricaria chamomilla* flower on testosterone and gonadotropins hormone in adult male rat. *Pars Journal of Medical Sciences*. 2015;12:37-41. [In Persian].
21. Johari H, Sharifi E, Mardan M, Kafizadeh F, Hemayatkhah V, Kargar H, *et al.* The effects of a hydroalcoholic extract of *Matricaria chamomilla* flower on the pituitary-gonadal axis and ovaries of rats. *Int J Endocrinol Metab* 2011;9:330-4. doi: 10.5812/kowsar.1726913X.1822.
22. Abdi S, Salehnia M, Hosseinkhani S. Quality of oocytes derived from vitrified ovarian follicles cultured in two- and three-dimensional culture system in the presence and absence of kit ligand. *Biopreserv Biobank* 2016;14:279-88. doi: 10.1089/bio.2015.0069.
23. Xu M, West E, Shea LD, Woodruff TK. Identification of a stage-specific permissive *in vitro* culture environment for follicle growth and oocyte development. *Biol Reprod* 2006;75:916-23. doi: 10.1095/biolreprod.106.054833.
24. Salehnia M, Pajokh M, Ghorbanmehr N. Short term organ culture of mouse ovary in the medium supplemented with bone morphogenetic protein 15 and follicle stimulating hormone: A Morphological, hormonal and molecular study. *J Reprod Infertil* 2016;17:199-207.
25. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:e45. doi: 10.1093/nar/29.9.e45.
26. Abedelahi A, Salehnia M, Allameh AA, Davoodi D. Sodium selenite

- improves the *in vitro* follicular development by reducing the reactive oxygen species level and increasing the total antioxidant capacity and glutathione peroxidase activity. *Hum Reprod* 2010;25:977-85. doi: 10.1093/humrep/deq002.
27. Baerwald AR, Adams GP, Pierson RA. Ovarian antral folliculogenesis during the human menstrual cycle: A review. *Hum Reprod Update* 2012;18:73-91. doi: 10.1093/humupd/dmr039.
28. Palma GA, Argañaraz ME, Barrera AD, Rodler D, Mutto AA, Sinowatz F, *et al.* Biology and biotechnology of follicle development. *Sci World J* 2012;2012:938138. doi: 10.1100/2012/938138.
29. Capcarova M, Petruska P, Zbynovska K, Kolesarova A, Sirotkin AV. Changes in antioxidant status of porcine ovarian granulosa cells after quercetin and T-2 toxin treatment. *J Environ Sci Health B* 2015;50:201-6. doi: 10.1080/03601234.2015.982425.
30. Murray AA, Molinek MD, Baker SJ, Kojima FN, Smith MF, Hillier SG, *et al.* Role of ascorbic acid in promoting follicle integrity and survival in intact mouse ovarian follicles *in vitro*. *Reproduction* 2001;121:89-96. doi: 10.1530/rep.0.1210089.
31. Oktay K, Newton H, Aubard Y, Salha O, Gosden RG. Cryopreservation of immature human oocytes and ovarian tissue: An emerging technology? *Fertil Steril* 1998;69:1-7. doi: 10.1016/S0015-0282(97)00207-0.
32. Scarlet D, Walter I, Hlavaty J, Aurich C. Expression and immunolocalisation of follicle-stimulating hormone receptors in gonads of newborn and adult female horses. *Reprod Fertil Dev* 2016;28:1340-8. doi: 10.1071/RD14392.
33. Oktay K, Schenken RS, Nelson JF. Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat. *Biol Reprod* 1995;53:295-301. doi: 10.1095/biolreprod53.2.295.
34. Channing CP, Schaerf FW, Anderson LD, Tsafiriri A. Ovarian follicular and luteal physiology. *Int Rev Physiol* 1980;22:117-201.
35. Drummond AE. The role of steroids in follicular growth. *Reprod Biol Endocrinol* 2006;4:16. doi: 10.1186/1477-7827-4-16.
36. Chaffin CL, Vandevoort CA. Follicle growth, ovulation, and luteal formation in primates and rodents: A comparative perspective. *Exp Biol Med (Maywood)* 2013;238:539-48. doi: 10.1177/1535370213489437.
37. Strauss JF 3<sup>rd</sup>, Kallen CB, Christenson LK, Watari H, Devoto L, Arakane F, *et al.* The steroidogenic acute regulatory protein (StAR): A window into the complexities of intracellular cholesterol trafficking. *Recent Prog Horm Res* 1999;54:369-94.
38. Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev* 2004;25:947-70. doi: 10.1210/er.2003-0030.
39. Conley AJ, Bird IM. The role of cytochrome P450 17 alpha-hydroxylase and 3 beta-hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the delta 5 and delta 4 pathways of steroidogenesis in mammals. *Biol Reprod* 1997;56:789-99.
40. Tiemann U, Schneider F, Vanselow J, Tomek W. *In vitro* exposure of porcine granulosa cells to the phytoestrogens genistein and daidzein: Effects on the biosynthesis of reproductive steroid hormones. *Reprod Toxicol* 2007;24:317-25. doi: 10.1016/j.reprotox.2007.07.008.
41. Jefferson WN, Patisaul HB, Williams CJ. Reproductive consequences of developmental phytoestrogen exposure. *Reproduction* 2012;143:247-60. doi: 10.1530/REP-11-0369.
42. Basini G, Bussolati S, Santini SE, Grasselli F. The impact of the phyto-oestrogen genistein on swine granulosa cell function. *J Anim Physiol Anim Nutr (Berl)* 2010;94:e374-82. doi: 10.1111/j.1439-0396.2010.01025.x.
43. Wang F, Shing M, Huen Y, Tsang SY, Xue H. Neuroactive flavonoids interacting with GABAA receptor complex. *Curr Drug Targets CNS Neurol Disord* 2005;4:575-85. doi: 10.2174/156800705774322030.
44. Brozic P, Smuc T, Gobec S, Rizner TL. Phytoestrogens as inhibitors of the human progesterone metabolizing enzyme AKR1C1. *Mol Cell Endocrinol* 2006;259:30-42. doi: 10.1016/j.mce.2006.08.001.
45. Sanderson JT, Boerma J, Lansbergen GW, van den Berg M. Induction and inhibition of aromatase (CYP19) activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. *Toxicol Appl Pharmacol* 2002;182:44-54. doi: 10.1006/taap.2002.9420.
46. Balunas MJ, Su B, Brueggemeier RW, Kinghorn AD. Natural products as aromatase inhibitors. *Anti Cancer Agents Med Chem Formerly Curr Med Chem Anti Cancer Agents* 2008;8:646-82. doi: 10.2174/187152008785133092.
47. Jefferson WN, Padilla-Banks E, Newbold RR. Adverse effects on female development and reproduction in CD-1 mice following neonatal exposure to the phytoestrogen genistein at environmentally relevant doses. *Biol Reprod* 2005;73:798-806. doi: 10.1095/biolreprod.105.041277.
48. Karbalay-Doust S, Noorafshan A, Dehghani F, Panjehshahin MR, Monabati A. Effects of hydroalcoholic extract of *Matricaria chamomilla* on serum testosterone and estradiol levels, spermatozoon quality, and tail length in rat. *Iran J Med Sci* 2015;35:122-8.
49. Kang JT, Moon JH, Choi JY, Park SJ, Kim SJ, Saadeldin IM, *et al.* Effect of antioxidant flavonoids (Quercetin and taxifolin) on *in vitro* maturation of porcine oocytes. *Asian-Australas J Anim Sci* 2016;29:352-8. doi: 10.5713/ajas.15.0341.
50. Patel S, Peretz J, Pan YX, Helferich WG, Flaws JA. Genistein exposure inhibits growth and alters steroidogenesis in adult mouse antral follicles. *Toxicol Appl Pharmacol* 2016;293:53-62. doi: 10.1016/j.taap.2015.12.026.
51. Lacey M, Bohday J, Fonseka SM, Ullah AI, Whitehead SA. Dose-response effects of phytoestrogens on the activity and expression of 3beta-hydroxysteroid dehydrogenase and aromatase in human granulosa-luteal cells. *J Steroid Biochem Mol Biol* 2005;96:279-86. doi: 10.1016/j.jsbmb.2005.03.006.
52. Rice S, Mason HD, Whitehead SA. Phytoestrogens and their low dose combinations inhibit mRNA expression and activity of aromatase in human granulosa-luteal cells. *J Steroid Biochem Mol Biol* 2006;101:216-25. doi: 10.1016/j.jsbmb.2006.06.021.
53. Kang JT, Kwon DK, Park SJ, Kim SJ, Moon JH, Koo OJ, *et al.* Quercetin improves the *in vitro* development of porcine oocytes by decreasing reactive oxygen species levels. *J Vet Sci* 2013;14:15-20. doi: 10.4142/jvs.2013.14.1.15.
54. Santini SE, Basini G, Bussolati S, Grasselli F. The phytoestrogen quercetin impairs steroidogenesis and angiogenesis in swine granulosa cells *in vitro*. *J Biomed Biotechnol* 2009;2009:419891. doi: 10.1155/2009/419891.
55. Pietta PG. Flavonoids as antioxidants. *J Nat Prod* 2000;63:1035-42. doi: 10.1021/np9904509.
56. Ziyen L, Yongmei Z, Nan Z, Ning T, Baolin L. Evaluation of the anti-inflammatory activity of luteolin in experimental animal models. *Planta Med* 2007;73:221-6. doi: 10.1055/s-2007-967122.