

Promotion of Oocyte Maturation and Embryonic Competence in Sheep by *Viola alba* subsp. *sintenisii*-Derived Bioactives

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Article

Keywords: *Viola alba* subsp. *Sintenisii*, Hydro phenolic extract, In vitro maturation (IVM), cumulus-oocyte complexes (COCs), in vitro Fertilization (IVF)

Posted Date: June 18th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-6277304/v1>

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Additional Declarations: No competing interests reported.

Abstract

The impact of a hydrophenolic extract derived from *Viola alba subsp. sintenisii* (L.). established on national Live collection of Viola- Zanjan, Iran (LCV) (36.6870267, 48.3881580) on the in vitro maturation (IVM) and fertilization of sheep cumulus-oocyte complexes (COCs) was investigated. Chemical analysis using LC-MS and GC-MS revealed the presence of biologically active compounds, including cyclotides, phenolic acids, and flavonoids, which have known roles in modulating cellular responses. COCs matured with the extract exhibited enhanced cumulus expansion, as evidenced by morphological observations and elevated expression of *fshr* (follicle-stimulating hormone receptor) and *gdf9* (growth differentiation factor 9) genes. Specifically, the 50 µL concentration of the extract significantly upregulated these gene expressions, suggesting optimal follicular support and oocyte developmental competence. Furthermore, Rhodamine 123 staining showed that mitochondrial activity and distribution in oocytes significantly improved in the extract-treated groups, indicating enhanced energy metabolism and cytoplasmic maturation. Collectively, these findings highlight the efficacy of *V. alba subsp. sintenisii* extract VE in enhancing the IVM process, likely through the action of its bioactive compounds on key molecular and cellular pathways. These results provide a foundation for integrating natural compounds into assisted reproductive technologies to improve livestock production efficiency.

Introduction

Oocyte maturation in sheep, as in other mammals, is a critical biological process that determines the quality of the egg and ultimately affects reproductive success. This process involves the completion of meiosis, cytoplasmic maturation, and the preparation of the oocyte for fertilization and early embryonic development. In sheep, these processes are influenced by intrinsic factors (e.g., genetics) and extrinsic factors (e.g., the ovarian microenvironment and hormones). Research has shown that oocytes mature in a complex interaction with their surrounding cumulus cells, with these cells supporting the oocyte through the stages of nuclear and cytoplasmic maturation ¹.

One of the key effects of effective and beneficial substances during maturation is the altered expression pattern of certain genes, including an upregulation of *gdf9* and *fshr*. The *gdf9* (Growth Differentiation Factor 9) gene is vital for oocyte maturation, as it regulates follicular development and the function of surrounding granulosa cells. Primarily expressed in oocytes, GDF9 plays a key role in promoting their growth and differentiation, making them competent for fertilization^{2,3}. One of its essential functions is stimulating the expression of *fshr* (Follicle-Stimulating Hormone Receptor), a receptor crucial for folliculogenesis ³. This interaction enhances follicular survival and maturation, with GDF9 driving the expansion of cumulus cells, which is necessary for the proper development of the oocyte. Together, GDF9 and *fshr* are integral in ensuring oocyte quality and fertility.²⁻⁴

Recent studies have demonstrated that resveratrol, a polyphenol found in Marjoram and also found in *Viola* species⁵, can significantly reduce oxidative stress during the in vitro maturation of sheep oocytes by

lowering reactive oxygen species (ROS) levels and enhancing glutathione production, thereby offering protection during the embryo vitrification process^{6–9}.

In recent research, the hormone ghrelin was shown to significantly influence sheep oocyte maturation by modulating signaling pathways related to the cell cycle and oxidative phosphorylation, highlighting its potential to alter early embryonic development and improve our understanding of the hormonal and signaling environments that favor oocyte maturation¹⁰. Flavonoids are a diverse group of plant-derived polyphenolic compounds known for their antioxidant, anti-inflammatory, and cell-signaling modulating properties. Quercetin and kaempferol, both flavonoids, have been shown to exert positive effects on the in vitro maturation (IVM) of oocytes by enhancing antioxidant activity, reducing oxidative stress, and improving cellular viability. Studies suggest that these compounds contribute to the regulation of intracellular signaling pathways, such as those involving MAPK and PI3K, which are crucial for oocyte maturation. Their antioxidant properties help protect oocytes from damage during culture, improving overall maturation rates and oocyte quality^{11,12}

Anti-inflammatory mechanisms play a crucial role in the in vitro maturation (IVM) of oocytes by maintaining a balanced cytokine environment and reducing oxidative stress, both of which are essential for proper oocyte development. Inflammation within the ovarian microenvironment can negatively impact oocyte quality and leading to impaired maturation and developmental competence¹³. As an anti-inflammatory factor, cortisol, a glucocorticoid hormone, plays a dual role in the in vitro maturation (IVM) of oocytes by modulating inflammatory responses and reducing oxidative stress. While physiological levels of cortisol can support oocyte maturation by regulating cytokine activity and maintaining cellular homeostasis.¹⁴

The use of natural extracts in enhancing oocyte maturation is gaining interest due to their rich bioactive compound content, which can influence key reproductive processes. Viola is an important forest plant due to its diverse flowering systems, as well as its ornamental and medicinal significance. Medicinal violets are generally wild and natural.¹⁵

The Violaceae comprises 500–600 predominately tropical and temperate species in 25 currently recognized genera. Viola section is one of the largest groups of the Violaceae family. Infra generic classification has varied, but recent phylogenetic analysis indicates that the genus can be subdivided into two subgenera and 16 sections worldwide^{16–19}. The most important medicinal species of Viola genus are *V. arvensis*, *V. baoshanensis*, *V. odorata*, *V. caspia* and *V. sintenisii*^{19–21}. According to published scientific reports, most of the dense habitats of Iranian violets are located in the forest areas of Golestan, Mazandaran and Gilan provinces, which are among the three dominant and hot habitats in Iran.^{18,19,21} Notably, *V. sintenisii* extract contains a spectrum of bioactive compounds—including phenolic acids, flavonoids, and cyclotides—and has shown promise in this area. These compounds have been identified to play critical roles in cellular signaling pathways that govern the maturation of oocytes. Phenolic acids and flavonoids, for example, are known to exert antioxidant effects that protect cellular

integrity during the maturation process, while cyclotides have been shown to modulate protein interactions and signaling cascades critical for cellular differentiation and development.^{6,10,22}

The use of VE in influencing these maturation processes introduces an intriguing dimension to reproductive biotechnology. Studies have highlighted that certain plant extracts can modulate reproductive functions, potentially by altering hormonal balances or by acting directly on the cellular components involved in oocyte development. For instance, substances like ghrelin have been studied for their effects on oocyte maturation in sheep, where they have been found to influence cellular pathways that control the maturation process.

The maturation of oocytes in sheep is a complex and finely regulated process, influenced by both intrinsic and extrinsic factors. Key genes, such as *gdf9* and *fshr*, play pivotal roles in regulating follicular development and ensuring oocyte competence for fertilization. Additionally, various beneficial substances, including resveratrol, flavonoids, cortisol, and bioactive compounds from plant extracts like *Viola*, offer promising therapeutic potential by enhancing antioxidant activity, modulating inflammatory responses, and influencing critical signaling pathways involved in oocyte maturation. These findings highlight the importance of understanding the molecular and environmental factors that contribute to oocyte quality, which can have significant implications for reproductive health and assisted reproductive technologies. Further research into the biochemical and physiological mechanisms governing oocyte maturation is essential for improving fertility treatments and advancing the field of reproductive biotechnology.

Material and Methods

Preparation of the extract with hydrophenolic solvent and its fractionation

The plant material used in this study, *Viola alba* subsp. *sintenisii* (L.), was obtained from the National Collection of Live Nettles (*Urtica dioica* L.) at the Research Institute of Modern Biological Techniques, University of Zanjan, Zanjan, Iran (36.6870267°N, 48.3881580°E). The species was formally identified by Dr. Ali Amarlou, a botanist at the University of Zanjan. A voucher specimen (accession number VA-ZNU-2023-01) was deposited at the herbarium of the Research Institute of Modern Biological Techniques for future reference (Fig. 1).

Dried and powdered aerial parts of the plant (100 g) were macerated with a solvent mixture of ethanol and water (8:2). The resulting extract was concentrated using a rotary evaporator under reduced pressure to yield a residue of 11.3 g. This residue was then dissolved in water to achieve the desired final concentrations.⁵⁸

GC-mass and LC-mass Analysis

GC-mass : Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the essential oil was performed by injecting 1 μL of the sample into a GC-MS system equipped with an HP-5MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm). The oven temperature was initially set at 60°C, then increased to 240°C at a rate of 3°C/min. Helium was used as the carrier gas at a flow rate of 1.4 mL/min. The split injection mode was employed with a 1:50 split ratio. The injector temperature was maintained at 250°C, and the detector was set to 280°C. Identification of compounds was based on retention indices and mass spectra, and quantification was performed using appropriate calibration standards⁵⁹.

LC-mass

The Liquid Chromatography- Mass Spectrometry (LC-MS) analysis was performed using a BEH C18 column (2.1 mm $\times$ 100 mm, 1.7 μm) with a binary gradient elution system. The mobile phases consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The gradient program started with 5% solvent B, increased to 95% over 10 minutes, held at 95% for 5 minutes, and then returned to 5% over 1 minute, with a total run time of 16 minutes. The flow rate was set at 0.3 mL/min. The mass spectrometer was operated in positive ion mode with an electrospray ionization (ESI) source. The capillary voltage was set at 3.5 kV, the cone voltage at 30 V, and the source temperature at 120°C. The desolvation temperature was 350°C, with a desolvation gas flow of 800 L/h and a cone gas flow of 50 L/h. Data acquisition was performed in full scan mode (m/z 100–1000) with a scan time of 0.5 seconds. The obtained data were analyzed using appropriate software to identify and quantify the compounds present in the extract⁶⁰.

Ovary Collection and Transportation:

Ovaries were obtained from slaughtered ewes at a local abattoir located near the University of Zanjan in western Iran (36.59188314826283, 48.75693092883606). Immediately after collection, the ovaries were rinsed with sterile phosphate-buffered saline (PBS) (Sigma Aldrich, Massachusetts, United States) containing 1X penicillin-streptomycin (BIO-IDEA, Tehran-Iran, 100X) to minimize microbial contamination. The samples were then transported to the embryo culture laboratory in a thermally insulated container maintained at 37°C to preserve their physiological integrity. The transportation process was carefully monitored, ensuring all ovarian samples reached the laboratory within three hours of collection.

IVM media and procedure

Ovaries collected from slaughtered ewes at a local abattoir near the University of Zanjan, western Iran, are transported to the laboratory under controlled conditions to maintain a stable temperature of approximately 37°C. They are placed in phosphate-buffered saline (PBS) supplemented with 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin and stored in a thermally insulated flask to preserve optimal conditions during transportation. The temperature of both the solution and the ovaries is maintained at 37°C throughout transportation.

Upon arrival at the laboratory, the ovaries are washed 3–4 times with pre-warmed (37°C) PBS containing 50 IU/ml penicillin and 50 $\mu\text{g}/\text{ml}$ of streptomycin to remove contaminants and prepare them for follicular

aspiration.

For follicular aspiration, a 25 ml syringe is used along with pre-warmed aspiration (HTCM,10% FBS (BIOSERA, France), 50 IU/ml Heparin (Darou Pakhsh, Tehran, Iran)) and oocyte washing media (HTCM,10% FBS), which have been previously prepared and maintained at the appropriate temperature in a sterile incubator(Table 1). A small volume of aspiration medium is first drawn into the syringe, followed by the aspiration of follicles of suitable diameter. The aspirated contents are then transferred into a sterile Falcon tube placed in a water bath to maintain temperature stability (Table 1).

Table 1

HTCM serves as the base medium for both aspiration and washing media, supplemented with specific additives. The aspiration medium contains 10% (FBS) and 50 IU/mL heparin, while the washing medium includes 10% FBS. Osmolarity measurements were performed using an osmometer (Gonotec® Osmomat 3000-Logan Utah), which requires a 15-minute pre-activation period to ensure accuracy.

HTCM			
Material	Brand	Concentration	Osmolarity: 275–285 pH: 7.2–7.4
M199	Gibco, Massachusetts, U.S.	0.475 g	
NaHCo3	Sigma Aldrich, Massachusetts, United States	0.005 M	
Na Pyruvate	Sigma Aldrich, Massachusetts, United States	0.0002 M	
HEPES (free acid)	Sigma Aldrich, Massachusetts, United States	0.1191 g	
HEPES (Na salt)	Sigma Aldrich, Massachusetts, United States	0.1301g	
Pen/strep	BIO-IDEA, Tehran-Iran	1X	
water		To 50ml	

After aspiration, the follicular fluid is allowed to settle in the Falcon tube for approximately 15 minutes, enabling the cumulus-oocyte complexes (COCs) to sediment. The supernatant is carefully removed using a 1000 µl micropipette, and droplets of the remaining sediment are placed in a Petri dish. Under a microscope, COCs with at least three layers of dense cumulus cells are selected and washed by sequential transfer through four droplets of oocyte washing medium to ensure thorough cleansing.

Grading of COCs Based on Morphological Characteristics:

COCs are graded based on the number of cumulus cell layers, ooplasm homogeneity, and oocyte size. COCs with five or more layers of cumulus cells are classified as Grade A, those with three to four layers as Grade B, and those with two or fewer layers as Grade C. Grade D includes degenerated oocytes and cumulus cells. Grades A and B are considered normal, while Grades C and D are classified as abnormal (Fig. 4).

Brilliant Cresyl Blue Staining Test:

Due to laboratory conditions and the use of the Brilliant Cresyl Blue (BCB) (Pallav) brand, a study was conducted to evaluate three BCB concentrations—13, 26, and 52 µg/mL—in three replicates, based on previous reports suggesting effective concentrations between 13 and 26 µg/mL⁶¹. Since the initial staining with BCB was unsuccessful, a preliminary experiment was performed to determine the optimal concentration for staining sheep COCs. The results demonstrate that a concentration of 26 µg/mL BCB was optimal for distinguishing between mature and immature oocytes. At a lower concentration of 13 µg/mL, the staining intensity was insufficient, making it difficult to accurately identify maturation status. In contrast, at a higher concentration of 52 µg/mL, excessive staining was observed, leading to increased background signal and reduced contrast between oocytes at different maturation stages. These findings highlight the importance of selecting an appropriate BCB concentration to ensure reliable assessment of oocyte developmental competence (Fig. 5).

After washing the COCs three times in modified phosphate-buffered saline (mPBS), which was supplemented with 0.5% (w/v) BSA (Merk, Germany), the oocytes were stained with BCB, diluted in mPBS⁶². The oocytes were incubated for 90 minutes at 38.6°C in a humidified atmosphere and 5% CO₂ (incubator: Binder, Germany). Following incubation, the oocytes were washed twice and categorized based on their cytoplasmic coloration. Oocytes exhibiting blue cytoplasmic staining were classified as BCB⁺ and those without blue coloration were classified as BCB⁻. The control group COCs were treated under the same conditions for 90 minutes without exposure to BCB.

In Vitro Maturation of Oocytes:

The maturation medium consisted of TCM buffer (Table 2) supplemented with 10% FBS (v/v), 0/1 IU/ml human menopausal gonadotropin (HMG) (B.POYESH DAROU, Isfahan-Iran), 1 µg/mL 17β-estradiol (Sigma Aldrich, Massachusetts, United States). Oocytes were transferred in groups (20 COCs per droplet) into 50 µL droplets of the maturation medium and incubated for 24 hours at 38.6°C in a humidified atmosphere containing 5% CO₂, under mineral oil (Sigma Aldrich, Massachusetts, United States) (Table 2).

Table 2

TCM serves as the base medium for in vitro maturation (IVM) and sperm capacitation. The IVM medium is supplemented with 10% (v/v) fetal bovine serum (FBS), 0.1 IU/mL human menopausal gonadotropin (HMG), and 1 µg/mL 17β-estradiol to support oocyte maturation. Additionally, TCM is used for sperm capacitation via the swim-up method; however, in this application, it does not contain any additives beyond the base medium.

TCM		
Material	Concentration	Osmolarity: 275–285 pH: 7.2–7.4
M199	0.475g	
NaHCO ₃	0.025 M	
Na Pyruvate	0.0002 M	
Pen/strep	1X	
water	To 50mL	

For in vitro maturation (IVM), 200 µl droplets of maturation medium are prepared in a Petri dish and covered with mineral oil. These droplets are equilibrated in an incubator at 38°C with 7% CO₂ for at least six hours before use to stabilize temperature and pH.

Following the washing steps, the COCs are transferred into the maturation droplets and incubated under the same conditions for 24 hours to facilitate in vitro maturation

RNA Extraction and RT-PCR:

Total RNA was isolated using a RiboX Kit (GENEALL Biotechnology, Seoul, Korea) according to the manufacturer's instructions with some modification, briefly, to extract RNA, homogenize 5–10 x 10⁶ cells in 1 ml of RiboEx™ reagent. Remove the culture media and add 1 ml of RiboEx™ per 10 cm² of culture dish area. Pass the lysate through a pipette several times and incubate for 5 minutes at room temperature. Add 0.2 ml of chloroform per 1 ml of RiboEx™ and shake vigorously for 15 seconds, followed by a 2-minute incubation at room temperature. Centrifuge at 12,000 x g for 15 minutes at 4°C, and transfer the aqueous phase to a fresh tube. Add 0.5 ml of isopropanol per 1 ml of RiboEx™ used and mix gently by inversion 3–5 times. Incubate for 10 minutes at room temperature, then centrifuge at 12,000 x g for 10 minutes at 4°C, discarding the supernatant. Wash the RNA pellet with 1 ml of 75% ethanol and centrifuge at 7,500 x g for 5 minutes at 4°C. Discard the ethanol, air-dry the pellet for 5 minutes, and dissolve the RNA in DEPC-treated water or 0.5% SDS solution by incubating at 56°C for 10–15 minutes.

First-strand cDNA was synthesized using the cDNA synthesis kit (Dena Zist Asia, Iran) and an oligo dT primer as follows. Mix 1 µg total RNA and 1 µL random six polymer primer, RNase-free water is made up

to 14.5 μ L at 70°C for 10 min, and experiences ice bath for 10 min. 0.5 μ L RNAase I, 5 $\times$ buffer 2 μ L, 10 mmol/L dNTP 2 μ L are added in for 42°C for 40 min

Primers for the *gdf9*, *fshr* and *gapdh* genes were designed using CLC Main Workbench software (CLC bio Co., Aarhus, Denmark) and Allele ID 7.5 (Premier Biosoft, Palo Alto, CA) (Table 3). Exon junction or separated exon strategies were employed to design primers, minimizing the risk of mispairing during PCR. For quantitative PCR (qPCR), 5 HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia) was used. The final reaction volume was 20 μ L, consisting of 10 pmol/ μ L of specific forward and reverse primers, 4 μ L of EvaGreen master mix (5X), and 1 μ L of cDNA. The reactions were performed using the Rotor-Gene 3000 (Corbett research, Australia) with the following cycling program: initial denaturation for 5 minutes at 95°C, followed by 45 cycles of 15 seconds each at 95°C, 20 seconds at 52 and 56°C for *gdf9* and *fshr* respectively, and 20 seconds at 72°C. A melting curve analysis was included, ramping from 65°C to 99°C in 0.5°C increments, with a 5-second wait at each step. Each PCR product displayed a single peak in the melting curve analysis (supplementary Fig. 1). All reactions were performed in triplicate (Table 3).

Table 3

This table presents the sequences of primers designed for RT-qPCR, along with their respective annealing temperatures (Ta, °C), expected PCR fragment sizes (bp), and corresponding accession numbers.

Target	Accession number	Fwd & Rev sequences	Amplicon(bp)	Ta(c)
<i>gdf9</i>	NC_056058	GAC GCC ACC TCT ACA ACA TTT AAC AGG AAA GGG AAA AGA A	200	56
<i>Fshr</i>	NC_056056	ATG ATG TCT TGG AAG TGA TAG CGA TGT ATA GCA GGT TGT T	93	54
<i>Gapdh</i>	NC_056056	GAG AAA CCT GCC AAG TAT TCA GTG TAG CCT AGA ATG	86	52

Rhodamin123

The 1 μ M Rhodamine 123 solution was prepared by diluting a 1 mM Rhodamine 123 stock solution (0.4 mg of Rhodamine 123 (Sigma-Aldrich, Massachusetts, United States) dissolved in 1 mL DMSO (Sigma-Aldrich, Massachusetts, United States)) with PBS. Prior to use, the dye solution was incubated at 37°C. Oocytes were then incubated in the dye solution at 37°C for 30 minutes, washed twice with PBS, and mounted on a microscope slide, which was covered with a coverslip. The samples were analyzed using an inverted fluorescent microscope (Nikon, Japan), with excitation at 508 nm and emission at 528 nm (Fig. 9).

In Vitro Fertilization (IVF) and Sperm preparation:

After removing the IVM medium drops, the matured COCs are washed 4 times in new Rinsing TALP(R-TALP) with 5mg/ml BSA, 50 µl/ml Na pyruvate stock(Prepared by dissolving in normal saline) drops for matured eggs, and then transferred to Fertilization TALP(F-TALP) with 5mg/ml BSA, 11µl/ml Na pyruvate stock, 10µl/ml heparin) drops that were added at least 6 hours ago and placed in the incubator, and remain in the incubator until sperm preparation.

Semen was obtained from a mature ram housed at the University of Zanjan's animal research facility. Collection was performed using a specialized collection device under controlled environmental conditions to ensure optimal semen quality. The ram was handled following ethical guidelines, with the collection process conducted by trained personnel to minimize stress (Thanks to Mr. Yaser Moghadam). Post-collection, the semen was immediately assessed for motility, viability, and concentration before being processed for further use in in vitro fertilization experiments. A volume of 2mL of fresh semen is transferred to a Falcon tube and centrifuged at 4,000 rpm for 20 minutes at 4°C. The supernatant is then removed using a sampler, and 500 µL of TCM (Table 2) medium is gently added to the sediment along the inner wall of the Falcon tube. Using the swim-up method, the tube is placed at a 45° angle in a CO₂-free incubator at 37°C for 45 minutes. After incubation, approximately 10 µL of the solution containing capacitated spermatozoa are collected. Depending on the number of capacitated spermatozoa, the sample may be diluted with IVF medium before being added to the IVF drops containing oocytes. (At a 1:100 ratio)

Embryo Culture Medium (IVC) intravaginal culture:

24 hours after sperm addition, the cells are removed from the IVF medium, and the cumulus layers are separated by gently vortexing until the cumulus cell layers detach. The embryos are then washed four times by IVC HEPES buffer (SOF) washing (Table 4) then transferred to the IVC SOF medium(Table 5), which has been pre-dropped and placed in the incubator for 6 hours prior to use (Table 4,5).

Table 4

Composition of IVC HEPES buffer (SOF) washing solution, including stock solutions and their final concentrations. Stock S contains NaCl (1 M), KCl (0.07 M), KH_2PO_4 (0.01 M), $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$ (0.01 M), and Na lactate (0.07 M). Stock NaHCO_3 consists of NaHCO_3 (0.5 M) dissolved in water. Stock HEPES contains HEPES (free acid, 0.2 M) and HEPES (Na salt, 0.2 M). The solution is prepared to maintain an osmolarity of 275–285 mOsm and a pH of 7.2–7.4.

IVC HEPES buffer (SOF) washing		
Material	Concentration	Osmolarity: 275–285 pH: 7.2–7.4
Stock S	10%	
Stock NaHCO_3	1%	
Stock $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (100X)	1%	
Stock HEPES	5%	
Na Pyruvate	0.0003 M	
Pen/strep	1X	
water	Up to 50 mL	

Table 5

Composition of IVC SOF medium, including stock solutions and their final concentrations. Stock S contains NaCl (1 M), KCl (0.07 M), KH_2PO_4 (0.01 M), $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$ (0.01 M), and Na lactate (0.07 M). Stock NaHCO_3 consists of NaHCO_3 (0.5 M) dissolved in water. Stock Glucose contains Glucose (0.15 M) dissolved in water. The medium is prepared to maintain an osmolarity of 265–275 mOsm and a pH of 7.2–7.4.

IVC SOF		
Material	Concentration	Osmolarity: 265–275 pH: 7.2–7.4
Stock S	10%	
Stock NaHCO_3	5%	
Stock $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (100X)	1%	
Stock glucose	1%	
BME-eaas(50x)	2%	
MEM-neaas (100x)	1%	
L-glutamine	0.004 M	
Na Pyruvate	0.0003 M	
BSA-FAF	1951.21 M	
Pen/strep	1X	
water	Up to 10 mL	

Embryo culture medium replacement (medium refresh):

This procedure is performed every 48 hours after the day of embryo culture (or 72 hours after IVF, i.e. on the third embryonic day) and using IVC-SOF medium (10% CSS).

Ethics declarations

All procedures involving cell, tissue, and sperm sampling, as well as cell culture, were conducted in accordance with ethical guidelines and approved by the Ethical Committee of Zanjan University, Iran (Approval Number: ZNU.REC.1402.006).

Result

GC-MS Analysis

The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of *Viola alba subsp. sintenisii* extract VE revealed a chemically complex profile, detecting a total of 38 volatile compounds, which together accounted for 99.998% of the extract's total composition. The three most abundant constituents were methyl salicylate (49.55%), furfuryl alcohol (5.42%), and hexadecanoic acid (4.17%), suggesting a

potential role in the extract's bioactivity. The compounds exhibited a broad distribution of concentrations, with an average abundance of $2.63 \pm 7.79\%$, emphasizing the chemical diversity of the extract. This variability suggests that multiple bioactive components might contribute to its observed effects on oocyte maturation (Fig. 2, Table 6).

Table 6

GC-MS Analysis of Volatile Compounds in *Viola alba subsp. sintenisii* Extract. This table presents the identified volatile compounds in the hydro-phenolic extract of *V. alba subsp. sintenisii*, analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). A total of 38 volatile compounds were detected, collectively accounting for 99.998% of the extract's composition. The three most abundant compounds were methyl salicylate (49.553%), furfuryl alcohol (5.419%), and hexadecanoic acid (4.171%), highlighting their potential role in the extract's bioactivity. The average abundance of volatile compounds was $2.63 \pm 7.79\%$, indicating a wide distribution of compound concentrations within the extract.

Peak IDs	RT(min)	Abundance(%)	Peak IDs	RT(min)	Abundance(%)
methyl salicylate	4.87	49.553	Benzyl alcohol	19.11	1.122
Furfuryl alcohol	15.94	5.419	β -pinene	6.81	1.003
Hexadecanoic acid	21.93	4.171	α -pinene	5.28	0.985
Octanoic acid	20.89	3.181	Propenoic acid	15.51	0.963
Hexanoic acid	18.57	2.951	Guaiacol	18.61	0.8
Butyrolactone	15.01	2.774	Propanoic acid	13.52	0.791
Isobutanoic acid	14.24	1.963	β -Myrcene	4.68	0.683
2-Methyl-3-butanol	3.61	1.872	Nonanoic acid	21.43	0.675
Octadecanoic acid	22.06	1.789	Eugenol	21.22	0.606
3-Penten-2-ol	4.26	1.683	Phenol	20.44	0.604
Butanoic acid	15.46	1.631	Styrene	8.28	0.498
Acetoin	9.36	1.624	Sabinene	4.52	0.466
dodecanoic acid	8.04	1.46	Limonene	7.39	0.456
α -Terpinene	7.18	1.451	Acetic acid	12.26	0.418
(E)-3-Hexenoic acid	19.78	1.44	Pentanoic acid	16.21	0.413
Hydroxyacetone	9.44	1.42	Phenethyl alcohol	19.74	0.405
Furfural	12.62	1.405	γ -Terpinene	7.91	0.39
Camphene	6.01	1.322	(Z)-2-Methyl-2-buten-1-ol	10.18	0.25
m-xylene	4.22	1.171	Ethyl 4-ethoxybenzoate	21.72	0.19

LC-MS Analysis

The Liquid Chromatography-Mass Spectrometry (LC-MS) analysis confirmed the extract’s biochemical richness, identifying 13 phenolic compounds. Among these, rutin (1968.13 mg/kg), quercetin-3-6-O-acetyl-β-glucopiranoside (875.30 mg/kg), and kaempferol-3-/6-O-acetyl-β-glucopiranoside (754.93 mg/kg) were the most abundant. The average concentration of phenolic compounds was 530.94 ± 493.28 mg/kg, indicating a wide range of compound abundance. Given the well-documented antioxidant and pharmacological roles of these phenolic constituents, their presence in the extract suggests a significant potential for influencing cellular signaling pathways involved in oocyte maturation (Fig. 3, Table 7).

Table 7

LC-MS Analysis of Phenolic Compounds in VE. This table presents the identified phenolic compounds in the hydro-phenolic extract of *V. alba subsp. sintenisii*, analyzed using Liquid Chromatography-Mass Spectrometry (LC-MS). A total of 13 phenolic compounds were detected, with the highest concentrations observed for Rutin (1968.13 mg/kg), Quercetin-3-6-O-acetyl-β-glucopiranoside (875.30 mg/kg), and Kaempferol-3-/6-O-acetyl-β-glucopiranoside (754.93 mg/kg). The average concentration of phenolic compounds was 530.94 ± 493.28 mg/kg, highlighting the variability in compound abundance within the extract. These bioactive molecules contribute to the antioxidant and pharmacological properties of the extract.

Peak IDs	RT (min)	LOD (mg/kg)	LOQ (mg/kg)	Recovery (%)	Concentration (mg/kg)
Rutin	6.61	1968.13	93	787.93	393.8
Quercetin-3-6-O-acetyl-/-β-glucopiranoside	8.88	875.3	100	350.38	175.76
Kaempferol-3-/6-O-acetyl-/-b-glucopiranoside	11.37	754.93	91	302.67	151.96
Isoquercitrin	7.05	720.67	93	288.63	144.55
Narcissin	9.55	626.65	89	251.37	125.64
Dattelic acid	5.77	624.24	95	250.43	125.59
Quercetin	13.02	355.95	101	143.31	71.2
Isorhamnetin-3-O-β-glucoside	9.85	285.77	101	114.96	57.76
Chlorogenic acid	4.11	198.61	91	80.44	40.49
Astragalin	7.96	194.54	89	78.76	38.92
Isorhamnetin-3-/6-Oacetyl-/-β-glucopiranoside	12.54	123.32	93	50.07	25.11
Isorhamnetin	14.87	104.8	97	42.64	21.59
Kaempferol	13.95	69.38	97	28.49	14.08

Grading of Cumulus-Oocyte Complexes (COCs)

To ensure experimental consistency, COCs were categorized based on their morphological characteristics, including cumulus cell layer number, ooplasm homogeneity, and oocyte size. Grade A COCs, characterized by five or more cumulus cell layers, uniform ooplasm, and optimal oocyte size, were considered of the highest quality. Grade B COCs, with three to four cumulus cell layers and minor variations in ooplasm homogeneity, were also deemed suitable for in vitro maturation (IVM). In contrast, Grade C COCs, displaying only two or fewer cumulus layers, irregular ooplasm, and reduced oocyte size, along with Grade D COCs exhibiting signs of degeneration, were excluded from further experiments. This rigorous selection process ensured that only developmentally competent oocytes were used for treatment with VE, minimizing experimental variability (Fig. 4).

Optimization of BCB Staining for COC Selection

To distinguish between mature and immature oocytes, the Brilliant Cresyl Blue (BCB) staining protocol was optimized by testing three different concentrations (13, 26, and 52 µg/mL). The 13 µg/mL concentration resulted in insufficient staining intensity, making it difficult to accurately assess oocyte maturity. Conversely, the 52 µg/mL concentration caused excessive staining, leading to uniform coloration across all COCs and reducing the ability to differentiate between mature and immature oocytes. The 26 µg/mL concentration proved optimal, effectively distinguishing BCB-positive (mature) from BCB-negative (immature) oocytes. This concentration was subsequently used to ensure the selection of metabolically active oocytes for further treatment with VE. The sampling process from the slaughterhouse was repeated at least 20 times to obtain a consistent population of COCs, which were then exposed to the extract at three concentrations (13, 26, and 52 µg/mL). Following 24 hours of maturation, various assays, including Rhodamine 123 staining for mitochondrial activity and qPCR for gene expression analysis, were performed. Additionally, a subset of COCs was subjected to in vitro fertilization (IVF) to evaluate embryonic development (Figs. 5 and 6).

Relative Gene Expression of *fshr* and *gdf9*

The effect of (VE) on the expression of key follicular development genes was assessed by quantifying *fshr* (follicle-stimulating hormone receptor) and *gdf9* (growth differentiation factor 9) expression using qPCR. After 24 hours of exposure, COCs treated with 50 µg/mL of the extract exhibited a significant upregulation of both *fshr* and *gdf9* ($p < 0.01$) compared to the control group. However, higher concentrations (100 and 200 µg/mL) did not result in further upregulation, suggesting a dose-dependent effect, with 50 µg/mL being the optimal concentration for enhancing oocyte developmental competence (Fig. 7).

Mitochondrial Activity and Distribution in COCs

To examine mitochondrial function, Rhodamine 123 (RH123) staining was used to assess mitochondrial activity and distribution in COCs treated with 50, 100, and 200 µg/mL of the extract. Fluorescent

microscopic analysis showed no significant differences in mitochondrial spatial distribution across the treatment groups ($p > 0.05$), indicating that the extract did not affect mitochondrial localization. However, qualitative observations suggested that COCs treated with 50 µg/mL exhibited brighter and more uniform fluorescence intensity, suggesting enhanced mitochondrial function and improved energy metabolism (Fig. 8).

COC Morphology Post-IVM with *V. alba* Extract

Morphological assessment of COCs following in vitro maturation (IVM) revealed that cumulus expansion was not significantly affected by treatment with VE at 50, 100, or 200 µg/mL. Quantitative analysis using ImageJ/Fiji software confirmed that cumulus expansion remained consistent across all treatment groups compared to controls. Importantly, treated COCs maintained normal structural integrity, indicating that the extract did not negatively impact oocyte or cumulus cell viability (Fig. 9).

sheep Embryo Development Post-IVF

To evaluate the developmental potential of oocytes matured VE, embryos were cultured under optimal in vitro conditions for seven days following fertilization. The early pre-implantation development of ovine embryos followed a well-defined sequential process, beginning with the two-cell stage on Day 1, where the zygote underwent its first mitotic division, forming two blastomeres within the zona pellucida.

By Day 2, the embryo progressed to the four-cell stage, continuing synchronous cleavage while maintaining totipotency. This was followed by the sixteen-cell stage on Day 3, characterized by increased cell-cell adhesion and the formation of tight junctions, which are crucial for subsequent development. On Day 4, further cleavage divisions resulted in the thirty-two-cell stage, marking the onset of compaction, a key transition leading to the morula stage.

By Day 5, the embryo reached the morula stage, where intercellular adhesion strengthened, setting the foundation for the formation of the blastocoel cavity. Finally, by Day 7, the blastocyst stage was achieved through cavitation, leading to the differentiation of two distinct cell populations: the inner cell mass (ICM), which gives rise to the fetus, and the trophectoderm, which contributes to placenta formation.

These progressive embryonic stages demonstrate that embryos derived from *V. alba* extract-treated oocytes followed a normal developmental trajectory, culminating in fully formed blastocysts ready for implantation (Fig. 10). This finding suggests that the extract does not negatively impact fertilization success or early embryogenesis, further supporting its potential role in enhancing reproductive outcomes.

Discussion

Our study demonstrated a significant upregulation of *gdf9* and *fshr* genes in sheep cumulus-oocyte complexes (COCs) following in vitro exposure to *Viola alba subsp. sintenisii* extract (VE), particularly at

the concentration of 50 µg/mL. These findings align with previous studies reporting that plant-derived bioactive compounds influence ovarian gene expression and improve developmental competence of oocytes through antioxidative and regulatory pathways. This modulation likely stems from bioactive substances identified in our extract via LC-MS, including quercetin, kaempferol, chlorogenic acid, isorhamnetin, and rutin, which are known for their roles in reducing oxidative stress and enhancing cellular function during in vitro maturation (IVM)^{23–44}

The pronounced upregulation of *gdf9* observed in our study aligns closely with prior reports where flavonoids such as kaempferol and quercetin significantly enhanced *gdf9* expression in sheep preantral follicles and granulosa cells cultured in vitro. These flavonoids reduce intracellular reactive oxygen species (ROS), protecting granulosa cells and oocytes from oxidative damage and promoting a favorable cellular environment for gene expression^{23,30,35,37,42,45–47}. Specifically, kaempferol was reported to stimulate ovarian follicle activation by modulating the PI3K/Akt signaling pathway, enhancing follicular survival and improving oocyte developmental competence^{41,42}. Quercetin, recognized for its potent antioxidant properties, stabilizes mitochondrial function, reduces apoptosis through modulation of the MAPK and PI3K/Akt signaling pathways, and supports follicular development and steroidogenesis in vitro^{32,35,37,42,45,47,48}.

Chlorogenic acid (CGA), another significant compound identified in our extract, demonstrates protective effects by safeguarding mitochondrial integrity, reducing apoptosis, and enhancing blastocyst formation rates under stressful in vitro conditions. These antioxidant properties likely contribute directly to the enhanced expression of developmental genes observed in our study^{25,26,33,49,50}. Improved mitochondrial membrane potential and decreased ROS levels in sheep and porcine oocytes treated with CGA highlight its protective role during stressful IVM conditions^{26,33,50}.

Isorhamnetin identified in our LC-MS analysis has previously been reported to protect porcine oocytes from oxidative stress and mitochondrial dysfunction via the PI3K/Akt signaling pathway, promoting oocyte maturation and reducing apoptosis^{29,51}. Rutin, another identified flavonoid, has demonstrated potent antioxidant effects, enhancing mitochondrial function and reducing apoptosis in sheep oocytes. It also improved developmental competence post-vitrification by decreasing oxidative stress and protecting mitochondrial integrity^{38–40,43,52}.

The observed dose-dependent effects, with optimal gene upregulation at 50 µg/mL and reduced effectiveness at higher concentrations (100 and 200 µg/mL), could be explained by the hormetic effect common to antioxidants like flavonoids and chlorogenic acid. Moderate concentrations effectively neutralize ROS without impairing physiological signaling pathways necessary for normal oocyte maturation, whereas higher concentrations may disrupt cellular redox balance^{23,26,35,42,50,53}. Previous studies similarly noted optimal beneficial effects at moderate doses of flavonoids and chlorogenic acid on follicular viability, gene expression, and embryo development, with higher concentrations linked to toxicity or reduced efficacy^{23,30,35,36,42,45}.

Our findings suggest that gene expression upregulation is dose-dependent, facilitated by balanced modulation between oxidative and antioxidative processes by flavonoids, chlorogenic acid, isorhamnetin, and rutin. The optimal concentration of 50 µg/mL aligns with previous reports demonstrating beneficial effects of moderate doses of these compounds during IVM^{23,26,30,42,50}.

Interestingly, despite the clear effects on gene expression and developmental competence, our study found no significant changes in mitochondrial distribution when using rhodamine123 staining. Such discrepancies might be attributed to differences in microscopy technique, resolution, fluorescent probe characteristics, or brand of Rhodamine 123 used. Studies have indicated variations in mitochondrial staining patterns depending on dye concentration, incubation time, or imaging conditions, potentially affecting visualization of subtle mitochondrial distribution changes^{54–57}.

In summary, our results underscore the significant role of bioactive compounds present in *Viola alba* subsp. *sintenisii* extract—particularly flavonoids, chlorogenic acid, isorhamnetin, and rutin—in modulating gene expression profiles critical for successful in vitro oocyte development. These compounds exert their beneficial effects through mechanisms including the reduction of oxidative stress, enhancement of mitochondrial function, regulation of apoptotic pathways, and modulation of key signaling cascades. Notably, beyond promoting oocyte maturation, treatment with the extract also led to a marked increase in the rate of successful blastocyst formation, indicating a positive influence on subsequent embryonic development and implantation potential. The observed dose-dependent response further emphasizes the necessity of precise concentration optimization within IVM protocols. Collectively, these insights not only deepen our understanding of how plant-derived antioxidants contribute to reproductive biotechnology but also provide a robust foundation for the integration of natural bioactives into embryo production strategies aimed at improving fertility outcomes.

Declarations

Competing interests

The authors declare no competing interests.

Author Contribution

Elahe Gholamian: Conducted the experiments, prepared and wrote the original draft of the manuscript, developed the methodology with the materials and laboratory setup. Ali Amarlou, Farzan Taheri, and Parvin Mehrabi: Conducted botanical work, prepared herbal extracts, and performed LC-MS/GC-MS analysis. Fatemeh Seyed Monfared Zanjani: Assisted with the experiments, wrote, reviewed, and edited the manuscript, developed the methodology with the materials and laboratory setup. Reza Asadpour: Preparing the technicians, contributed to methodology development, and validated the study. Parsa Dorani: Collected samples, reviewed and edited the manuscript, and contributed to the discussion. Elahe

Imani and Maral Majidi: Collected samples and prepared media. Performed data analysis. Abbas Bahari: Supervised the study, contributed to conceptualization, methodology, software, and data analysis, and interpreted the data.

Acknowledgment

We would like to express our gratitude to Dr. Davood Zahmatkesh and Mr. yasser moghadami for their valuable assistance in preparing a fresh semen sample in this study. Their expertise and support greatly contributed to the success of our research.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Figures



Figure 1

Morphological characteristic of *V. alba* subsp. *sintenisii*. established on LCV.

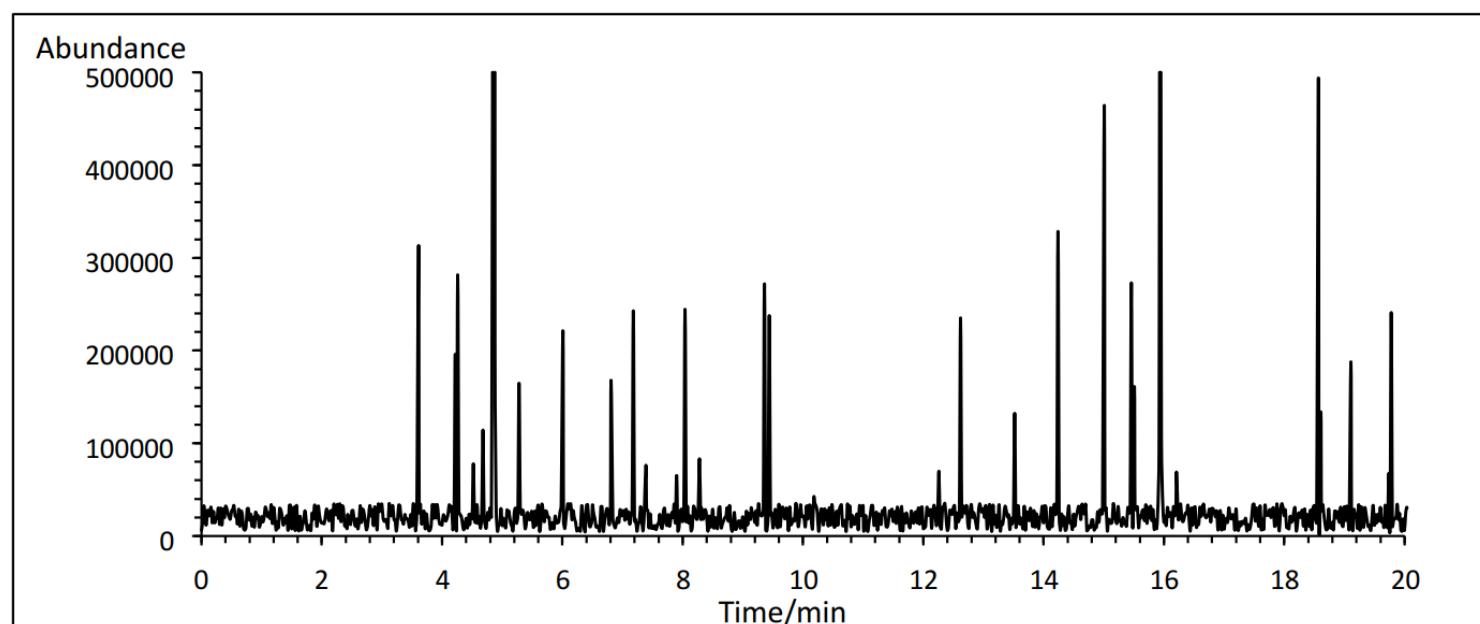


Figure 2

GC-MS Chromatogram of *Viola alba* subsp. *sintenisii* Extract VE: This chromatogram represents the Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the hydro-phenolic extract of *V. alba* subsp. *sintenisii*. The x-axis denotes the retention time (min), while the y-axis represents abundance, corresponding to the relative intensity of detected compounds. Several prominent peaks indicate the presence of major volatile constituents, with methyl salicylate (49.553%), furfuryl alcohol (5.419%), and hexadecanoic acid (4.171%) being the most abundant compounds. The chromatogram reflects the complex chemical profile of the extract, confirming the diversity of bioactive components.

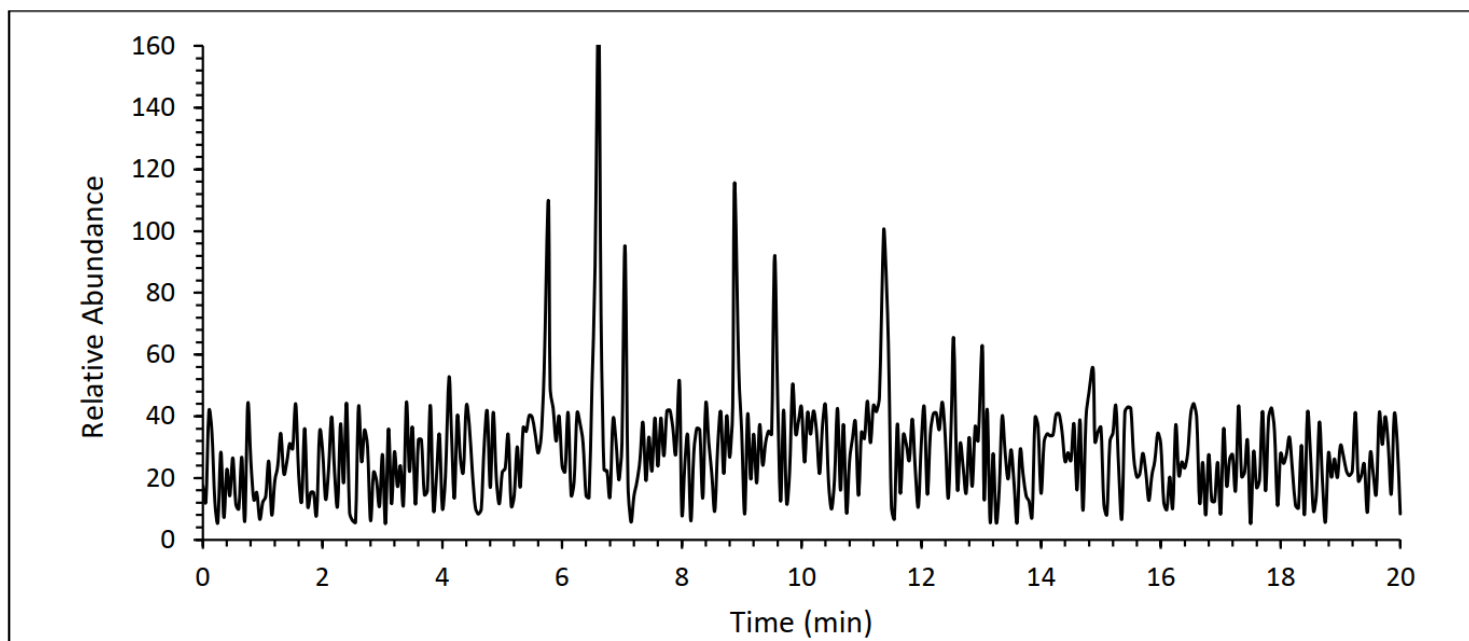


Figure 3

LC-MS Chromatogram of VE. This chromatogram represents the Liquid Chromatography-Mass Spectrometry (LC-MS) analysis of the hydro-phenolic extract of *V. alba subsp. sintenisii*. The x-axis denotes the retention time (min), while the y-axis represents relative abundance, indicating the intensity of detected phenolic compounds. The peaks correspond to the presence of various bioactive compounds, with Rutin (1968.13 mg/kg), Quercetin-3-6-O-acetyl- β -glucopiranoside (875.30 mg/kg), and Kaempferol-3-/6-O-acetyl- β -glucopiranoside (754.93 mg/kg) being the most abundant. The chromatographic profile confirms the diversity of phenolic constituents within the extract.

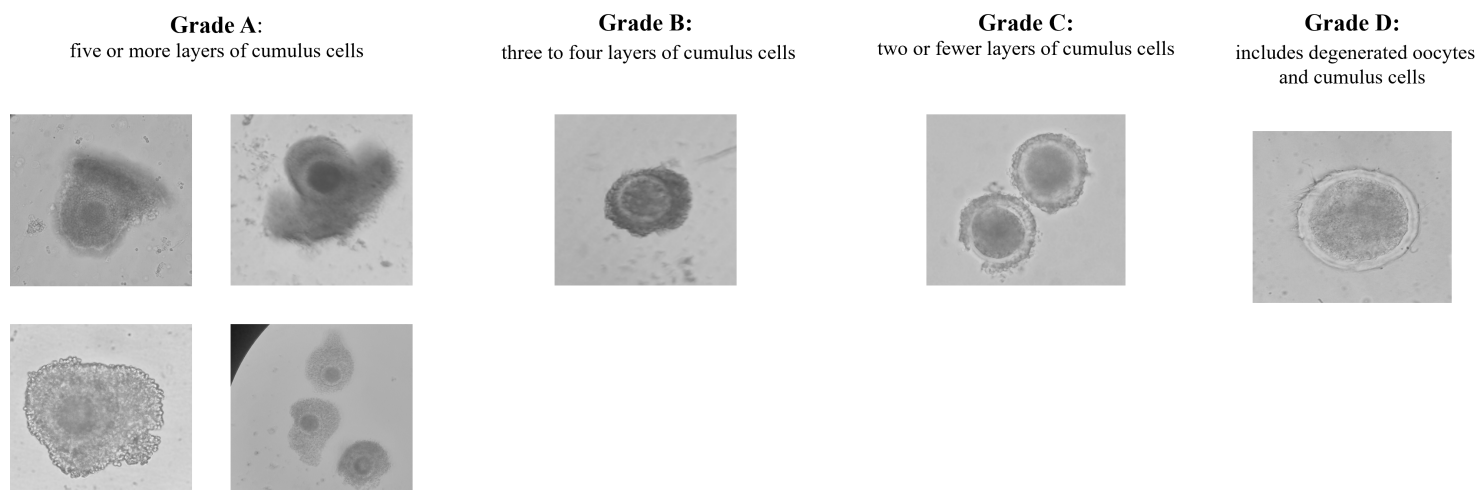


Figure 4

Grading of cumulus-oocyte complexes (COCs) based on morphological characteristics. COCs are classified according to the number of cumulus cell layers, ooplasm homogeneity, and oocyte size. Grade A COCs have five or more layers of cumulus cells, while Grade B has three to four layers. Grade C

includes COCs with two or fewer cumulus layers, and Grade D consists of degenerated oocytes and cumulus cells. Grades A and B are considered normal, whereas Grades C and D are abnormal. Representative images depict each grade, illustrating the progressive loss of cumulus cell layers and changes in oocyte morphology.

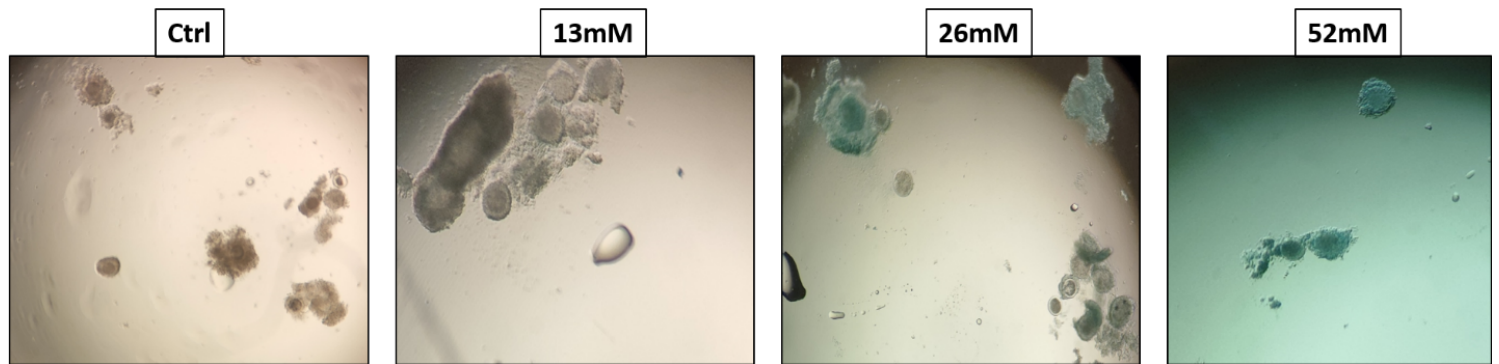


Figure 5

Optimization of BCB staining concentration for selecting mature sheep COCs. COCs were stained with varying concentrations of BCB (Ctrl, 13, 26, and 52 $\mu\text{g}/\text{mL}$) to determine the optimal concentration for distinguishing mature (BCB positive) oocytes. The results indicate that 26 $\mu\text{g}/\text{mL}$ BCB provided the best differentiation between mature and immature oocytes, as lower concentrations (13 $\mu\text{g}/\text{mL}$) showed insufficient staining, while higher concentrations (52 $\mu\text{g}/\text{mL}$) resulted in uniform staining of all COCs, compromising the ability to differentiate maturation status.

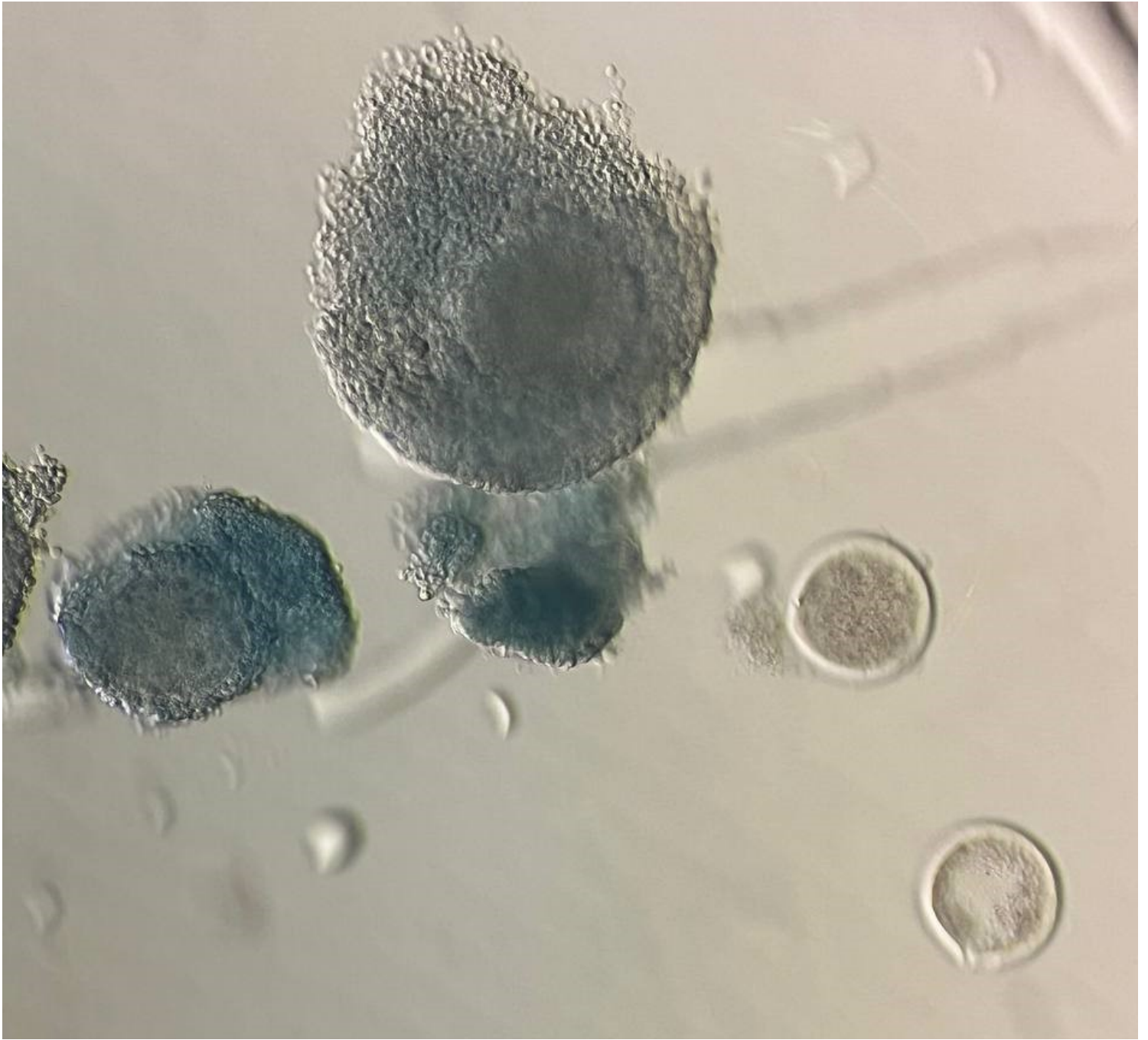


Figure 6

BCB Staining: After optimizing the BCB staining concentration at 26 $\mu\text{g/mL}$, only BCB-positive cumulus-oocyte complexes (COCs) were selected for further analysis, ensuring a homogenous population of developmentally competent oocytes. BCB-negative COCs were excluded to minimize variability and focus on the potential effects of VE on metabolically active oocytes. The selected BCB-positive COCs were then exposed to three different concentrations of VE to assess its impact on mitochondrial activity, oocyte maturation, and overall cellular health.

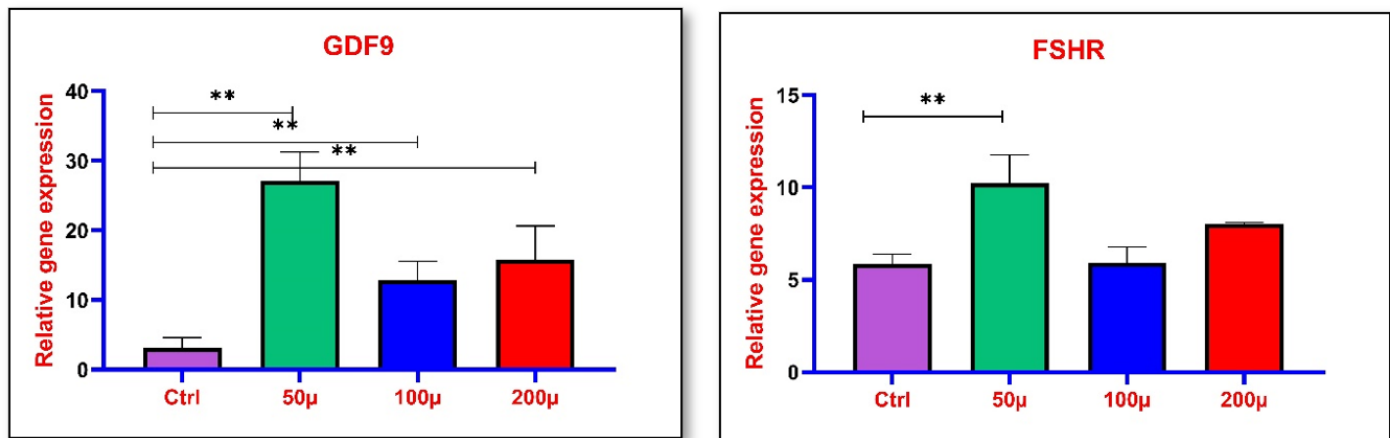


Figure 7

Relative gene expression of *fshr* and *gdf9* in sheep COCs after 24 hours of exposure to VE. COCs were challenged with 50, 100, and 200 µg/mL of (EV), prepared by dissolving 5 mg of dry matter in 1 mL of double-distilled water (ddH₂O), under the conditions specified in the Materials and Methods section. After 24 hours, total RNA was extracted, and the expression levels of two key maturation genes, *fshr* (A) and *gdf9* (B), were assessed by real-time qPCR. The 50 µg/mL concentration showed significantly higher expression of both genes compared to the control (Ctrl) and other concentrations, highlighting its potential role in promoting COC maturation. Data are presented as mean ± SEM, with statistical significance indicated by $p < 0.01$.

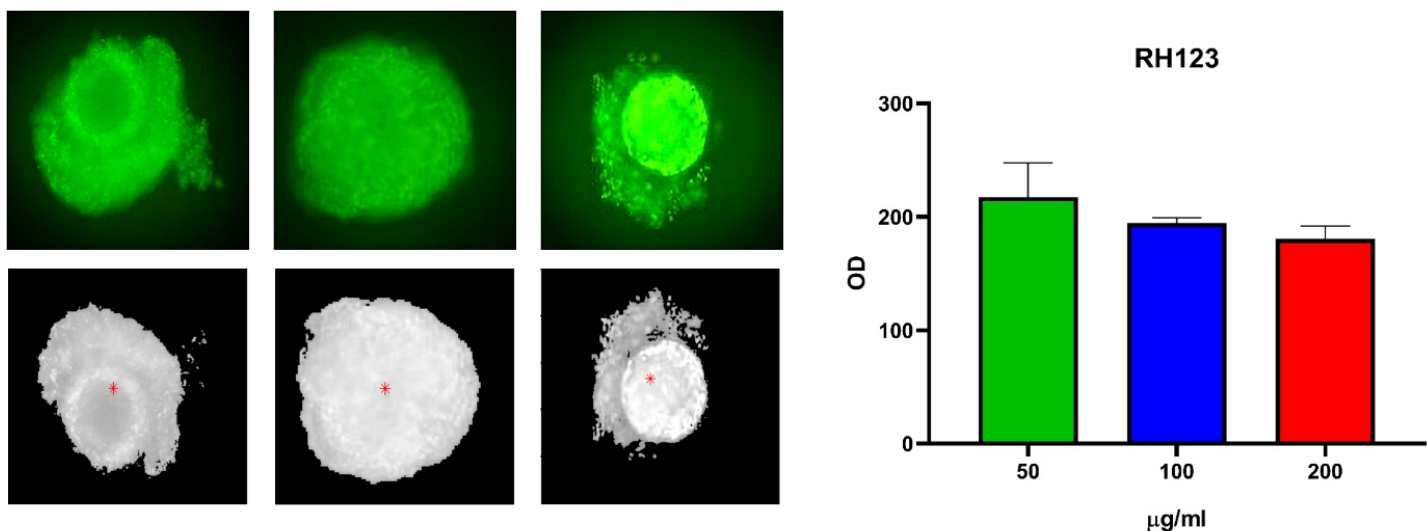


Figure 8

Quantitative analysis of mitochondrial distribution in sheep cumulus-oocyte complexes (COCs) stained with Rhodamine 123 (RH123). Fluorescent microscopic images were processed using image analysis software to assess mitochondrial distribution patterns. Image segmentation was performed to isolate individual COCs, followed by intensity-based thresholding to distinguish mitochondrial-rich regions.

Fluorescence intensity and spatial distribution were quantified using region-of-interest (ROI) analysis, and data were normalized to account for background fluorescence. Statistical analysis (ANOVA followed by post-hoc tests) revealed no significant differences in mitochondrial distribution among the three treatment groups ($p > 0.05$). The results suggest that different concentrations of VE did not induce detectable alterations in mitochondrial localization within COCs.

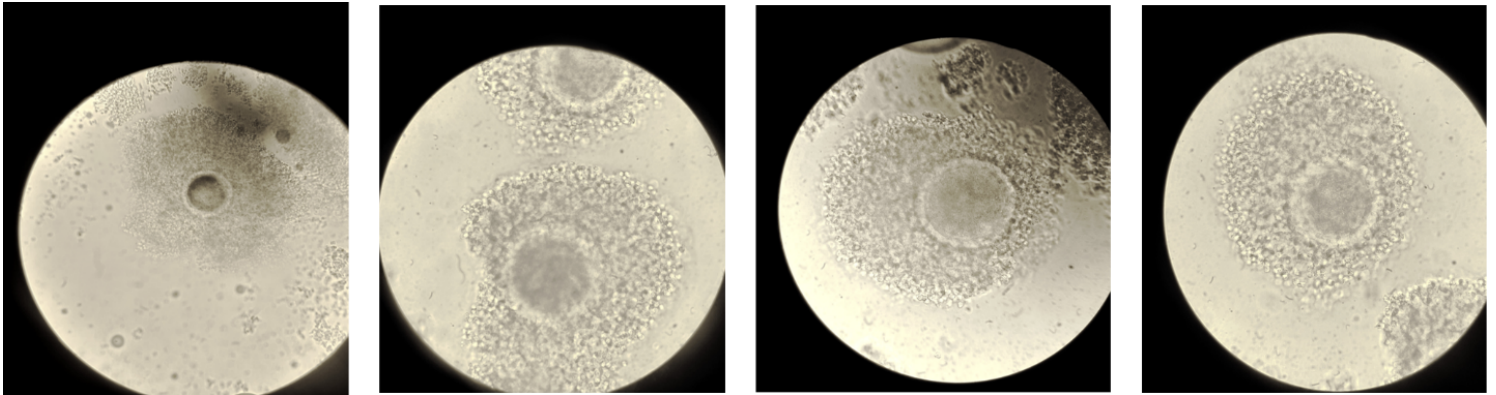


Figure 9

Morphological evaluation of sheep cumulus-oocyte complexes (COCs) after in vitro maturation (IVM) with VE.

COCs were exposed to maturation media supplemented with (VA) at concentrations of 50, 100, and 200 µg/mL, alongside a control group. Cumulus cell expansion, a key indicator of oocyte maturation, was assessed using ImageJ/Fiji software. Quantitative analysis revealed no significant differences in cumulus expansion between the treatment and control groups, suggesting (VA) did not affect cumulus cell expansion under these conditions.

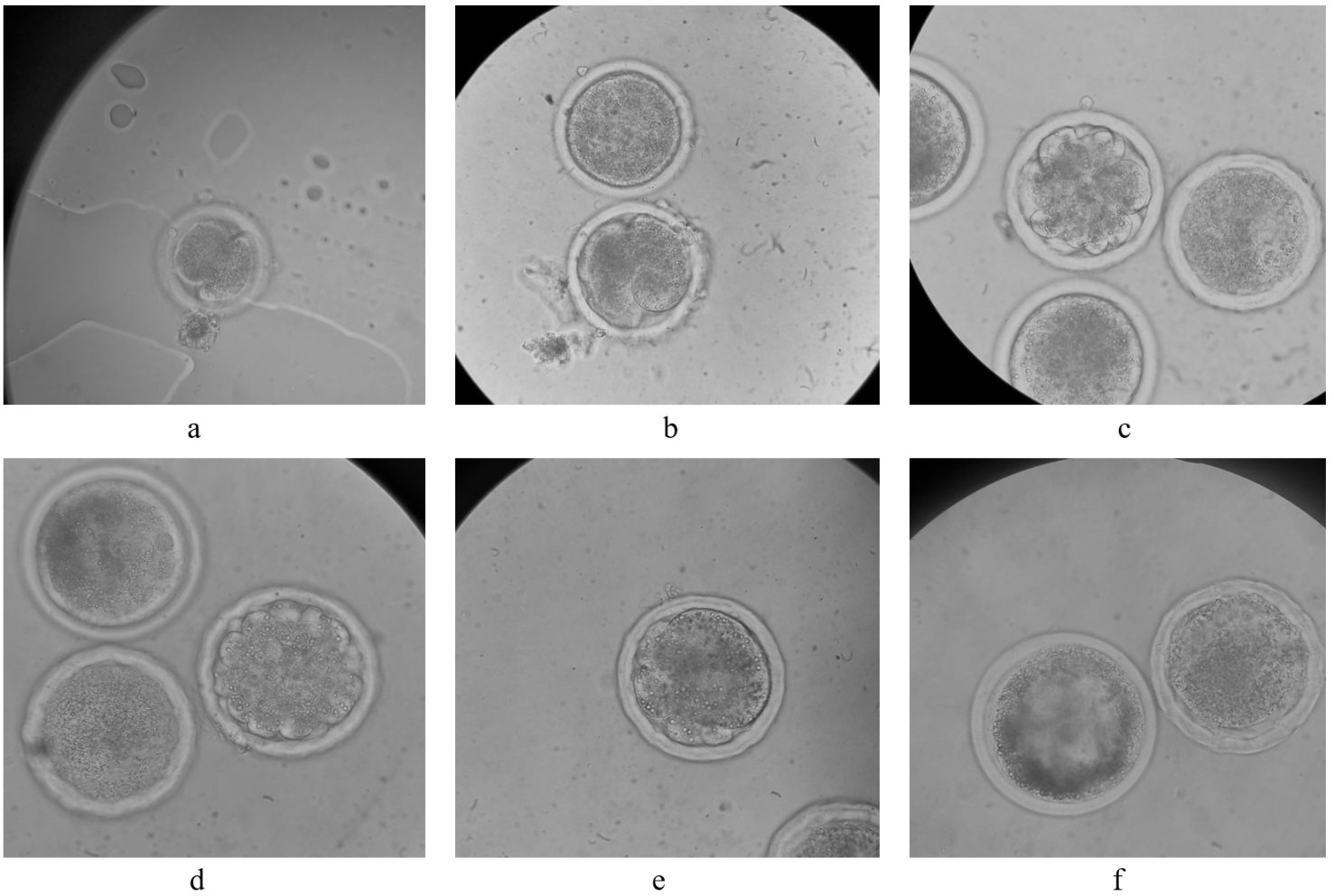


Figure 10

In Vitro Fertilization (IVF) Developmental Stages in Ovine Embryos

Representative microscopic images showing the sequential developmental stages of ovine embryos cultured in vitro from fertilization to the blastocyst stage. (A) Two-cell stage (Day 1), (B) Four-cell stage (Day 2), (C) Sixteen-cell stage (Day 3), (D) Thirty-two-cell stage (Day 4), (E) Morula stage (Day 5), and (F) Blastocyst stage (Day 7). Embryos were cultured under optimized in vitro conditions to assess developmental competence following fertilization.