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Effect of the acidified extract of *Moringa oleifera* leaves as a supplement in the *in vitro* culture medium of sheep preantral follicles

Efeito do extrato acidificado das folhas de *Moringa oleifera* como suplemento no meio de cultivo *in vitro* de folículos pré-antrais ovinos

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Highlights

M. oleifera to the *in vitro* culture of ovine follicles improved oocyte growth.
M. oleifera extract to the *in vitro* culture of ovine follicles improved GSH nivels.
Plant extract can be a alternative supplement for the in vitro growth of follicles.

Abstract

This study was conducted to evaluate the effects of the acidified extract of *M. oleifera* leaves as a supplement into the base medium for *in vitro* culture of sheep isolated secondary follicles. Follicles were isolated and cultured for 12 days in α-MEM$^+$ (supplemented with bovine serum albumin, insulin, glutamine, hypoxanthine, transferrin, selenium, and ascorbic acid) with or without 0.1; 0.2 or 0.4 mg/ml of the acidified extract of *M. oleifera.* Follicle morphology, antral cavity formation, follicular and oocyte diameter, glutathione (GSH) concentration, mitochondrial activity and meiotic resumption were evaluated. After 12

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days of culture, there was no significant difference among treatments in relation to follicular morphology, antral cavity formation, diameter and mitochondrial activity. Nevertheless, oocytes from follicles cultured in α-MEM⁺ showed greater GSH concentration than media containing *M. oleifera* extract. Furthermore, the concentration of 0.4 mg/ml *M. oleifera* extract significantly increased the percentage of fully grown oocyte (≥ 110 µm) when compared to the other treatments. In conclusion, the concentration of 0.4 mg/ml *M. oleifera* extract as a supplement of the culture medium, maintained the survival, and increased the percentage of fully grown oocytes.
Key words: Phenolic compound. *Moringa oleifera.* Oocyte. Ovine. Preantral follicle.

Resumo

Este estudo foi conduzido para avaliar os efeitos do extrato acidificado de folhas de *M. oleifera* como suplemento ao meio base para cultivo in vitro de folículos secundários isolados de ovinos. Os folículos foram isolados e cultivados por 12 dias em α-MEM⁺ (suplementado com albumina sérica bovina, insulina, glutamina, hipoxantina, transferrina, selênio e ácido ascórbico) com ou sem 0,1; 0,2 ou 0,4 mg/ml do extrato acidificado de *M. oleifera.* Foram avaliados morfologia folicular, formação da cavidade antral, diâmetro folicular e oocitário, concentração de glutationa (GSH), atividade mitocondrial e retomada meiótica. Após 12 dias de cultivo, não houve diferença significativa entre os tratamentos em relação à morfologia folicular, formação da cavidade antral, diâmetro e atividade mitocondrial. No entanto, oócitos de folículos cultivados em α-MEM⁺ apresentaram maior concentração de GSH do que meios contendo extrato de *M. oleifera.* Além disso, a concentração de 0,4 mg/ml de extrato de *M. oleifera* aumentou significativamente a porcentagem de oócitos totalmente crescidos (≥ 110 µm) quando comparado aos demais tratamentos. Em conclusão, a concentração de 0,4 mg/ml de extrato de *M. oleifera* como suplemento do meio de cultura manteve a sobrevivência e aumentou a porcentagem de oócitos totalmente crescidos.
Palavras-chave: Composto fenólico. Folículo pré-antral. *Moringa oleifera.* Oócito. Ovino.

Introduction

The *in vitro* culture of secondary follicles is an important approach to obtain a larger number of meiotically competent oocytesto embryo production (Macedo et al., 2019; Kona et al., 2021). However, in larger species, such as sheep and goat, the production of metaphase II (MII) oocytes and embryos from in vitro grown secondary follicles is still low (Sá et al., 2020). One of the factors that may contribute to poor oocyte quality is the generation of oxidative stress by the overproduction of reactive oxygen species (ROS) under in vitro environmental conditions (Yang et al., 2017; Ding et al., 2020). In this sense, medicinal plant extracts with antioxidant properties, such as *Amburana cearensis, Morus nigra* and *Justicia insularis,* have been used as supplement of the standard medium or as alternative culture mediato improve quality and development of oocytes from secondary follicles cultured *in vitro* (Gouveia et al., 2016; Cavalcante et al., 2018; Mbemya et al., 2018).

Moringa oleifera Lam (Moringaceae family), known as moringa or white acacia, is a tree originally found in northwestern India, but currently cultured in tropical and subtropical regions of the world (Lakshmidevamma

et al., 2021). *M. oleifera* leaf contains high amounts of protein, vitamin, mineral, and fatty acid, which can be used as an alternative nutritional source for livestock animal (Qwele et al., 2013; Su & Chen, 2020). Furthermore, it has been used in folk medicine for the treatment of a wide range of diseases due their various pharmacological activities like hypotensive, hypocholesterolemic, anti-inflammatory, antidiabetic, antibiotic, antioxidant, and nutraceutical properties (Fahey, 2017). The main therapeutic effects of *M. oleifera* are derived from its phenolic compounds with antioxidant properties, such as quercetin, isorhamnetin, gallic acid and kaempferol (Martono et al., 2019; Asgari-Kafrani et al., 2020; Oldoni et al., 2021). These secondary metabolites can removeor reduce ROS production, increasing the activity of antioxidants endogenous, such as reduced glutathione (GSH), the main intracellular component for preventing damage caused by oxidative stress in oocytes (Mukherjee et al., 2014), and improving mitochondrial function, thus preventing cellular damage from oxidative stress (Luqman et al., 2012; Asgari-Kafrani et al., 2020; González-Burgos et al., 2021).

Oral administration of *M. oleifera* leaf aqueous extract decreased insulin levels and increased the number and diameter of secondary and antral follicles in a rat model of polycystic ovary syndrome (PCOS) with insulin-resistant (Amelia et al., 2018). In other study, oral administration of methanolic extract of *M. oleifera* leaves in adult rats for three consecutive estrous cycles improved the follicular development and decreased atresia (Bogapathi et al., 2021). Furthermore, the addition of ethanolic extract of *M. oleifera* leaves to the *in vitro* maturation medium of sheep cumulus-oocyte complexes improved the synthesis of essential proteins for the maturation process, intracellular calcium concentration, and MII oocyte rates when compared to the medium without the extract (Barakat al., 2015).

Considering its antioxidant properties, distribution all over the world, and low-cost compared to chemical products, it could be speculated that the *M. oleifera* leaves extract would protect ovarian follicles from oxidative damage and stimulate their development during *in vitro* culture. However, there are no reports on the effects of the addition of *M. oleifera* extract to the *in vitro* culture medium of ovarian follicles in any species. Thus, this study was conducted to evaluate the effects of the acidified extract of *M. oleifera* leaves as a supplement into the base medium for *in vitro* culture of sheep isolated secondary follicles.

Material and Methods

Unless indicated otherwise, media, supplements, and chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). As this study used the ovaries disposed by the slaughterhouse and did not involve the experimentation with live animals, approval was not sought from the Ethics Committee on Animal Use.

Plant material and extract preparation

Fresh leaves of *M. oleifera* were collected in Juazeiro (9°25'17.9" South, longitude 40°28'52.7" West of Greenwich, Bahia, Brazil). A voucher specimen (23819) is deposited at the Herbarium São Francisco

Valley of the Federal University of São Francisco Valley. To obtain the acidified aqueous ethanol extract of *M. oleifera* leaves, the dried and pulverized plant material was subjected to fractional maceration with acidic aqueous ethanol (ethanol:water:acetic acid, 70:30:1, v/v/v) with a solid:solvent ratio of 1:10. Five extractions were performed with intervals of 72 h between each extraction, and subsequently the solution was filtered and concentrated in vacuum rotary evaporator SL-126 (SOLAB Científica, São Paulo, Brazil). After evaporation of the solvent, the *M. oleifera* extract was obtained. The extract was stored at 4ºC until further use. It is important to highlight that as the extract was obtained from fresh leaves of *M. oleifera,* in a specific region of Bahia, differences may be observed when the study is repeated in other locations due to the different environmental conditions of *M. oleifera* growth.

Source of ovarian tissue

Ovaries (n = 40) from adult mixed-breed sheep were collected at a slaughterhouse and used for *in vitro* culture with different *M. oleifera* concentrations (n = 25). Additional pairs (n=15) were collected and cultured in control and best extract treatment and later the oocytes were placed for *in vitro* maturation assessment.

Immediately post-mortem, the ovaries were washed once in 70% alcohol and then twice in 0.9% saline solution supplemented with antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). Next, the ovaries were transported within 1 h to the laboratory in Corning®tubes (Sarstedt, Newton, NC, USA) containing 0.9% saline solution with antibiotics at 4°C (G.A. Silva et al., 2021a).

Isolation, selection, and in vitro culture of ovine secondary follicles

Isolation, selection, culture, and follicle evaluation were performed according to (Santos et al., 2019). Ovarian cortical slices (1-2 mm thick) were cut and subsequently placed in a holding medium consisting of minimum essential medium (MEM) with HEPES and antibiotics. Secondary follicles (220-240 µm) were visualized under a stereomicroscope (Nikon, Tokyo, Japan) and mechanically isolated by microdissection using 26-gauge (26-G) needles. Approximately, 8 to 12 secondary follicles were isolated from each pair of ovine ovaries. The follicles selected for the *in vitro* culture had an intact basement membrane, two or more layers of granulosa cells, and a visible and healthy oocyte. Isolated follicles were pooled and then randomly allocated to the treatment groups, with approximately 50 follicles per group.

The follicles were evaluated for survival (morphology), growth (diameter) and formation of the antral cavity. The evaluation of the follicles was performed blindly by an experienced investigator. Survival was evaluated by morphology, follicles were individually classified as normal when an intact and centrally located oocyte was present and surrounded by granulosa cells well organized in one or more layers and without a pyknotic nucleus, surrounded by an intact basement membrane with ovarian follicles considered atretic when present condensation of the chromatin of the oocyte or granulosa cells. In morphologically normal follicles, the following parameters were evaluated: antral cavity formation, follicular growth. For evaluation of antrum formation rates, the emergence

of a translucent cavity visible between the layers of granulosa cells filled with of follicular. Follicular growth was assessed by measuring follicular diameter with the aid of a micrometric eyepiece, coupled directly to a stereomicroscope, measured from of the basement membrane and including two perpendicular measurements of each follicle; taxes of total growth, calculated as the diameter of normal follicles on day 12 minus the follicle diameter on day 0; daily growth rate, divided by the days of cultivation *in vitro* (12 days) and oocyte growth, with approximately 50 follicles per group.

Follicles were cultured individually in 100 µl droplets of culture medium under mineral oil in Petri dishes (60 x 15 mm, Corning®) at 39°C under 5% CO_2 for 12 days (Santos et al., 2019; Nascimento et al., 2019). The base medium consisted of α-MEM (pH 7.2-7.4) supplemented with 3.0 mg/ml bovine serum albumin (BSA), 10 ng/ml insulin, 2 mM glutamine, 2 mM hypoxanthine, 5.5 µg/ml transferrin, 5.0 ng/mL selenium and 50 µg/ml ascorbic acid (referred as α-MEM$^+$). For the experimental conditions, the selected follicles were cultured in α-MEM$^+$ (control medium) or in this medium supplemented with 0.1, 0.2 or 0.4 mg/ml of acidified extract of *M. oleifera* (Ajagun-Ogunleye & Ebuehi, 2020). Every two days, 60 µl of the culture media was replaced with fresh media in each droplet. Partial exchange of the culture medium is necessary to provide nutrients without completely removing substances produced in the different compartments of the follicle from the culture medium (Araújo et al., 2011).

Morphological evaluation of follicle development

Follicular morphology was evaluated through visualization using an stereomicroscope every 6 days. Only those follicles showing an intact basement membrane, with bright and homogeneous granulosa cells and absence of morphological signs of atresia were classified as morphologically normal follicles. Atretic follicles were those containing a darkened oocyte and surrounding granulosa cells or a misshapen, retracted or vacuolated oocyte, condensation of oocyte chromatin or granulosa cells, pyknotic nucleus and disorganized granulosa cells detached from the basement membrane.

The rupture of the basement membrane was also observed and characterized as oocyte extrusion. Furthermore, the following endpoints were assessed in the morphologically normal follicles: antral cavity formation, defined as the emergence of a visible translucent cavity within the granulosa cell layers; diameter, which was measured from the basement membrane using a pre-calibrated ocular micrometer attached to a stereomicroscope (Nikon) at 100x magnification. At the end of the culture, the healthy follicles were carefully and mechanically opened with 26 G needles under a stereomicroscope (Nikon) for oocyte recovery. The percentage of fully-grown oocytes (i.e., oocyte ≥110 µm) was calculated as the number of acceptable quality oocytes recovered from the total number of cultured follicles (x 100).

Assessment of GSH concentrations and mitochondrial activity

After *in vitro* culture, the oocytes were recovered, and intracellular GSH concentrations and mitochondrial activity were determined as previously described (G.A. Silva et al., 2021a). Briefly, oocytes were incubated in the dark for 30 min in phosphate-buffered saline (PBS; pH 7.2) supplemented with 10 mM 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (CellTracker® Blue; Invitrogen Corporation, Carlsbad, CA, USA) and 100 nM MitoTracker Red (MitoTracker® Red, CMXRos, Molecular Probes, Melbourne, Victoria, Australia) at 39ºC to detect GSH levels as blue fluorescence and mitochondrial activity as red fluorescence. The blue coloration of GSH indicates the activity of antioxidant enzyme GSH, capable of removing toxic compounds from cells and producing leukotrienes, the red color indicates the presence of active mitochondria, responsible for the generation of energy (ATP) to carry out activities cytoplasmic (Almansa-Ordonez et al., 2020).

After incubation, the oocytes were washed with PBS, and the fluorescence was observed under an epifluorescence microscope (Nikon) with UV filters (370 nm for GSH and 579 nm for mitochondrial activity). Fluorescence intensities of the oocytes were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Maturation of ovine oocytes from in vitro cultured secondary follicles

In vitro maturation (IVM) was performed in the oocytes derived from *in vitro* grown secondary follicles after 12 days of culture in α-MEM$^+$ (control medium) or in the treatment that obtained the highest percentage of fully grown oocytes (medium containing 0.4 mg/mL *M. oleifera* extract). For IVM, additional pairs of ovine ovaries (*n* = 15 ovaries) were collected, washed, transported to the laboratory, and cultured as described above. After culture, the cumulus-oocyte complexes (COCs) enclosed in healthy follicles were mechanically collected with 26-G needles under a stereomicroscope. Only oocytes ≥110 µm of diameter with a homogeneous cytoplasm and surrounded by at least one compact layer of cumulus cells were selected for IVM. The COCs were transferred to drops of 100 µL of maturation medium composed of tissue culture medium 199 (TCM 199) supplemented with 10% fetal calf serum (FCS), 1 µg/ml follicle stimulating hormone (human recombinant FSH; Gonal-F; Serono Laboratórios, São Paulo, Brazil) and 1 µg/mL luteinizing hormone (LH; ovine pituitary) and incubated for 24 h under 5% CO_2 in the air at 39ºC. After IVM, the oocytes were denuded and incubated in drops of PBS containing 10 mM Hoechst 33342 for 15 min at room temperature in the darkened area and visualized under epifluorescence microscopy (Nikon). The chromatin configuration was analyzed and classified in intact germinal vesicle (GV), meiotic resumption (including germinal vesicle breakdown [GVBD], and metaphase I [MI]) or nuclear maturation (metaphase II [MII]).

Ferric reducing antioxidant power (FRAP)

The pure extract, the control medium and the medium with the addition of 0.4 mg/ml of *Moringa oleifera* extract were subjected to antioxidant capacity analysis

using the FRAP method. Ferric reducing antioxidant power was determined using the method described by de (K. F. Carvalho et al. (2020b). The FRAP reagent was freshly prepared from acetate buffer 0.3 M (pH 3.6), 2,4,6-tripyridyl-s-triazine (TPTZ) 10 mM in 40 mM HCl, and FeCl3:6H2O 20 mM solutions. The three solutions were mixed together in the ratio 10:1:1 (v:v:v). Then, 900 µl of freshly-prepared FRAP reagent were mixed with 30 µl of properly diluted samples and 90 µl of distilled water. The mixture was heated at 37 °C and left at this temperature during the reaction. After 20 min, the absorbance was measured at 593 nm. The FRAP value was expressed as µmol Fe+2/100 g sample based on a calibration curve prepared using FeSO4 as standard.

Statistical analysis

Data regarding follicular survival, extrusion, antrum formation and fully grown oocytes were expressed as percentages and compared using the Chi-squared test. Data for follicular diameter, GSH levels and mitochondrial activity were evaluated using the D 'Agostine test to verify the normal distribution of residues and homogeneity of variances. The data were subsequently evaluated using the Kruskal-Wallis non-parametric test for comparisons. When significant differences were observed among treatments, the means were compared using the Student-Newman-Keuls test and data were expressed as means ± standard error mean (SEM). Data from meiotic resumption after IVM were expressed as percentages and compared by the Fisher exact test. Statistical significance was defined as $P < 0.05$.

Results and Discussion

Follicular morphology and development after in vitro culture

Morphological analysis: Follicles were individually classified as normal when showed that normal secondary follicles have oocytes surrounded by well organized and normal granulosa cells, surrounded by an intact basement membrane an intact oocyte was present and without a pyknotic nucleus. (Figure 1A). At day 6 of culture, most follicles at all treatments displayed a small antral cavity (Figure 1B), and few atretic follicles were observed (Figure 1C). The percentage of morphologically normal follicles significantly decreased from day 0 to day 12 in α-MEM$^+$ (control medium) and 0.1 mg/mL *M. oleifera* extract, whereas the follicular survival was maintained ($P > 0.05$) throughout the *in vitro* culture period in both 0.2 and 0.4 mg/mL *M. oleifera* (Figure 2). At the end of culture (day 12), no difference ($P > 0.05$) on the percentage of normal follicles was observed among treatments (88.1%, 84.6%, 89.2% and 94% for α-MEM$^+$, and 0.1, 0.2 and 0.4 mg/mL *M. oleifera* extract, respectively). Similarly, no significant difference was observed among treatments regarding extrusion rate (0.0, 0.0, 16.6 and 4,5% for α-MEM$^+$, and 0.1, 0.2 and 0.4 mg/mL *M. oleifera* extract, respectively).

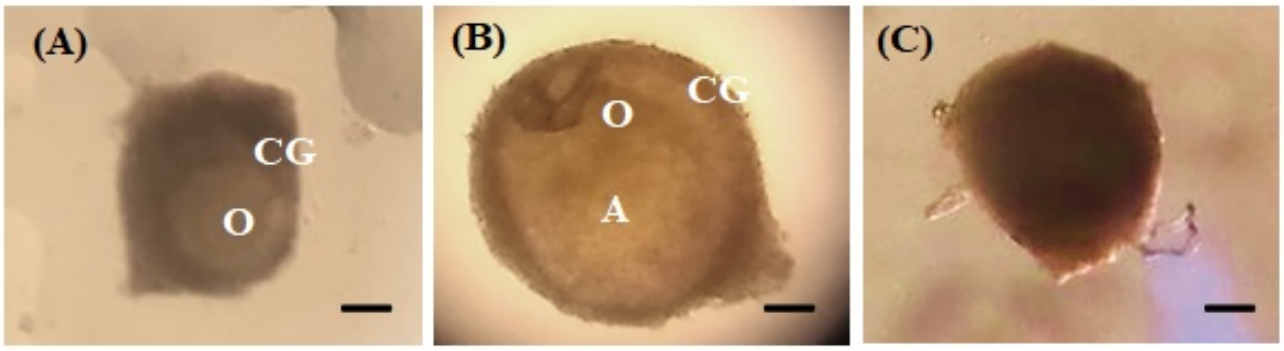

Figure 1. Morphologically normal secondary follicle on day 0 (A); normal antral follicleon day 6 (B); and atretic follicle after 12 days of culture (C). O: Oocyte, CG: Granulosa cells.

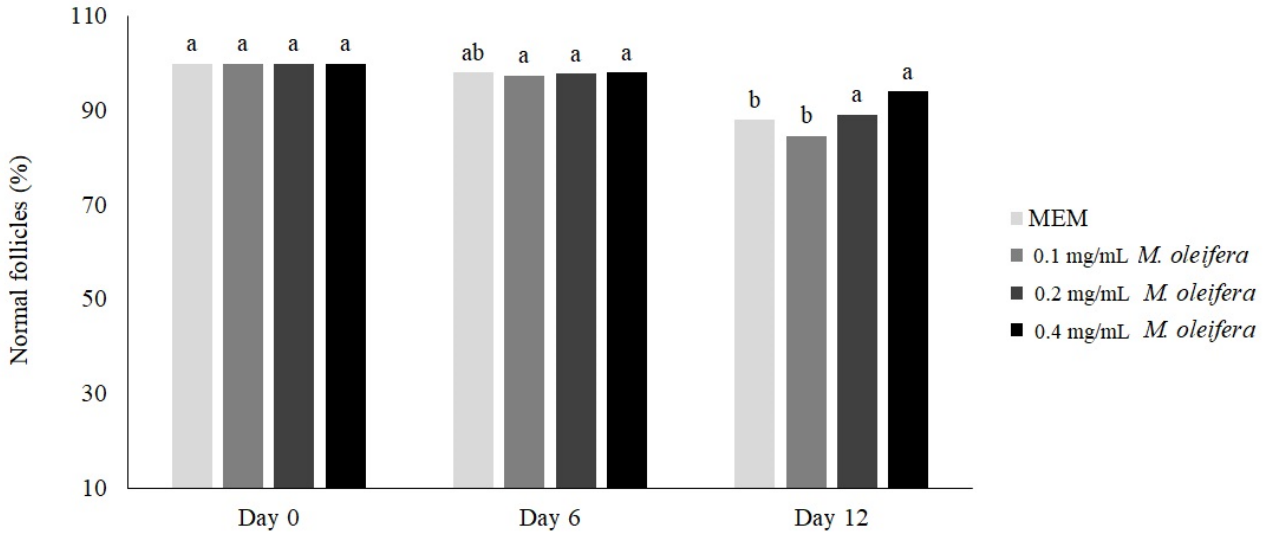

Figure 2. Morphologically normal follicles after *in vitro* culture in α-MEM⁺ and in different concentrations of *Moringa oleifera* leaves extract (0.1, 0.2 and 0.4 mg/mL). ([a,b])indicates difference between the days of culture in the same treatment ($p<0.05$).

All the treatments induced a progressive and significant increase in the antrum formation throughout the period of culture (Figure 3). After 12 days of culture, there was no significant difference in the antrum formation (86.0%,82.5%, 86.9% and 92.0% for α-MEM⁺, and 0.1, 0.2 and 0.4 mg/mL *M. oleifera* extract, respectively) (Figure 3) and in follicular diameter (Figure 4) among treatments. However, at the end of culture, medium containing 0.4 mg/ml *M. oleifera* extract significantly increased the percentage of fully grown oocytes (82.5%) compared to the other treatments (72.5%, 57.1% and 69.4% for α-MEM⁺, 0.1 and 0.2 mg/ml *M. oleifera* extract, respectively; Figure 5).

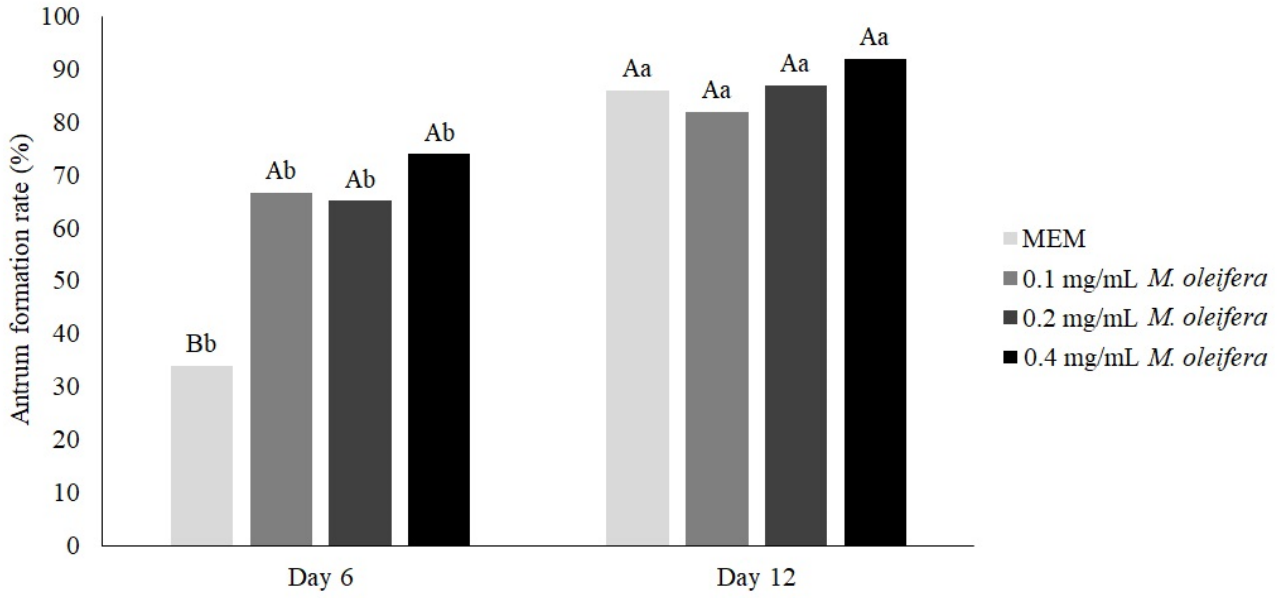

Figure 3. Percentages of antral cavity formation in follicles cultured in α-MEM⁺ or different concentrations of *Moringa oleifera* leaves extract (0.1, 0.2 and 0.4 mg/mL). (^{A,B}) indicates significant difference between treatments on the same day of culture. (^{a,b}) indicates difference between the days of culture in the same treatment ($p<0.05$).

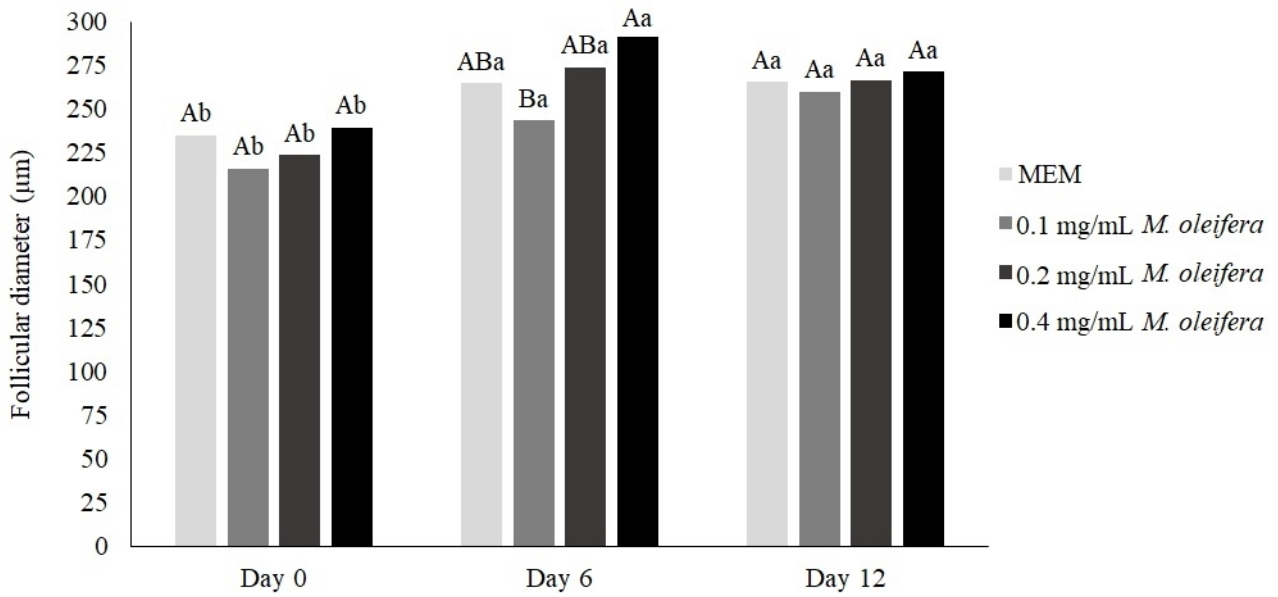

Figure 4. Diameter (µm) of follicles cultured for 12 days in α-MEM⁺ or different concentrations of *Moringa oleifera* leaves extract (0.1, 0.2 and 0.4 mg/mL). (^{A,B}) indicates significant difference between treatments on the same day of culture. (^{a,b}) indicates difference between the days of culture in the same treatment ($p<0.05$).

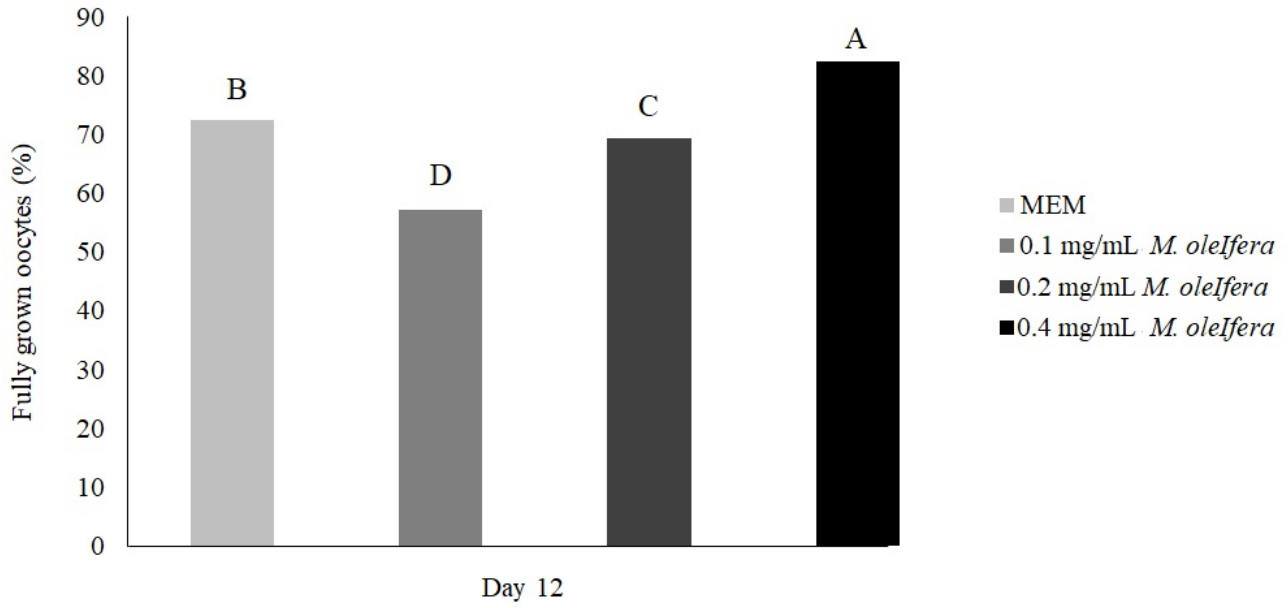

Figure 5. Fully grown oocytes after *in vitro* culture in α-MEM^{+} and in different concentrations of *Moringa oleifera* leaves extract (0.1, 0.2 and 0.4 mg/mL). (^{A,B,C,D}) indicates significant difference between treatments on the same day of culture ($p<0.05$).

Intracellular GSH concentrations and mitochondrial activity after in vitro culture

After 12 days of culture, oocytes from follicles cultured in the control medium (α-MEM^{+}) showed greater ($P < 0.05$) intracellular GSH concentrations than media containing *M. oleifera* extract (Figure 6). Regarding mitochondrial activity, there was no significant difference among treatments.

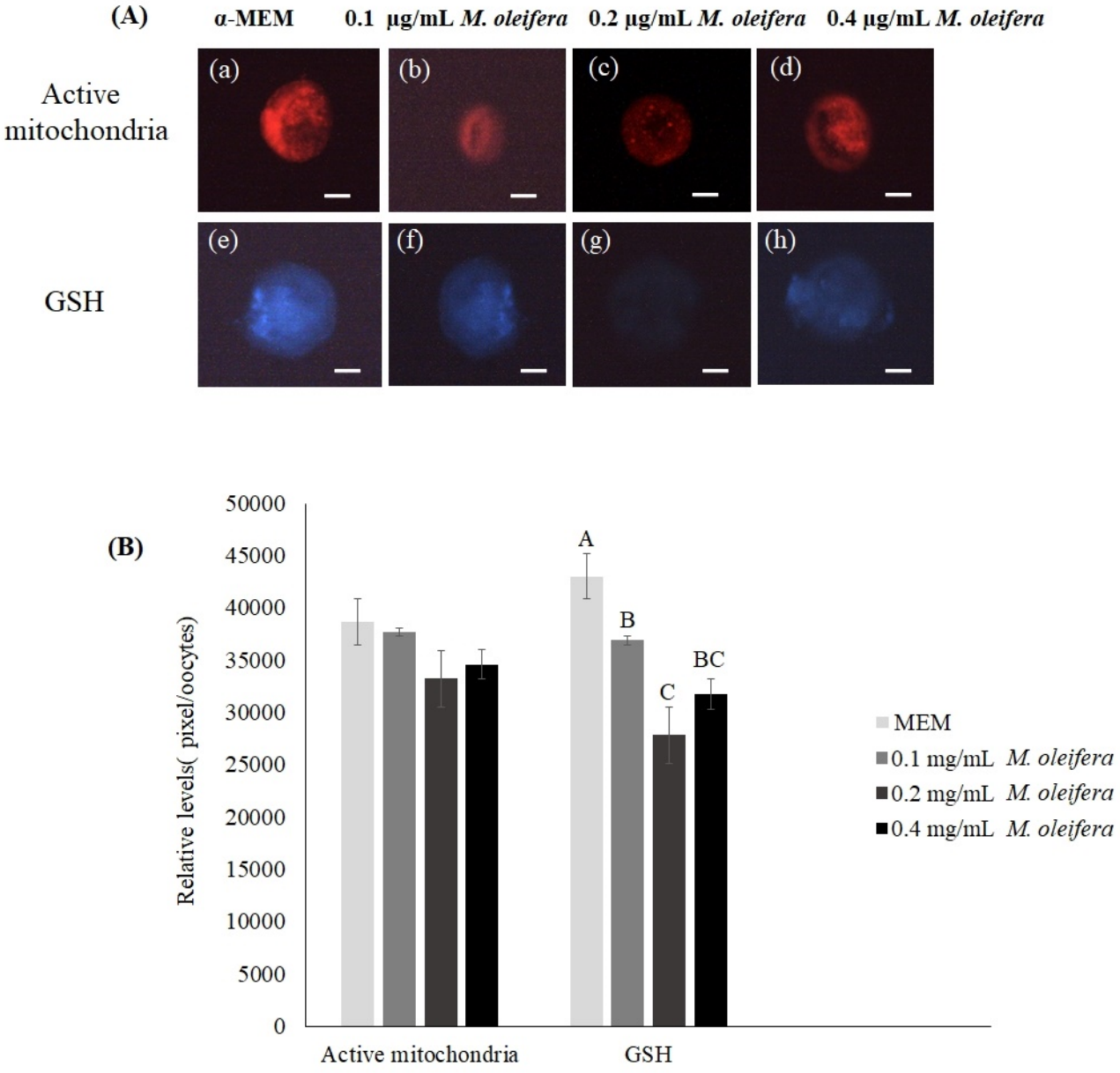

Figure 6. Photomicrographic images of epifluorescence of sheep oocytes cultured *in vitro.* (a) Oocytes were coraned with MitoTracker Red to detect intracellular levels of mitochondrial. Oocytes cultured in α-MEM⁺ (A) or with 0.1 (B), 0.2 (C) or 0.4 µg/ml (D) acidified extract from moringa leaves Scale bar 50 µm. (b) Intracellular levels of mitochondrial activity in sheep oocytes after 12 days of in vitro cultivation in α-MEM⁺ and in different concentrations of acidified extract from moringa leaves (0.1, 0.2 and 0.4 mg/mL). (^{A,B}) indicate significant differences between treatments.

Chromatin configuration after IVM

IVM was performed in the oocytes derived from *in vitro* grown secondary follicles after culture in α-MEM^{+} (control medium) or in medium containing 0.4 mg/mL *M. oleifera* extract. Both treatments showed a similar (P > 0.05) percentage of meiotic resumption rate (28% and 29% for α-MEM^{+} and 0.4 mg/ml *M. oleifera* extract, respectively).

Ferric reducing antioxidant power (FRAP)

FRAP assay displayed values of 244.53, 7.7 and 15.33 µmol Fe^{+2}/100 g for pure *M. oleifera* extract, α-MEM^{+} and 0.4 mg/mL *M. oleifera* extract, respectively.

This study constitutes the first report demonstrating the effects of acidified extract of *M. oleifera* leaves as a supplement of the medium for the *in vitro* culture of secondary follicles. After 12 days of culture, addition of 0.4 mg/mL *M. oleifera* extract to the medium resulted in the highest percentage of fully grown oocytes (≥ 110 µm) compared to other treatments. This is an important finding because previous studies have shown that there is a positive correlation between oocyte size and meiotic competence (Crozet et al., 2000; Cavilla et al., 2008; F. A. L Carvalho et al., 2020a), with better results being obtained when oocytes greater than 110-120 µm were used, as reported in bovine (Huang et al., 2013), caprine (Crozet et al., 2000; Cadenas et al., 2018) and ovine (R. F. Silva et al., 2021b).

Although we did not observe significant differences in meiosis resumption between 0.4 mg/ml *M. oleifera* extract and α-MEM^{+} treatments, we believe that a greater maturation rate could be obtained after 18 days instead of 12 days of culture as suggested by (Cadenas et al., 2018). Due to their larger diameter, follicles from day 18 of culture are supposed to have a greater number of granulosa and cumulus cells, which could help to improve oocyte meiotic competence (Cadenas et al., 2018). Furthermore, evidence has shown that longer culture periods are needed to support follicle development and enhance size and gene expression profile in oocytes from secondary follicles grown *in vitro* (Paulino et al., 2022).

It is noteworthy that our percentage of fully grown oocytes (82.5%) was greater than those obtained previously with plant-based culture medium or plant-derived antioxidants on *in vitro* culture of sheep secondary follicles 32.69% for kaempferol: (Santos et al., 2019), 36.3% for *J. insularis* extract: (Mbemya et al., 2018), 36.6% for protocatechuic acid: (Menezes et al., 2017), 40% for gallic acid: (G. A. Silva et al., 2021b); 50% for rutin: (Lins et al., 2017); 55% for *Morus nigra* extract: (Cavalcante et al., 2018). Furthermore, it is important to highlight that the method of obtaining the plant extract influences its composition and effectiveness. The method of extraction using acidified aqueous ethanol as a solvent has a higher efficiency and maintains a greater concentration of phenolic acids, anthocyanins, flavonoids, phenolic compounds when compared to other extraction methods (Piovesana & Noreña, 2019). In fact, chromatographic characterization associated with the use of mass spectrometry identified 32 secondary metabolites in the acidified extract of *M. oleifera* leaves used in the present study, such as quercetin, kaempferol and rutin (Silva et al. unpublished data). Furthermore, the

antioxidant capacity by the FRAP method of the medium that received 0.4 mg/ml of *Moringa oleifera* extract was approximately double compared to the control medium of this work, which corroborates with *in vitro* studies have demonstrated that these compounds can prevent damage in ovarian cells because of their cytoprotective and antioxidant properties. Cerezetti et al. (2021), using 25 µg/ml of quercetin in the culture medium of bovine ovarian fragments, found a greater capacity to reduce ferric ions by the FRAP method when compared to the control.

The supplementation with quercetin in the vitrification medium reduced ROS accumulation, amplified antioxidant activity and improved viability and maturation rates as well as developmental competence of sheep oocytes (Davoodian et al., 2021). In bovine granulosa cells, quercetin reduced the response to oxidative stress mediated by the Nrf2 regulator at the transcriptional and post-transcriptional level (Khadrawy et al., 2020). Furthermore, in the *in vitro* culture of bovine ovarian tissue using quercetin supplementation maintain the integrity of the development of ovarian follicles in *in vitro* culture for 5 and 10 days and improved antioxidant potential after 10 days of culture (Cerezetti et al., 2021). In goat oocytes, quercetin was able to replace the antioxidant cysteamine in the *in vitro* maturation medium, as well as increased oocyte mitochondrial activity, ensuring greater cell viability and quality (A. A. A. Silva et al., 2018). Furthermore, kaempferol maintained follicular survival during *in vitro* culture of ovine secondary follicles and increased the expression of metabolically active mitochondria (Santos et al., 2019), promoted primordial follicle activation and cell proliferation through the PI3K/AKT pathway and reduced DNA fragmentation of *in vitro* cultured sheep preantral follicles (Santos et al., 2019). Additionally, rutin was effective in replacing three antioxidants added to the medium (transferrin, selenium, and ascorbic acid), maintaining follicular viability in the *in vitro* development of sheep secondary follicles (Lins et al., 2017). Ascorbic acid is one of the antioxidants that, when added to the *in vitro* culture medium of bovine pre-antral ovarian follicles, was efficient in maintaining the morphological integrity, as well as the stability of reactive oxygen species (Bergamo et al., 2021).

Supplementation of the *in vitro* culture medium with rutin reduced apoptosis, promoted activation and growth of sheep primordial follicles (Lins et al., 2021).Therefore, it can be suggested that the antioxidant compounds, including quercetin, kaempferol and rutin, present in the *M. oleifera* leaves extract may act in association to maintain follicle viability and enhance oocyte development from sheep secondary follicles cultured *in vitro,* contributing to obtain greater rates of fully grown oocytes to IVM.

In the current study, *M. oleifera* extract had no additive effect on follicular survival, antrum formation and diameter after 12 days of culture. This result may be explained by the composition of the control medium (α-MEM) that contains non-essential amino acids, carbohydrates, sodium pyruvate, lipoic acid, biotin, vitamins, and DNA precursors, which help to maintain the follicular viability and growth (Mbemya et al., 2017; Menezes et al., 2017; Santos et al., 2019). We believe that a longer culture period (18 days) may be necessary to note a significant change in follicular viability and growth (Cadenas

et al., 2018). It is important to highlight that regardless of the concentration, *M. oleifera* extract did not present toxicity to the follicles, maintaining follicular survival similar to that observed in the control medium.

Unexpectedly, the GSH concentrations were highly expressed in the oocytes cultured in α-MEM⁺. Previous studies described that oxidative stress could lead to an increase in the GSH concentrations and overwhelm the ability of GSH to quench ROS (Mosharov et al., 2000; Lievre et al., 2001). Therefore, in the current study, we suggest that the high GSH concentrations observed in oocytes cultured in the control medium may represent a compensatory response of the cells to protect against the *in vitro* oxidative stress. However, this compensation was not effective as evidenced by a greater oocyte growth rate in medium supplemented with 0.4 mg/mL *M. oleifera* extract.

Conclusion

The addition of 0.4 mg/ml of *M. oleifera* leaf extract to the *in vitro* culture medium of ovine secondary follicles maintained follicular survival and improved oocyte growth. This provides a greater yield of oocytes that can be used for IVM, increasing the efficiency of *in vitro* cultivation of isolated secondary follicles. This extract can be a cheap and effective alternative supplement for the *in vitro* development of sheep secondary follicles. More studies are needed to obtain standardized extracts, which could be used as a commercial supplement in the future, and to improve the maturation rates of oocytes cultured *in vitro.*

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