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***CAMPUS* DE SOBRAL - CURSO DE MEDICINA**
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FRANCISCO FREIRE CAETANO FILHO

AVALIAÇÃO DOS EFEITOS DO ÓLEO ESSENCIAL DE *Lippia sidoides Cham.* E DO TIMOL DURANTE O CULTIVO *IN VITRO* DE FOLÍCULOS PRÉ-ANTRAIS INCLUSOS EM TECIDO OVARIANO BOVINO

SOBRAL
2023

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Biotecnologia, da Universidade Federal do Ceará – *Campus* Sobral, como requisito parcial para a obtenção do Título de Mestre em Biotecnologia. Área de concentração: Biotecnologia. Linha de Pesquisa: Análises Integrativas de Sistemas Biológicos. Área Temática: Fisiologia Reprodutiva

Orientador: Prof. Dr. José Roberto Viana Silva

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"É justo que muito custe, o que muito vale"

(Santa Tereza D'Ávilla)

RESUMO

O objetivo deste trabalho foi avaliar os efeitos da adição do óleo essencial de *Lippia sidoides* (OELS) e seu composto ativo timol durante o cultivo *in vitro* de folículos pré-antrais inclusos em tecido ovariano bovino, a fim de avaliar a ativação, desenvolvimento, viabilidade e integridade morfológica folicular. Bem como, a configuração da matriz extracelular (MEC), expressão de mRNA para *SOD, CAT, PRDX6 e GPX1* e quantificação do ambiente antioxidante através do contéudo de Tiol e atividade das enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPX). Para a execução deste estudo, todos os produtos químicos, incluindo o óleo essencial de *Lippia sidoides* e o Timol, foram adquiridos de forma comercial pela Laszlo e Sigma-Aldrich Brasil Ltda., respectivamente. Posteriormente, realizou-se o cultivo *in situ*, onde o córtex ovariano foi dividido em fragmentos de aproximadamente 3x3x1 mm que foram, em seguida, cultivados por 6 dias em placas de 24 poços contendo 0,5 mL de meio α-MEM+ sozinho ou suplementado com diferentes concentrações do óleo essencial de *Lippia sidoides* (400, 800, 1.600, 3.200 µg/mL) ou do timol (400, 800, 1.600, 3.200 µg/mL µg/mL). A cada 2 dias foi feita a troca parcial (60%) do meio de cultivo. Ao final do período de cultivo, parte dos fragmentos foram fixados e submetidos as análises histológicas, e parte dos fragmentos congelados a -80ºC para as análises bioquímicas e moleculares. Os resultados mostraram que a adiação de 400µg e 800µg/mL de OELS e do timol ao meio de cultivo apresentaram melhores taxas de folículos morfologicamente normais quando comparado aos demais tratamentos testados. Sendo a concentração de 400µg/mL do OELS e do timol a que promoveu melhores taxas de ativação e desenvolvimento folicular, além de elevar os níveis de colágeno e a densidade do estroma em ambos os experimentos. Os resultados de expressão gênica mostraram que concentração de 800µg//mL no tratamento com timol reduziu os níveis de mRNA para *SOD*, *CAT* e *PRDX6* e não influenciou na expressão da *GPX1*. Já na análise com OELS a concentração de 800µg/mL manteve a expressão para *SOD*, *CAT* e *PRDX*6 enquanto aumentou a expressão de *GPX1* em relação ao controle cultivado. A respeito do perfil de Tiol e atividade antioxidante das enzimas CAT, SOD e GPX, no tratamento com timol apenas a concentração de 800µg/mL causou o aumento dos níveis de CAT e redução nos níveis de SOD, mas não influenciou nos valores de Tiol e GPX enquanto no tratamento com OELS apenas a concentração de 400µg/mL do óleo aumentou os níveis de GPX, mas não influenciou na atividade das demais enzimas. Em conclusão, a presença do Timol e do óleo essencial de *Lippia sidoides* ao meio de cultivo *in vitro* promove a manutenção da viabilidade folicular, induz uma maior ativação e desenvolvimento dos folículos, atenuando e modulando o estresse oxidativo.

Palavras-chave: Cultivo *in vitro.* Folículos pré-antrais. Estresse oxidativo. Antioxidante. *Lippia sidoides*

ABSTRACT

The aim of this study was to evaluate the effects of adding *Lippia sidoides* essential oil (LSEO) and its active compound thymol during the in vitro culture of preantral follicles included in bovine ovarian tissue, in order to assess follicle activation, development, viability and morphological integrity. As well as the configuration of the extracellular matrix (ECM), expression of mRNA for *SOD*, *CAT*, *PRDX6* and *GPX1* and quantification of the antioxidant environment through thiol content and activity of the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). To carry out this study, all the chemical products, including *Lippia sidoides* essential oil and Thymol, were purchased commercially by Laszlo and Sigma-Aldrich Brasil Ltda, respectively. Subsequently, in situ culture was carried out, where the ovarian cortex was divided into fragments of approximately 3x3x1 mm which were then cultured for 6 days in 24-well plates containing 0.5 mL of α-MEM+ medium alone or supplemented with different concentrations of *Lippia sidoides* essential oil (400, 800, 1,600, 3,200 µg/mL) or thymol (400, 800, 1,600, 3,200 µg/mL µg/mL). The culture medium was partially changed (60%) every 2 days. At the end of the cultivation period, part of the fragments were fixed and subjected to histological analysis, and part of the fragments were frozen at -80ºC for biochemical and molecular analysis. The results showed that the addition of 400µg and 800µg/mL of LSEO and thymol to the culture medium showed better rates of morphologically normal follicles when compared to the other treatments tested. The 400µg/mL concentration of LSEO and thymol promoted the best rates of follicle activation and development, as well as increasing collagen levels and stromal density in both experiments. The gene expression results showed that the concentration of 800µg//mL in the thymol treatment reduced the mRNA levels for *SOD, CAT* and *PRDX6* and did not influence the expression of GPX1. In the analysis with LSEO, the concentration of 800µg/mL maintained the expression of *SOD, CAT* and *PRDX6* while increasing the expression of *GPX1* in relation to the cultured control. With regard to the thiol profile and the antioxidant activity of the CAT, SOD and GPX enzymes, in the treatment with thymol only the 800µg/mL concentration caused an increase in CAT levels and a reduction in SOD levels, but did not influence the thiol and GPX values, while in the treatment with LSEO only the 400µg/mL concentration of the oil increased GPX levels, but did not influence the activity of the other enzymes. In conclusion, the presence of Thymol and *Lippia sidoides* essential oil in the *in vitro* culture medium promotes the maintenance of follicular viability, induces greater activation and development of follicles, and attenuates and modulates oxidative stress.

Key words: *In vitro* culture. Pre-antral follicles. Oxidative stress. Antioxidants. Lippia *sidoides*

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LISTA DE SIGLAS E SIMBOLOS

AROH	Antioxidantes Fenólicos
ARTs	Técnicas de Reprodução Assistida
BMP-4	Proteína Morfogênica Óssea 4
CAT	Catalase
cMOS	Ciclina-Dependente Quinase
COCs	Complexos Cumulus Oócitos
DCM	Diclorometano
E2	Estradiol
ECM	Matriz Extracelular
EGF	Fator de Crescimento Epidermal
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária
EROS	Espécies Reativas de Oxigênio
FAO	Organização das Nações Unidas para Agricultura e Alimentação
FDA	Agência de Alimentos e Medicamentos dos Estados Unidos
FIV	Fertilização *In Vitro*
GDF-9	Fator de Crescimento Diferenciação 9
GSH	Glutationa
GSH-Px	Glutationa Peroxidase
H2O2	Peroxido de Hidrogênio
HAT	Transferência de Átomos de Hidrogênio
IA	Inseminação Artificial
IGF-1	Fator de Crescimento Semelhante a Insulina
IMI	Imidacloprida
LSEO	Óleo Essencial de *Lippia sidoides*
MAPA	Ministério da Agricultura, Pecuária e Abastecimento
MDA	Malondialdeído
MEP	Metileritritol Fosfato
mRNA	RNA Mensageiro
NAC	N-Acetilcisteína
OEs	Óleos Essenciais
ONU	Organizações das Nações Unidas

OPU	Aspiração Folicular Guiada por Ultrassom
P4	Progesterona
PIV	Produção *In Vitro* de Embriões
POF	Insuficiência Ovariana Prematura
PRDX1	Peroxirredoxina 1
PRDX6	Peroxiredoxina 6
SET	Transferência de Elétrons
SOD	Superóxido Dismutase
TE	Transferência de Embriões
TNF-α	Fator de Necrose Tumoral Alfa
USDA	Departamento de Agricultura dos Estados Unidos

LISTA DE SÍMBOLOS

%	Porcentagem
©	Copyright
®	Marca Registrada
cm	Centímetro
nm	Nanometro
PM	Petametro
µg	Micrograma
µL	Microlitro
µm	Micrômetro
M	Molar
g	Grama
mg	Miligrama
mM	Milimolar
nM	Nanomolar
Ng	Nanograma
ºC	Grau Celsius
<	Menor que
α	Alfa
β	Beta
h	Hora
min	Minuto
s	Segundo
mm	Milímetro
O_2	Oxigênio
H_2O_2	Peróxido de hidrogênio
OH	Hidroxila
$O_2^{\circ-}$.	Ânion superóxido
CO_2	Dióxido de carbono

SUMÁRIO

1. INTRODUÇÃO

Levantamento recente da Organização da Nações Unidas (ONU) apontou que o planeta deve atingir, até o ano de 2050, a marca de nove bilhões de pessoas. Assim, o grande desafio para o futuro será produzir alimento em quantidade suficiente para atender a essa crescente demanda populacional (ONU, 2022). Nesse cenário, o gado é um componente crítico de sistemas agrícolas sustentáveis e representa uma estratégia chave para a produção de alimento em todo o mundo (LARDY; CATON, 2012; REYNOLDS *et al*., 2015).

O Brasil, que em 2022 concentrou o maior volume de exportações de carne bovina, continua a ser um dos maiores produtores mundiais de bovinos (EMBRAPA, 2022). Neste contexto, o uso de biotécnicas reprodutivas tais como a inseminação artificial (IA), a transferência de embriões (TE) e a fertilização *in vitro* (FIV) podem contribuir positivamente para aumentar ainda mais a produtividade nas fazendas de gado, uma vez que possibilitam produzir mais rápida e eficientemente animais de alto valor genético, além de contribuir para a preservação de material genético raro ou em risco de extinção (GONCALVES, 2022).

Entre as biotécnicas reprodutivas, a produção *in vitro* de embriões (PIV) tornou-se, na última década, uma tecnologia amplamente utilizada na indústria pecuária mundial (ZHU *et al*., 2018) e tem como princípio mais básico o aumento do potencial reprodutivo. Tradicionalmente, esta biotécnica envolve etapas que incluem a obtenção e maturação de oócitos, a fecundação e o cultivo dos zigotos até estágios de desenvolvimento adequado à transferência para receptoras ou congelamento para uso futuro. Entretanto, o rendimento da PIV ainda é insatisfatório, ficando ao redor de 40% de embriões produzidos sobre o número de oócitos inseminados (EMBRAPA, 2022). Neste sentido, o refinamento da técnica associado à preocupação em relação à disponibilidade, quantidade maior de folículos e a qualidade oocitária tem sido alvos de novos estudos (BIZARRO *et al*., 2021).

Na espécie bovina, a população folicular ao nascimento está em torno de 235.000 folículos por ovário, sendo em sua maioria (cerca de 90%) folículos da categoria pré-antral (ERICKSON, 1966; SILVA-SANTOS *et al*., 2011; FIGUEIREDO *et al*., 2018, SENEDA *et al.*, 2021). Assim, esse *pool* de folículos pré-antrais pode potencialmente fornecer um grande número de oócitos viáveis aptos a diversas técnicas de reprodução assistida (ARTs) (FISCH B; ABIR, 2018; BUS *et al*., 2019). Neste sentido, o cultivo *in vitro* de tecido ovariano tem se mostrado uma biotécnica estratégica para o aproveitamento dessa população folicular além de contribuir para aprimorar nossa compreensão acerca da dinâmica fisiológica de folículos pré-

antrais em bovinos e outras espécies (BRAW-TAL; YOSSEFI, 1997; FIGUEIREDO *et al*., 2007).

Entretanto, apesar do significativo avanço obtido, o cultivo *in vitro* de tecido ovariano ainda apresenta limitações. Dentre elas, a ocorrência de estresse oxidativo constitui uma importante barreira. Sob condições de cultivo, o estresse oxidativo é gerado a partir da produção exacerbada de radicais livres , sendo esse fenômeno favorecido por fatores como manipulação, luz, variações de temperatura e pH, suplementação do meio e níveis de oxigênio durante o cultivo (LINS *et al*., 2017; ZHANG *et al*., 2019).Com isso, a ocorrência de danos ao DNA e membranas celulares, disfunção mitocondrial com depleção da produção de ATP como resultado do estresse oxidativo podem perturbar severamente a homeostase redox celular, contribuindo para a baixa qualidade de folículos e oócitos ao final do cultivo (ANAZETTI; MELO, 2007). Por essas razões, a suplementação dos meios de cultivo com substâncias antioxidantes tem sido proposta como alternativa para o aprimoramento dos sistemas de cultivo *in vitro* de tecido ovariano em espécies domésticas, como os bovinos (HEGGENDORN *et al*., 2020). Com esse propósito, a utilização de bioprodutos de origem vegetal, com potencial antioxidante têm sido objeto de importante estudo na área. Entre as substâncias antioxidantes obtidas a partir de plantas têm chamado atenção os óleos essenciais, caracterizados por possuírem uma ampla variedade de componentes bioativos, incluindo moléculas antioxidantes, razão pela qual têm atraído cada vez mais o interesse de pesquisadores (CARNEIRO *et al*., 2017).

Nesse contexto, a espécie *Lippia sidoides Cham.* conhecida popularmente como "Alecrim-pimenta" têm sido objeto de estudos que investigam as funcionalidades dos seus óleos essenciais (RODRIGUES *et al*., 2009; DE MORAIS *et al*., 2016). Estudos anteriores realizados com essa planta, identificaram no seu óleo essencial a presença de monoterpenos, sesquiterpenos, ésteres de ácidos graxos e álcoois aldeidados (MONTEIRO *et al*., 2021; FELIX *et al*., 2022). Adicionalmente, o principal constituinte químico do óleo essencial *Lippia sidoides* é o monoterpeno Timol que tem sido amplamente associado às atividades biológicas deste óleo (FIGUEIREDO, 2017). O Timol, um composto fenólico, é conhecido por possuir atividade antimicrobiana, anti-inflamatórias, anticâncer e antioxidante. Seus potenciais antioxidantes já foram investigados *in vitro* em vários tipos celulares, como linfócitos humanos, fibroblastos e queratinócitos de hamster, células mononucleares do sangue periférico e células ovarianas de ratas e, de maneira geral, esse composto reduz consideravelmente o dano oxidativo ao DNA bem como diminui significativamente a concentração de espécies reativas de oxigênio (EROS)

intracelular, além de aumentar os níveis de glutationa (GSH) (AYDIN *et a*l., 2005; MAHRAN *et al*., 2019; SILVA-GALVÃO *et al*., 2020; GUNES-BAYIR *et al*., 2020). Em conjunto, esses estudos demonstram o potencial do óleo essencial de *Lippia sidoides* e do seu composto majoritário, o Timol, para compor o meio de cultivo de tecido ovariano em bovinos.

Para uma melhor compreensão da relevância desta dissertação, a revisão da literatura a seguir abordará aspectos relacinados à segurança alimentar e a utilização de biotécnicas reprodutivas aplicadas à bovinocultura; avanços e desafios no cultivo *in vitro* de folículos pré-antrais; estresse oxidativo e influência dos compostos antioxidantes durante o cultivo *in vitro* de folículos pré-antrais; óleos essenciais como fonte de agentes antioxidantes; aspectos químicos e atividade antioxidante do óleo essencial de *Lippia sidoides* e os aspectos químicos e mecanismos de ação antioxidante do Timol.

2. REVISÃO BIBLIOGRÁFICA

2.1. *A segurança alimentar e a utilização de biotécnicas reprodutivas aplicadas à bovinocultura*

Os desafios atuais na indústria da pecuária são numerosos e necessitam de remediação através de abordagens baseadas na ciência para prover alimento de qualidade às populações atuais bem como garantir que a produção alimentícia no futuro possa acompanhar adequadamente a demanda crescente por alimentos em todo o mundo. De acordo com o relatório sobre as perpectivas da população mundial, divulgado pelo Departamento de Assuntos Económicos e Sociais das Nações Unidas, a projeção é que até 2050 a população mundial tenha um acréscimo de mais 1,7 bilhão de pessoas (ONU, 2022) sendo, portanto, necessário um aumento de 60% na produção de alimentos nos próximos anos (FAO, 2015; SAATH e FACHINELLO, 2018). Paralelamente, o consumo de proteína de origem animal (carne, leite e derivados) deve saltar dos atuais 58% para 72% (SOARES, 2019). Diante deste cenário, garantir a segurança alimentar frente a escassez de recursos naturais constitui um grande desafio para o futuro. Consequentemente, existem necessidades significativas de avanços que garantam melhor eficiência, segurança e sustentabilidade da indústria agropecuária.

Atualmente, o Brasil é um dos maiores produtores mundiais de alimentos (EMBRAPA, 2022) sendo também responsável pelo terceiro maior consumo global de carne bovina, absorvendo internamente 80% de toda a produção nacional (USDA, 2018), com uma taxa de consumo de 24,8kg/pessoa/ano (SOARES; XIMENES, 2022). Neste sentido, as projeções da Secretaria de Política Agrícola do Ministério da Agricultura e Pecuária (Tabela.1) apontam para um aumento de 12,14% na produção de carne bovina no Brasil, seguido do acréscimo de 4,8 % no consumo doméstico interno somados a outros 16,3% no volume de exportações até 2033 (MAPA, 2023).

Tabela 1 - Projeção de produção, consumo e exportação de carne bovina – Brasil

Ano	Produção (mil t)	Consumo (mil t)	Exportação (mil t)
2023	9.065	6.266	2.883
2024	9.168	6.469	2.969
2025	9.134	6.228	3.055
2026	9.268	6.196	3.140

Tabela 1 - Projeção de produção, consumo e exportação de carne bovina – Brasil - Continua

2027	9.453	6.354	3.226
2028	9.575	6,422	3.312
2029	9.678	6.410	3.397
2030	9.805	6.435	3.483
2031	9.939	4.495	3.569
2032	10.064	6.593	3.655
2033	10.186	6.568	3.740

Fonte: Autor, Adaptado de Secretaria de Produção Agricola/ MAPA (2022).

Reunidos esses dados, e considerando que a produção pecuária contabiliza 18% do balanço global de alimentos em termos de ingestão calórica diária por pessoa (MOTTET *et al*., 2017) a expansão da pecuária nacional e o aumento da eficiência produtiva deve vir acompanhada da sustentabilidade da produção e conservação dos ecossistemas naturais (STRASSBURG *et al*., 2014). Além disso, dada a importância desse segmento para a alimentação humana, o Brasil em conjunto com o bloco sul foi convocado pela Organização das Nações Unidas (ONU) a responder por 40% da demanda mundial de alimentos nos próximos anos (SOARES, 2019).

Nesta linha de pensamento, através do melhoramento genético é possível que produtores aumentem rapidamente o número de bezerros produzidos por ano pecuário. Para isso, as biotécnicas da reprodução têm sido estratégicas para a intensificação sustentável dos rebanhos de gado comercial, aumento da produtividade e incremento do suprimento mundial de alimentos (SOARES, 2019). Dentre as biotecnologias atuais aplicadas em maior escala, se destacam a inseminação artificial (IA) largamente disseminada, inclusive em pequenas propriedades, sem grandes custos ao produtor (SALVADOR, 2019); a transferência de embriões clássica (TE) e a aspiração folicular guiada por ultrassom (OPU) seguida da produção *in vitro* de embriões (PIV) que juntas trouxeram transformações significativas para o setor econômico e pecuário nacional na última década (GONÇALVES *et al*., 2022). Neste último caso, através do método de punção folicular, tornou-se possível à recuperação de oócitos de fêmeas vivas destinadas à fecundação *in vitro* (FIV), possibilitando a multiplicação de animais de interesse econômico e a elevação da produção de bezerro por vaca/ano, em comparação a Transferência de Embriões (TE) clássica (RUMPF, 2007).

Apesar do relativo sucesso desta técnica, a incorporação da produção *in vitro* de embriões no Brasil ainda constitui um desafio para maximizar de forma acessível e sustentável

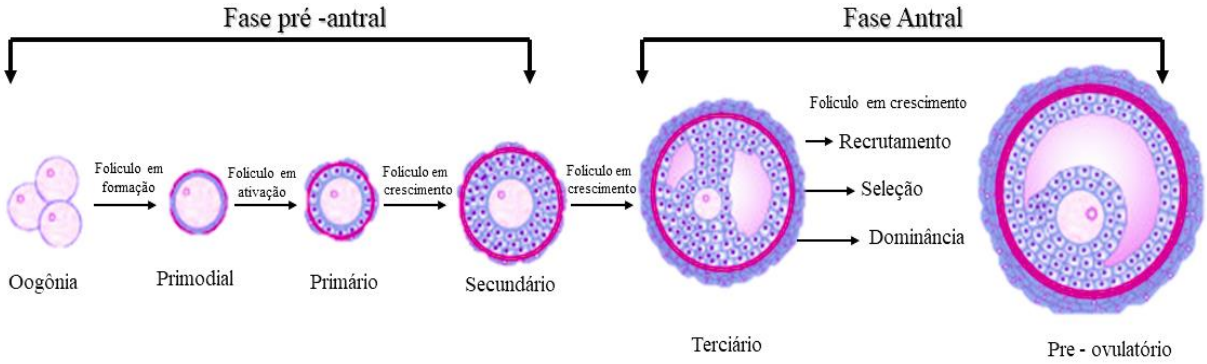
a eficiência reprodutiva dos rebanhos em pequenas propriedades e, consequentemente, elevar a produção de carne bovina dos atuais 9,5 milhões para 24,2 milhões de toneladas na próxima década (SOARES, 2019). Dentre as principais limitações, estão os baixos índices de blastocisto; a criotolerância limitada; variabilidade de resultados; perdas gestacionais; menor viabilidade dos oócitos obtidos por OPU de bezerras em relação aos de vacas e novilhas; o custo do embrião que é mais alto do que um embrião de TE e as barreiras ambientais à exportação (FERRÉ *et al*., 2020; MAPELLE, 2023, LIMA *et al*., 2023).

Nessa perspectiva, a utilização de biotecnicas reprodutivas que possibilitem aumentar o número de folículos destinados as técnicas de reprodução assistida, e constituírem no futuro uma fonte de óocitos competentes a fertilização *in vitro* tem ganhado destaque, como o cultivo de folículos pré-antrais inclusos em fragmentos do córtex ovariano.

2.2. *Avanços e desafios no cultivo in vitro de folículos pré-antrais.*

O cultivo *in vitro* de tecido ovariano constitui uma excelente ferramenta para aprimorar a compreensão do controle da foliculogênese (SILVA *et al*., 2020), sendo uma opção promissora para gerar no futuro um número significativo de oócitos maduros e revolucionar os atuais procedimentos de criação de animais, estabelecimento de bancos de oócitos, preservação de espécies ameaçadas de extinção e fertilização *in vitro* em mamíferos (BARBATO *et al*., 2023). No córtex ovariano é possível encontrar milhares de folículos em desenvolvimento, em sua maior parte (90%) classificados como pré-antrais., ou seja, folículos primordiais, primários e secundários.

Figura 1- Representação esquemática do desenvolvimento folicular completo.



Fonte: Autor, adaptado de Araújo *et al* (2014).

Além disso, esta biotécnica tem como vantagens a coleta de tecido ovariano em qualquer idade, estágio do ciclo ou ainda de animais que morrem subitamente, permitindo a obtenção de grande número de oócitos de qualquer fêmea (SHAW *et al,* 2000; SUTTON *et al*., 2021; COSTA *et al*., 2022) que podem ser criopreservados ou destinados ao cultivo *in vitro*.

Historicamente, a produção de descendentes vivos a partir de folículos primordiais cultivados *in vitro* foi alcançada com sucesso em camundongos, sendo relatada pela primeira vez há mais de trinta anos (EPPIG; SCHROEDER, 1989; CARROLL; GOSDEN, 1993). Em ruminantes, apesar dos estudos já realizados com cabras, búfalos e ovelhas os avanços ainda são limitados ao baixo número de oócitos maduros e embriões após o cultivo *in vitro* (SILVA et al., 2016; NASCIMENTO *et al*., 2023). Neste sentido, a espécie bovina é a que ainda enfrenta os maiores desafios para avançar no campo do cultivo *in vitro* de folículos pré-antrais e a melhor conquista, até o momento, limita-se a formação da cavidade antral após o crescimento *in vitro* de folículos primordias e intermediários (< 40 μm de diâmetro) inclusos em tecido ovariano (GUTIERREZ *et al*., 2000; MCLAUGHLIN *et al*., 2010, PAULINO *et al*., 2020).

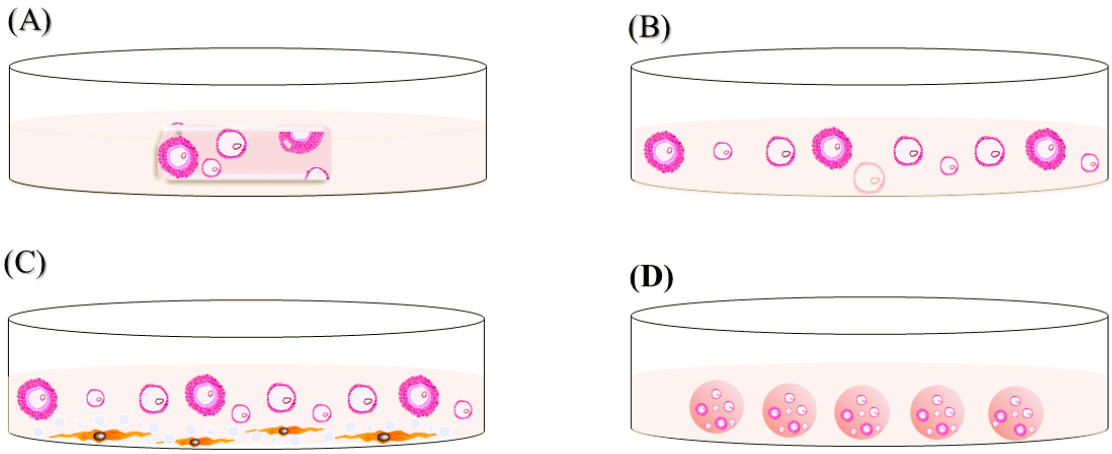
Aspectos como a duração da foliculogênese (~ 6 meses) para bovinos contra cerca de 20 dias em camundongos (PAULINO *et al*., 2020); as diferenças anatômicas e funcionais; a idade do material biológico inicial; o tamanho dos folículos em desenvolvimento e a consistência mecânica do tecido ovariano, dificultam a tradução dos resultados obtidos em camundongos para grandes mamíferos e humanos (BARBATO *et al*., 2023). Por esta razão, vários pesquisadores têm trabalhado para desenvolver sistemas de cultura eficientes (NASCIMENTO *et al*., 2023), uma vez que há um consenso de que o desenvolvimento de folículos primordiais para o estágio secundário só pode ser alcançado por meio da cultura do córtex ovariano intacto (YANG *et al*., 2021).

Dentre os sistemas de cultura *in vitro* disponíveis para os folículos pré-antrais, estão: 1) os que cultivam fragmentos corticais ovarianos (*in situ*), 2) os que cultivam os folículos isoladamente após sua remoção do tecido ovariano e 3) o recente método de cultura “de várias etapas”, que associa a cultura de fragmentos do tecido ovariano ou do ovário inteiro, seguida pelo isolamento dos folículos secundários e pela cultura destes separadamente (NASCIMENTO *et al*., 2023).

No primeiro caso, cultivo *in situ*, (Figura 2 - A), o cultivo simula as condições *in vivo*, ao permitir o contato entre folículo e as células do estroma ovariano, da teca e da granulosa mantendo uma forma tridimensional, semelhante à observada no ambiente natural. Já na segunda técnica (Figura 2 - B, C) o folículo, ao ser cultivado isoladamente, permite o

acompanhamento do desenvolvimento folicular individual, além de possibilitar maior contato do meio de cultivo contendo substâncias de interesse com a estrutura folicular (ARAÚJO *et al*., 2014). Nesse sistema de cultura, ainda é possivel realizar o cultivo de folículos isolados utilizando o modelo bidimensional (2D), no qual o folículo cresce diretamente sobre a placa de cultivo ou sobre uma monocamada de substrato, (matriz extracelular ou componentes do estroma ovariano e fibroblastos) ou o modelo tridimensional, no qual o folículo isolado é inserindo em uma matriz, como o hidrogel (BIZARRO *et al*., 2021).

Figura 2 - Representação esquemática dos sistemas tradicionais de cultivo *in vitro* de folículos pré-antrais. (A) Cultivo *in vitro* de folículos pré-antrais envoltos em tecido ovariano (*in situ*). (B) Cultivo *in vitro* de folículos pré-antrais iolados. (C) Cultivo *in vitro* no sistema bidimensional. (D) Cultivo *in vitro* no sistema tridimensional.

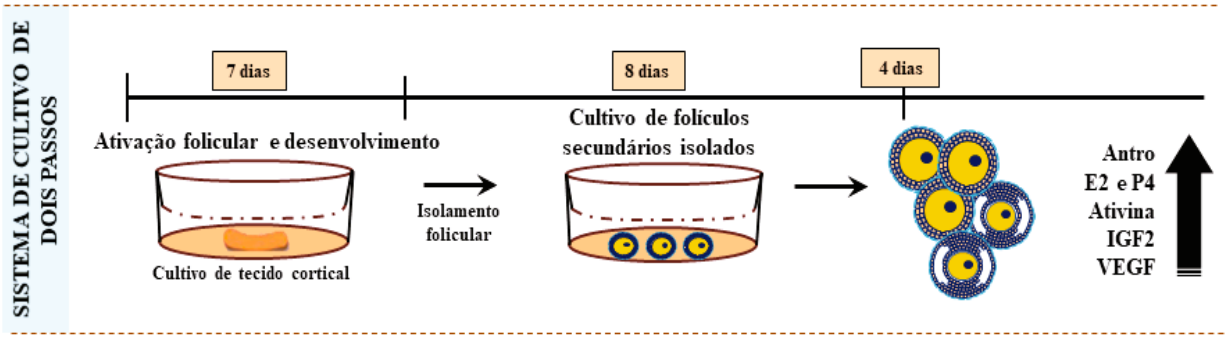


Fonte: Autor, adaptado de Bizarro -Silva (2021)

Por sua vez, no sistema de cultura folicular de várias etapas (Figura 3), a estratégia se baseia em simular ainda mais o ambiente fisiológico dos folículos em desenvolvimento (GREEN e SHIKANOV, 2016; SIMON *et al*., 2020). Utilizando folículos pré-antrais precoces, consiste inicialmente consiste em um cultivo *in situ* durante 7 dias, levando muitos folículos primordiais a ativarem e entrarem na fase de crescimento mantendo seu suporte de células somáticas até a fase pré-antral (MCLAUGHLIN *et al*., 2018). Ao final do cultivo *in situ*, é realizada a dissecação mecânica dos folículos pré-antrais que são, então, destinados individualmente ou em grupo a um novo cultivo durante 8 dias (XU *et al*., 2021), com posterior cultivo de Complexos cumulus óocitos (COCs) por mais 4 dias, que, quando viáveis são destinados a maturação *in vitro*. Utilizando esta técnica Xu *et al* (2021) demonstraram que grande parte de folículos humanos evoluíram até a fase antral, produzindo hormônios esteróides (E2 e P4) e mais da metade sobreviveram durante por mais de 3 semanas. Anteriormente,

Mclaughlin *et al* (2018) a partir de folículos com cavidade antral oriundos da etapa 2, recuperou e cultivou por mais 4 dias COCs contendo oócitos com diâmetro maior que 100 µm, identificando após 24 horas a presença de corpo polar e expansão de células cúmulos.

Figura 3 - Representação esquemática dos sistemas de cultivo *in vitro* de folículos pré-antrais várias etapas.



Fonte: Adaptado de Nascimento *et al* (2023)

Apesar dos expressivos avanços já obtidos, nenhuma maturação meiótica eficiente foi descrita para a espécies bovina até o momento (PAULINO *et al* 2020). Dentre os motivos que podem influenciar o sucesso da foliculogênese *in vitro*, além da procedência dos ovários e os métodos de isolamento utilizados (SILVA *et al*., 2015), se destaca a ocorrência do estresse oxidativo.Esta ação negativa, está atrelada a inúmeros fatores tais como: manipulação, exposição a luz, alterações de pH, cessação da proteção antioxidante sistêmica das células, exposição à concentração supra fisiológica de oxigênio (20%) e diferentes composições do meio (SA NAR *et al*., 2018).

Neste contexto, nos últimos anos, diversos estudos analisaram elaboração de um meio de cultivo que possibilite as condições necessárias ao desenvolvimento folicular. Castro et al. (2014) e mais tarde, Jimenes *et al* (2016) relataram que α-MEM seria mais eficaz na promoção do crescimento e manutenção da viabilidade folicular em tecidos ovarianos bovinos quando comparados aos meios TCM199,

Posteriormente, outras investigações avaliaram os efeitos da adição de diferentes suplementos ao meio de cultura de tecido ovariano bovino. Cunha *et al* (2017) mostraram que a presença da Proteína Morfogenética Óssea 4 (BMP-4) (100 ng BMP4 /mL) durante o cultivo dos folículos em fragmentos de tecido ovariano promoveu um aumento dos diâmetros folicular e oocitário de folículos primários e secundários após um período de 06 dias de cultivo *in vitro*, além de garantir a manutenção da viabilidade folicular. Em outro relatório, Silva *et al* (2017),

evidenciaram que a adição do Fator de Necrose Tumoral (TNF-α) (10ng/mL) durante o cultivo *in vitro* de folículos ovarianos bovinos diminui a sobrevivência folicular e aumenta o número de células apoptóticas no tecido do ovário após 06 dias de cultivo. Por sua vez, Paulino *et al* (2020) relataram que o EGF (10 ng/mL) promoveu o crescimento folicular e aumentou a expressão de mRNA para GDF-9 e cMOS ao tempo que a progesterona também aumentou a expressão de transcritos para GDF-9 além de ciclina B1 em oócitos de folículos bovinos cultivados por 18 dias, enquanto Nascimento *et al* (2022) ao exprorar os os efeitos da N-Acetilcisteína (NAC) durante o cultivo *in vitro* de folículos pré-antrais bovinos relatou que a NAC (1.0 mM) aumenta a viabilidade e o crescimento de folículos pré-antrais cultivados *in vitro*.Nesta mesma linha investigativa, Vasconcelos *et al* (2021) mostraram que a presença do Eugenol (4-allyl-2-methoxyphenol) no meio de cultivo aumentou a expressão de mRNA para a enzima GPX1, além de manter a viabilidade de folículos secundários bovinos enquanto. Já, Costa *et al* (2022) observaram que Aloe *vera* 10,0% melhora a distribuição da matriz extracelular em tecidos e aumenta a expressão do mRNA para *PRDX6* após 6 dias *in vitro*.

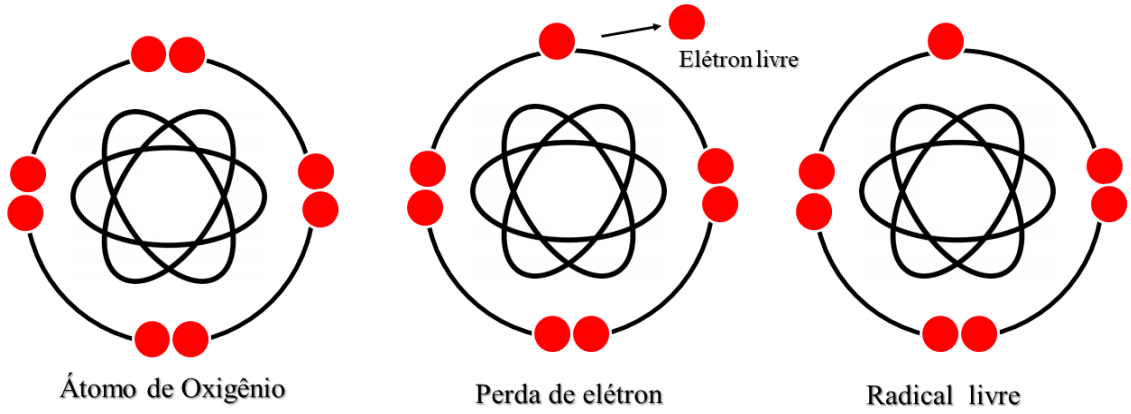
Em conjunto estes achados sugerem que um meio acrescido de substâncias tais como fatores de crescimento, aminoácidos e compostos vegetais, pode ser a chave para proteger os folículos contra a exarcebada produção das espécies reativas de oxigênio (EROS) durante as fases de ativação o desenvolvimento *in vitro* (RODRIGUES *et al*., 2009). Nesse contexto, metabolitos secundários extraídos de vegetais têm ganhado destaque por possuírem uma grande variedade de moléculas sequestradoras de radicais livres, como flavonoides, antocianinas, carotenoides, glutationina e vitaminas (MAIA, 2008). Essas características ampliam o interesse na utilização dessas substâncias como potenciais inibidores do estresse oxidativo em condições *in vitro.*

2.3. *Estresse oxidativo e influência dos compostos antioxidantes durante o cultivo in vitro de folículos pré-antrais.*

O cultivo *in vitro* do tecido ovariano apresenta inúmeras vantagens, uma vez que os folículos presentes podem ser mantidos no ambiente natural (COSTA *et al*., 2022). No entanto, independente do sistema utilizado, o estresse oxidativo durante o período de cultivo pode trazer danos à fisiologia folicular contribuindo para resultados insatisfatórios como uma menor competência folicular e produção de oócitos de baixa qualidade (ZHANG *et al*., 2019; CHAUDHARY *et al*., 2019; VON MENGDEN *et al*., 2020).

O estresse oxidativo ocorre devido aumento das espécies reativas de oxigênio (EROS) resultante do desequilíbrio entre a geração de compostos oxidantes e antioxidantes em nível celular (BARBERINO *et al*., 2016; LINS *et al*., 2017). Estas moléculas são formadas a partir das reações de redução do oxigênio (O_2) e constituem parte dos radicais livres, como por exemplo, o superóxido (O_2•-), radical hidroxil (OH•) que são caracterizadas pela perda de um ou mais elétron em sua última camada eletrônica (Figura 4) o que os torna instáveis e altamente reativos (HAWKINS *et al*., 2019). Suplementarmente, as espécies não radicalares como o H_2O_2 e O_3, mesmo não possuindo elétrons livres, podem reagir com moléculas ao seu redor ocasionando os mais variados efeitos adversos (TORTORA *et al*., 2012; MARTELLI, NUNES, 2014).

Figura 4 - Representação esquemática da formação de um radical livre, caracterizado pela perda de um elétron a partir de um átomo de oxigênio.



Fonte: Autor, adaptado de Vasconcelos *et al* (2022)

Em condições normais, as EROS são produzidas através do metabolismo celular e exercem importância em processos celulares, como maturação de oócitos (LONERGAN, FAIR 2016), esteroidogênese folicular e ovulação (ZHU *et al*., 2018). No entanto, em condições de cultivo *in vitro*, em níveis elevados, as EROS podem causar alterações na homeostase iônica intracelular e lipoperoxidação, além de danos em macromoléculas como DNA, proteínas, carboidratos e ativar vias de sinalização que provocam a morte celular (SHABAN *et al*., 2017; HE *et al*, 2016; WANG *et al*., 2017).

De fato, o cultivo *in vitro* de fragmentos de tecido ovariano ocasiona uma maior formação de espécies reativas (LINS, 2017; SA NAR *et al*, 2018), uma vez que manipulação de tecidos e células em condições *in vitro*, pode resultar na produção excessiva de EROS (MASSIGNAM et al., 2018). Nos folículos dentre os principais compartimentos intracelulares

afetados pelo estresse oxidativo, além das mitocôndrias que são as primeiras organelas a sofrerem degeneração, visto serem o local onde são produzidos os radicais de oxigênio (SHI, DANSEN, 2020; HARRINGTON *et al*, 2023) há uma reação inflamatória no oócito e nas células cumulus, induzindo um desequilíbrio no fator de crescimento e na produção de citocinas, provocando a apoptose dessas células, e, por conseguinte, à atresia folicular (ZHANG *et al*., 2016; CHAUDHARY *et al*., 2019).

Estudos anteriores, mostraram que a ocorrência do estresse oxidativo, além da apotose em células da granulosa resulta na redução dos níveis de estradiol e em atresia folicular (GHATEBI *et al*., 2019). Além disso, o desequilíbrio na produção de EROS é capaz de interromper a comunicação entre oócitos e células da granulosa (CHAUBE *et al*., 2014; ADELDUST *et al* 2015); aumentar a produção de subprodutos da peroxidação lipídica como o malondialdeído (MDA) levando à disfunção celular e por fim induzir várias vias de apoptose mitocondrial através da ativação de receptores de morte localizados nas membranas celulares, como a proteína quinase ativadora de mitógenos e da via das caspases (KASHKA *et al*., 2016; CHEN *et al*., 2017; BOCK *et al*., 2020).

Embora as células possuam um sistema de defesa antioxidante mediado por proteínas de ligação ao ferro e ao cobre, transferina, ferritina e albumina ou por menamismos enzimáticos (Tabela 2) várias substâncias antioxidantes têm sido usadas como suplemento em meios de cultura para diminuir a produção de espécies reativas de oxigênio (DE LIMA *et al*., 2017; TORRES-OSÓRIO *et al*., 2019; YANG, 2021) e melhorar o potencial reprodutivo dos gametas. Os antioxidantes são substâncias endógenas ou exógenas, que atuam inibindo ou retardando o início da oxidação do substrato devido sua capacidade de doar elétrons ao átomo desestruturado, atenuando os processos oxidativos e assim diminuindo os danos moleculares nas células (ROCHA *et al*., 2016; BORGES *et al*, 2019).

Tabela 2 - Sistema celular de defesa antioxidante

Antioxidantes	Ação biológica	Referência
Enzimáticos (Fontes endógenas)		
Superóxido dismutase (SOD)	Dependente de SOD-Cu/Zn (citoplasma), SOD-Mn (mitocôndria). Catalisa a conversão do radical superóxido (O2•) em peróxido de hidrogênio (H_2O_2)	He *et al*., (2016)
Catalase (CAT)	Catalisa a conversão de H_2O_2 ou hidroperóxidos	Cozza *et al* (2011)
Glutationa peroxidase (GPX)	Catalisa a redução do H_2O_2 a H_2O	Abedelahi *et al*.; (2010)
Peroxidorredoxina (PRDX)	Catalisa a redução de H2O2, hidroperóxidos fosfolipídicos e hidroperóxidos de cadeia curta através da ação da GPX	Fisher *et al* (2016)

Tabela 2 - Sistema celular de defesa antioxidante – Continua

Não enzimáticos (Fontes exógenas)		
β-caroteno,α-tocoferol, flavonoides,taninos, vitamina C, selênio e extratos de plantas	Proteção contra a oxidação de lipídeos e DNA, Atuam como agente redutores das EROs. Conferem proteção contra danos causados pela LDL-ox	Fito *et al* (2007), Carneiro *et al* (2017); Aquino *et al*(2017).

Fonte: Autor, adaptado de Barbosa *et al* (2010)

Diversos estudos analisararam a utilização de antioxidantes de origem natural, como meios de suplementação para o cultivo *in vitro* de tecido ovariano de animais domésticos (TAMURA *et al*, 2020; RESIDIWATI *et al*., 2021; BERGAMO *et al*., 2022). Nesse contexto, Barberino *et al* (2016) avaliariam os efeitos do extrato de Amburana *cearenses* sobre os níveis de GSH e atividade mitocondrial em folículos secundários de cabra. Em outro estudo, Menezes *et al* (2018) investigaram o efeito do extrato da mesma espécie como meio de preservação ou de cultivo de tecido ovariano ovino. Mbemya *et al* (2018) relataram a influência da suplementação do extrato de Juglans *insularis* ao meio de cultura mostrando efeitos semelhantes ao do FSH após a cultura de folículos secundários de ovinos. Além disso, Rocha *et al* (2018) demonstraram que o resveratrol foi capaz de manter parâmetros aceitáveis de EROS em tecido ovariano de bovinos. Suplementar a estes achados, a adição de substâncias antioxidantes derivadas de plantas (carvacrol, eugenol, rutina) derivados de plantas, ao meio de cultivo promoveram a ativação e mantiveram a viabilidade, morfologia e crescimento de células reprodutivas em camundongos, ovinos e bovinos, respectivamente (MAHRAN *et al*., 2019; OMIDPANAH *et al*., 2020; VASCONCELOS *et al*., 2021)

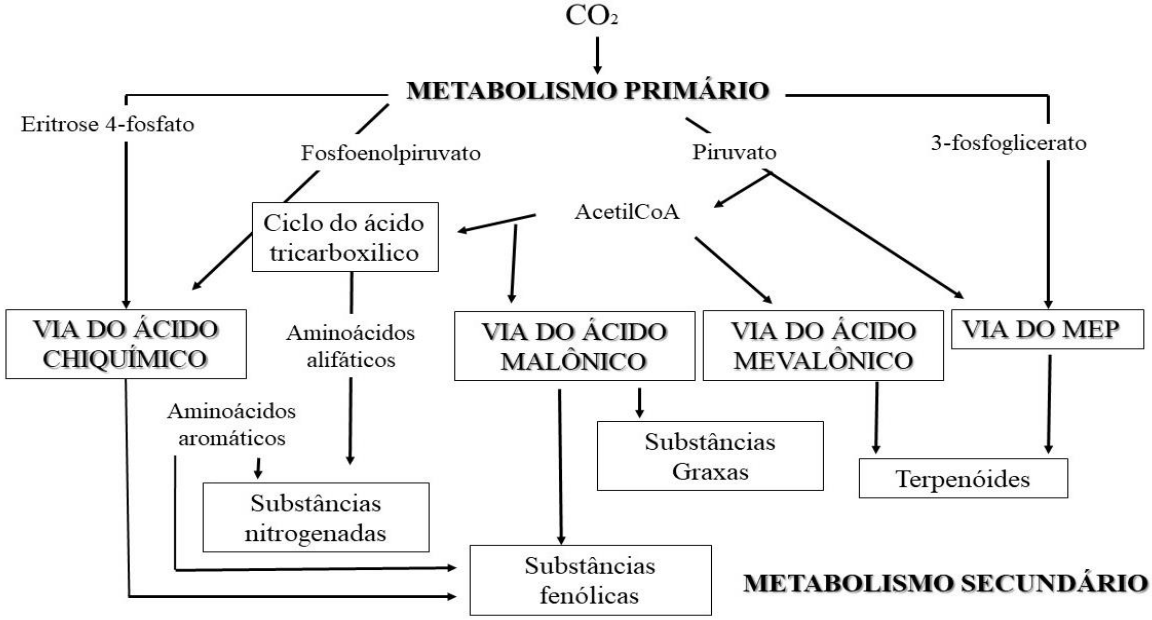
Por esta razão, o potencial da utilização desses compostos, incluindo o óleo essencial de *Lippia sidoides,* como suplementos de sistemas de cultivo *in vitro* de folículos ovarianos, tem despertado o interesse de pesquisadores, se fazendo necessário, portanto, conhecer as propriedades químicas que regulam os mecanismos de ação antioxidante destas substâncias.

2.4. *Óleos essenciais como fonte de agentes antioxidantes.*

Os óleos essenciais (OEs) são extratos líquidos complexos, voláteis, limpidos e lipofílicos com odor e cor característicos, obtidos de várias partes das plantas (NAZZARO *et al*., 2017; ALMEIDA *et al*., 2020; ABERS *et al*., 2021). Evidências apontam que os OEs exibem importantes atividades biológicas, tais como: antibacteriana, anticancerígena, anti-inflamatória, antifúngica e antioxidante (BAJALAN *et al*., 2017; KIM *et al*., 2019;

PINTATUM *et al*., 2020). Dentre os diferentes gupos de compostos orgânicos que formam os OEs, estão os monoterpenos, sesquiterpenos, fenilpropanoides, ésteres e outras substâncias de baixo peso molecular. (ALMEIDA *et al*., 2020, BORGES *et al*., 2020). Estes metabolitos secundários são produzidos nas plantas por diferentes vias, podendo ainda apresentar composição química diversificada variável entre espécies, bem como entre as partes de uma mesma planta que o produz (MIRANDA *et al*., 2016).

Figura 5. Esquema geral do metabolismo primário e as vias dos metabólitos secundários



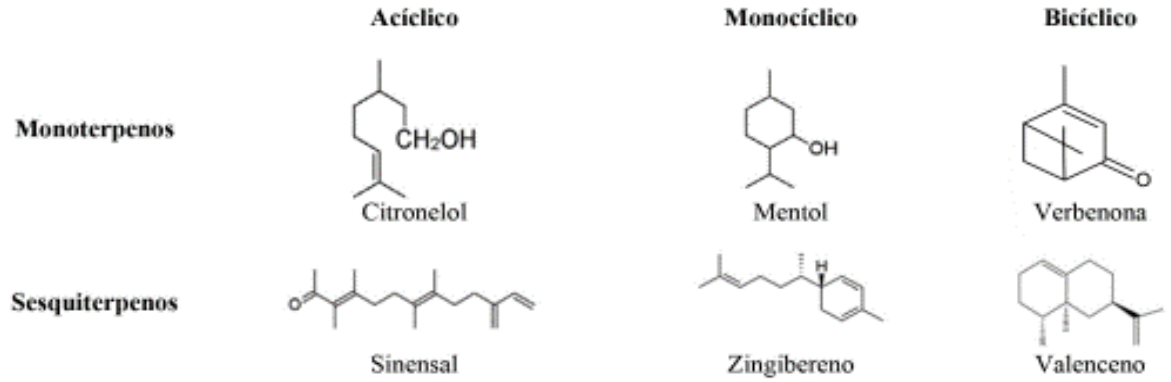
Fonte: Autor, adaptado de Santos (2015)

Sintetizados a partir do metabolismo secundário das plantas, os terpenos são formados pela condensação de unidades de isopreno (C5) e são representados dentre outras moléculas, pelos monoterpenos e sesquiterpenos (FELIPE; BICAS, 2017). Essas substâncias são sintetizados a partir da acetil-CoA, via rota do ácido mevalônico ou via rota do metileritritol fosfato (MEP) enquanto os compostos fenólicos são biossintetizados a partir de duas rotas principais: via ácido chiquímico e ácido malônico e os compostos nitrogenados, por sua vez, são produzidos a partir dos aminoácidos (MARTINS, 2012) como demonstrado anterriomente na Figura 5.

Monoterpenos e sequisterpenos (Figura 6) contituem a classe dos compostos quimicos mais encontradas nos óleos essenciais (SILVA, 2008). A primeira molécula possui 10 átomos de carbono sendo formada por 2 unidades de isopreno (C10H16), que podem formar vários esqueletos de carbono. Além disso, pode ser encontrada nas formas linear (acíclico), monocíclico e bicíclico. Já a segunda possui 15 átomos de carbono; sendo formada por 3

unidades de isopreno (C15H24) e pode ocorrer nas formas acíclica, monocíclica, bicíclica e tricíclica (DE GROOT; SCHMIDT,2016)

Figura 6 - Exemplos de monoterpenos e sesquiterpenos: acíclico, monocíclico e bicíclico



Fonte: Cruz (2021).

Dentre os constuintes dos OEs com eleveado potencial antioxidante além do geraniol, p-cimeno, mentol, eucaliptol e metil chavicol, destacam-se o timol e seu isômero carvacrol que contêm ligações duplas de carbono conjugadas, bem como uma hidroxila fenólica ligada ao anel bezeno (HAMIDPOUR, 2017). Enquanto antioxidantes fenólicos (AROH) essas moléculas podem agir através de dois mecanismos de ação, que podem ocorrer em paralelo, embora tenham taxas de reação diferentes (AQUINO *et al*., 2017). A primeira forma de ação se dar a partir da transferência de átomos de hidrogênio (HAT), onde o radical livre (R•) remove um átomo de hidrogênio do antioxidante (AROH), transformando-o no radical ArO•, resultando em um radical fenoxil não reativo. No segundo mecanismo, por meio da transferência de elétrons (SET), o antioxidante pode doar um elétron ao radical livre, formando, entre outros produtos, um cátion radical do antioxidante (ArO•+) que é estável e não reage com substratos (ROJANO *et al*., 2008).

Adicionalmente, a atuação destes compostos enquanto agentes redutores, confere a capacidade de ligação a íons metálicos, estimulando a atividade de enzimas como a catalase (CAT) e a superóxido-dismutase (SOD) interferindo assim nas reações de propagação e formação de radicais livres (AQUINO *et al*., 2017; GHAFFARI *et al.*, 2018). Por estas razões os óleos essenciais, são reconhecidos por terem inúmeras atividades biológicas, como atividades analgésicas, antioxidantes, antimicrobianas e anti-inflamatórias, entre outros (D.MICIĆ *et al*., 2021; NONATO *et al*., 2022) o que têm despertado cada vez mais o interesse dos pesquisadores em virtude do importante papel na medicina natural, comestologia, farmacologia e seus benefícios à saúde humana. (KARPINSKI, 2020)

2.6. Aspectos químicos e atividade antioxidante do óleo essencial de Lippia siodoides

A planta de *Lippia sidoides* ou Alecrim pimenta como é conhecido popularmente, pertence a família das *Verbenaceae*. É uma espécie endêmica e de uso medicinal bastante encontrada no Nordeste brasileiro, como nos estados do Rio Grande do Norte e Ceará (BRASIL, 2018). Com ramos providos de folhas aromáticas e flores diminutas e esbranquiçadas, esse arbusto de caule lenhoso pode atingir até três metros de altura (MORÃO, 2016; SOUSA *et al*., 2020). Dada a sua importância medicinal e potencial econômico sustentável o *Lippia sidoides* é considerado um potente gerador de produtos farmacológicos, integrando, portanto, o banco de informações sistematizadas da relação nacional de plantas mediciais de interesse do ao Sistema Único de Saúde (SANTOS *et al*., 2016; BRASIL, 2018)

Além do uso medicinal através da infusão de suas folhas como alternativa para o tratamento de infecções bucais e da garganta (SOUSA *et al*, 2020) e "problemas no tratamento de pele" (ALMEIDA *et al*., 2010), suas propriedades sedativa, analgésica, espasmolítica (TAVARES et al., 2005); antimicobriana (DE ASSIS *et a*l., 2022); antifúngica (ALMEIDA *et al*., 2020); antiparasitária (DE MELO *et al*., 2020) e antioxidante (ZANOTTO *et al*., 2023) já foram descritas na literatura, estando fortemente associadas a composição química do óleo essencial extraído de suas folhas e flores.

A maioria das pesquisas envolvendo a planta de *Lippia sidoides* avaliaram a composição e as atividades biológicas de seus extratos e óleo essencial (BRASIL, 2018). Neste contexto, inúmeros constituintes foram identificados no óleo essencial de *Lippia sidoides*, tais como: α-tujeno, sabineno β-pineno, mirceno, α-felandreno, α-terpineno, ρ-cimeno, limoneno, 1,8-cineol γ-terpineno, terpin-4-ol, α-terpineol, verbenona, metil-éter-timol, neral, timol, carvacrol, timol acetato, β-elemeno, (E)-cariofileno, α-humuleno, dehidro-aromadendrano γ-muuroleno, amorfa-4,7-(11) -dieno, 7-epi-α-selineno e óxido de cariofileno (ROZZA *et al*, 2013, PEREIRA *et al*., 2013; AQUINO *et al*., 2014). No entanto, os primeiros estudos de caracterização fitoquímica da espécie que analisaram a composição do óleo essencial de suas folhas, apontaram o timol (43,5%) seguido do α-felandreno (22,4%) como principais constituintes (CRAVEIRO, 1981).

Em observações posteriores, pode-se constatar que o principal constituinte na maioria dos casos foi o timol, sendo que sua concentração nos OEs estudados variou de 34% a 95% seguido do carvacrol, que quando majoritário no OE, mostrou variação de teor de 31,68 a 51,8% (BRASIL, 2018). De fato, estes monoterpenos fenólicos foram identificados como os compostos mais ambudantes em estudos realizados com espécimes de várias partes do Brasil.

No Nordeste, incluindo várias regiões do Ceará, o principal componente do óleo essencial de *Lippia sidoides* é o timol (CARVALHO *et al*., 2013; DE MORAIS *et al*.; 2016; MORÃO e*t al*.,2016). Já em análises conduzidas em plantas cultivadas na região Sudeste do Brasil, os componentes majoritários foram o carvacrol (LIMA *et al*., 2011) e 1,8-cineol, isoborneol e acetato de bornila (MORAIS *et al*., 2012), respectivamente.

Portanto, é provável que existam diferentes quimiotipos em relação à composição química de seu OE como demonstrado na (Tabela 6) que lista os constituintes majoritários encontrados no óleo essencial extraído das folhas de *Lippia sidoides* coletadas em diferentes lugares do Brasil.

Tabela 3 - Principais componentes (%) do óleo essencial das folhas de *Lippia sidoides*

Constituintes majoritários em (%)			Local de coleta	Referência
Timol (22,4)	1,8-Cineol (18,7)	Timol metil éter (5,3)	Mossoró (RN)	Costa *et al*., 2001
Timol (80,8)	p-Cimeno (8,6)	(E)-Cariofileno (5,1)	Nordeste do Brasil	Cavalcanti *et al*., 2010
Timol (43,5)	α-Felandreno (22,4)	(E)-Cariofileno (9,7)	Crato (CE)	Costa *et al*., 2015
Timol (56,7)	Carvacrol (16,7)	p-Cimeno (7,1)	Fortaleza (CE)	Botelho *et al*., 2007
Timol (78,4)	p-Cimeno (6,3)	(E)-Cariofileno (6,2)	Teresina (PI)	Medeiros *et al*., 2011
Timol (83,4)	(E)-Cariofileno (5,8)	p-Cimeno (7,1)	Viçosa (CE)	Aquino *et al*, 2014
Carvacrol (26,4)	1,8-Cineol (22,6)	p-Cimeno (9,9)	Itumirim (MG)	Guimarães *et al*., 2015
Carvacrol (31,7)	Cimeno (19,6)	1,8-Cineol (9,3)	Itumirim (MG)	Lima *et al*., 2011
Timol (84,9)	p-Cimeno (5,3	Timol metil éter (3,0)	Cariri (CE)	Mota *et al*., 2012
Timol (30,2)	Benzeno (14,5)	(E)-Cariofileno (11,8)	Montes Claros (MG)	Aquino *et al*., 2014

Fonte: Autor, adaptado de Guimarães *et al* (2015).

Diante da crescente aplicabilidade de plantas utilizadas na medicina natural, vários estudos relacionados à análise da atividade antioxidante do óleo essencial de *Lippia sidoides*, apresentaram resultados inovadores (SIQUEIRA - LIMA *et al*., 2017). Na indústria alimentícia, este OE tem sido usado para melhorar a qualidade e a conservação dos alimentos perecíveis (ISHKEH *et al*., 2019) em virtude do potencial de ação de monoterpenos fenólicos presentes na composição dessa espécie (CANTÚ-VALD'EZ *et al*., 2020). Ao avaliarem a capacidade antioxidante, Nonato *et al* (2022) relataram que esse OE apresentou porcentagem de inibição correspondentes a 99% nas concentrações de 500 e 1000 µg/mL e 58,53% na 1000 µg/mL, nos

ensaios ABTS e DPPH respectivamente. Anteriormente, Costa *et al* (2018) avaliaram o seu potencial na eliminação do radical DPPH e verificaram que todas as concentrações do óleo utilizadas no ensaio (6.400, 3.200, 1.600, 800, 400, 200 e 100μg/mL) apresentaram atividade antioxidante, não sendo observada diferença estatística entre as maiores doses (6.400, 3.200 μg/mL). Em outro trabalho, Monteiro (2007) verificou que a atividade antioxidante desse óleo, expressa como a concentração que inibe 50% do radical livre DPPH foi igual a 7,27g/mL.

Em outra abordagem, durante o cultivo *in vitro* de células reprodutivas de mamíferos, a capacidade antioxidante do óleo essencial de *Lippia sidoides* também foi investigada e trouxe resultados animadores sobre o aumento das concentrações de glutationa (TEIXEIRA *et al*., 2014); redução da taxa de apoptose celular durante a maturação *in vitro* de oócitos (PEREIRA, 2015) e nos parâmetros de desenvolvimento e qualidade de blastocistos bubalinos produzidos *in vitro* (SOLLECITO *et al*., 2019). Em geral estes achados, demonstraram o potencial antioxidante deste óleo essenciais, que pode ser atribuído à presença de componentes majoritários como o Timol.

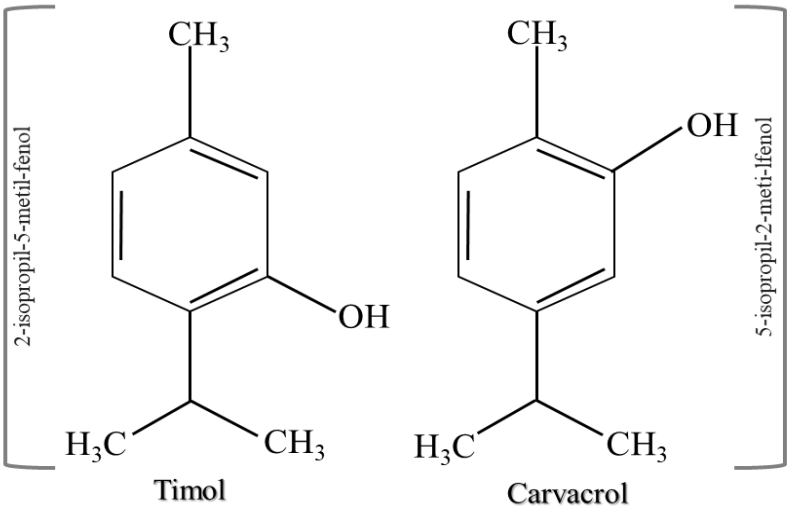
2.7. *Aspectos químicos e mecanismos de ação antioxidante do timol*

O Timol (2-isopropil-5- metilfenol) é um monoterpeno natural de estrutura aromática biossintetizado pela hidroxilação do p-cimeno após a aromatização do γ-terpineno em p-cimeno (SALEHI *et al*., 2018). Esta molécula pode ser obtida a partir de óleos essenciais de várias plantas (BRASIL,2018) ou ainda de forma sintética (THOZET; PERRIN,1980). O Timol é estruturalmente muito semelhante ao carvacrol variando apenas a posição do grupo hidroxila no anel fenólico (Figura 7). Com fórmula química $C_{10}H_{14}O$, é caracterizado como um meta-cresol em cuja molécula salienta-se um anel aromático trissubstituído com relação 1,2,4 e presença de hidroxila fenólica, além dos radicais metila e isopropila

O Timol é uma substância cristalina branca (Figura 8) de odor agradável, sensível à luz e ao calor e que permanece líquida a temperaturas consideravelmente mais baixas (SUN *et al*., 2021). Em contrapartida, em temperatura ambiente, encontra-se na forma de cristais (HOLLAND *et al*., 2014). Além disso, o Timol é ligeiramente solúvel em água em pH neutro e absorve radiação UV máxima em 274 nm (NORWITZ *et al*., 1986; WADE, REYNOLDS,1997), é dissolvido facilmente em alguns solventes orgânicos tais como diclorometano (DCM), acetato de etilo (AcOEt), metanol (MeOH) e álcool (KOWALCZYK *et al*., 2020; MESQUITA, 2017). Do ponto de vista biológico, por exibir toxicidade relativamente

baixa para as células de mamíferos (MARCHESE *et al*., 2016; CHAUHAN; KANG, 2014) o timol é considerado pela agência do departamento de saúde do governo norte-americano (FDA) como uma substância segura (NAGOOR MEERAN *et al*., 2017).

Figura 7 – Estrutura química do timol e carvacrol.



Fonte: Autor, adaptado de SHIYAB *et al* (2012)

Figura 8 – Imagem representativa de cristais de Timol e suas informações química

De fato, o Timol é um importante agente natural e tem gerado interesse na comunidade científica em estudos farmacológicos por seu potencial terapêutico no tratamento *in vivo* de algumas doenças tais como depressão, câncer, infertilidade (MEERAN *et al*., 2017). Estudos já relataram seus efeitos cardioproteror (GHOLAMI-AHANGARAN *et al*., 2022), neuroproteror (NOURMOHAMMADI et al.,2022), antiflamatório (LIU *et al*., 2022; AL-KHRASHI *et al*., 2021) além de sua recente utilização em terapias experimentais no tratamento contra o câncer (BOUHTIT *et al*., 2021; GHORANI *et al*., 2022; SERAJ *et al*., 2022).

O Timol vem sendo amplamente utilizado na medicina e na indústria farmacêutica e alimentar (CAO *et al*., 2021), devido ao seu potencial antibacteriano (HASSANNEJAD *et al*., 2019; RÚA *et al*., 2019; DOMINGUEZ-USCANGA *et al*., 2021; LEVENT *et al*., 2021); atividades analgésicas e antioxidante (MEERAN *et al*., 2017; ISLAM *et al*., 2018; GUVENÇ *et al*., 2018; CAO *et al*., 2021; SINGHAL *et al*., 2022; ALIZADEH; NAZARI, 2022). Além disso, nos últimos anos, o papel do Timol ganhou proeminência, uma vez que essa molécula tem bioatividade positiva na fisiologia e metabolismo animal (OMONIJO *et al*., 2019)

Em mamíferos vários estudos demonstraram os seus efeitos nos benefícios para a saúde animal (SHAO *et al*., 2023), produção (CASTAÑEDA-CORREA *et al*., 2019) e reprodução (TIJANI *et al*., 2023). Neste último caso, um dos efeitos mais estudados do Timol inclui a eliminação de radicais livres, aumentado as atividades de várias enzimas antioxidantes endógenas. Guvenc *et al* (2018) investigaram os parâmetros de qualidade espermática, parâmetros bioquímicos, níveis de malondialdeído (MDA), glutationa reduzida GSH), glutationa peroxidase (GSH-Px), catalase (CAT) em estudo com ratos. Os resultados mostraram que o timol diminuiu significativamente os níveis de MDA nos testículos, fígado e tecidos renais, ao tempo que aumentou os níveis de GSH e CAT em comparação com o grupo controle. Além disso, nesse estudo o timol melhorou a qualidade do esperma de ratos, bem como motilidade e concentração de espermatozóides em comparação ao grupo de controle

O Timol possui atividade semelhante à SOD na remoção de radicais superóxido *in vitro* em reações que gerem espécies reativas de oxigênio (KRUK *et al*., 2000). Ele também demonstrou atividade antioxidante moderada em células de fibroblastos de pulmão de hamster chinês V79 (UNDEGER et al., 2009). Além disso, Archana *et al* (2011) relataram que o Timol (25μ/mL) mostrou potente atividade antioxidante por meio da modulação das atividades de antioxidantes enzimáticos e diminuição da peroxidação lipídica em células de hamster chinês V79 induzidas por raios gama. Na linha celular Caco-2, o timol (250 μM) atenuou o estresse oxidativo induzido por H_2O_2 (CABELLO *et al*., 2015) e em neutrófilos humanos atenuou a produção de EROS a apresentou atividade inibitória da mieloperoxidase (PEREZ-ROSES *et al*., 2016). A atividade antioxidante do Timol sobre os ovários foi avaliada por Mahran *et al* (2019). Nesse estudo, os autores verificaram que o Timol (50 mg/kg) melhorou significativamente a capacidade antioxidante total nos ovários de ratas irradiadas com radiação gama de corpo inteiro. Em outro trabalho, com tecido testicular de ratos adultos foi demonstrado que o Timol alivia a toxicidade testicular induzida pelo imidaclopride (IMI) através da modulação do estress oxidativo por diferentes mecanismos (SABER *et al*., 2021). Nesse estudo,

ratos tratados com Timol exibiram uma elevação significativa de CAT testicular, SOD, e GSH em comparação com o grupo tratado com IMI.

Mahran *et al* (2019) exploraram os efeitos modulatórios do Timol na insuficiência ovariana prematura induzida por radiação (POI) *in vivo*, bem como os possíveis mecanismos subjacentes, particularmente o impacto na conversa cruzada entre TNF-α e a sinalização IGF-1 em POF. Neste estudo realizado com ratos, foram destacados as potenciais aplicações clínicas deste composto fenólico em fêmeas inférteis com baixa reserva ovariana e foi analisado o efeito radioprotector da CAR e do Timol da conversa cruzada entre TNF-α e IGF-1 em animais submetidos a radiação gama

De maneira geral, o mecanimos de ação antioxidante dos monoterpenos está ligada a sua estrutura química. De fato, os fenóis demonstram melhor atividade antioxidante, podendo doar elétrons ou hidrogênio interrompendo mecanismos de oxidação devido a presença de hidroxilas, logo, supõe-se que quanto mais hidroxilas na molécula maior seu potencial de ação (MORAIS *et al*., 2009). Embora, o Timol apresente apenas uma hidroxila, a presença do anel benzênico garante maior estabilidade a molécula doadora de elétrons e hidrogênio, acelerando o processo de eliminação de radicais livres (CRUZ, 2021).

3. JUSTIFICATIVA

Com o aumento exponencial da população global, um dos maiores desafios para as próximas décadas será produzir alimentos em quantidade suficiente para garantir a segurança alimentar da população mundial (ONU, 2022). Usando como parâmetro a recomendação de 0,8 g/kg de proteína por dia (WU, 2016) e a diferença entre a população humana atual e a esperada em 2050, a sociedade precisa produzir de forma sustentável aproximadamente 120 milhoes de kg de proteína adicional, equilibrada para as necessidades de aminoácidos dos seres humanos, para atender às demandas futuras (HUBBART *et al*., 2023). O Brasil que continua a ter um dos maiores rebanhos de gado do mundo, em conjunto com outros países do bloco sul deverá responder por 40% da demanda mundial de alimentos nos próximos anos (SOARES, 2019). Neste contexto, a utilização de biotécnicas da reprodução que permitam de forma significativa expandir a disponibilidade de gametas destinados a técnicas de reprodução assistida, podem contribuir favoravelmente para o aumento do ganho genético e melhoria da eficiência reprodutiva de rebanhos bovinos (GONÇALVES *et al*., 2022).

Dentre estas biotecnologias, o cultivo *in vitro* de tecido cortical ovariano de bovinos é uma excelente ferramenta para aprimorar o controle da foliculogênese e um passo estratégico para a otimização do desenvolvimento folicular (SILVA *et al*., 2020), uma vez que possibilita o resgate de um grande número de folículos pré-antrais existentes nos ovários (cerca de 90%) que constituem um valioso material genético que podem ser cultivados *in vitro* como fonte de oócitos. Além disso, pode contribuir para a preservação de espécies ameaçadas de extinção, bem como fornecer informações que possam subsidiar o tratamento da infertilidade em humanos (ARAÚJO *et al*., 2014; FISCH, ABIR, 2018; BUS *et al*., 2019). No entanto, a ocorrência do estresse oxidativo durante o cultivo *in vitro* de tecido ovariano ainda representa um importante fator limitante para a obtenção de folículos e oócitos plenamente competentes após o cultivo. (ZHANG *et al*., 2019; VON MENGDEN *et al*., 2020). No entanto, estudos recentes têm demonstrado que os efeitos deletérios do estresse oxidativo, podem ser reduzidos ou evitados mediante a adição de substâncias antioxidantes ao meio de cultivo, contribuindo favoravelmente para o desenvolvimento saudável de folículos cultivados *in vitro* (TORRES-OSÓRIO *et al*., 2019; YANG, 2021).

Nesse sentido, a utilização de compostos extraídos de plantas de biomas brasileiros, a exemplo dos óleos essenciais, que apresentem em sua composição moléculas capazes de neutralizar as espécies reativas de oxigênio são apontadas como alternativas promissoras para

o melhoramento do cultivo *in vitro* de folículos ovarianos de mamíferos (TAMULA *et al*., 2020; BERGAMO *et al*., 2022; COSTA *et al*., 2022; VASCONCELOS *et al*, 2022) uma vez que a ação antioxidante está entre os efeitos mais importantes para protocolos de cultivo de células e tecidos. Neste sentido, o Alecrim pimenta (*Lippia sidoides)*, reconhecido pelo Ministério da Saúde como um potente gerador de produtos farmacológicos (BRASIL, 2018), apresenta em seu óleo essencial monoterpenos, sesquiterpenos, fenilproponídes e outras substâncias de baixo peso molecular (BORGES *et al*., 2020), figurando como substância antioxidante de grande potencial para adição de meios de cultivo de tecido ovariano bovino. Além disso, podem-se encontrar nessa substância, moléculas com elevado potencial antioxidante, como o Timol em proporções de até 95%, amplamente estudado em diversos conjuntos celulares (MEERAN *et al*., 2017, CAO *et al*., 2021; SINGHAL *et al*., 2022), mas que, assim como óleo essencial de *Lippia sidoides,* carecem de investigação sobre seus efeitos antioxidantes durante o cultivo de tecido ovariano de ruminantes domésticos, fatores que reunidos justificam o pioneirismo desde trabalho no âmbito da biotecnologia reprodutiva com folículos ovarianos bovinos.

4. HIPÓTESES CIÊNTIFICAS

- A adição do óleo essencial de *Lippia sidoides* e do timol ao meio de cultivo *in vitro* de tecido ovariano bovino cultivado por 6 dias apresentam efeitos biológicos distintos.

- A adição do óleo essencial de *Lippia sidoides* e do timol em diferentes concentrações (400, 800, 1.600 e 3.200µg/mL) ao meio de cultivo *in vitro* de tecido ovariano bovino proporciona ativação e desenvolvimento folicular mantendo a morfologia celular adequada, preservando a integridade da matriz extracelular e das células do estroma

- A adição do óleo essencial de *Lippia sidoides* e do timol ao meio de cultivo aumenta os níveis de RNAs mensageiros para *SOD, CAT, PRDX6 e GPX1* no tecido ovariano bovino cultivado *in vitro*.

- A adição do óleo essencial de *Lippia sidoides* e do timol ao meio de cultivo promove uma maior expressão da atividade das enzimas antioxidantes SOD, CAT, GPX em tecido ovariano bovino cultivado *in vitro.*

5. OBJETIVOS

5.1. *Objetivo geral*

- Avaliar o efeito da adição do óleo essencial de *Lippia sidoides* e do timol em protocolos de cultivo *in vitro* de folículos pré-antrais inclusos em tecido ovariano bovino.

5.2. *Objetivos específicos*

- Avaliar a composição química do óleo essencial de *Lippia sidoides*, elencando seus constituintes majoritários.

- Avaliar o efeito de diferentes concentrações do óleo essencial de *Lippia sidoides* (3.200, 1.600, 800, 400 μg/mL) ou do timol (3.200, 1.600, 800, 400 μg/mL) no cultivo *in vitro* de tecido ovariano bovino sobre a morfologia da célula e do tecido, ativação e desenvolvimento folicular, integridade da matriz extracelular e densidade celular do estroma.

- Quantificar os níveis de RNAs mensageiros para *SOD, CAT*, *PRDX6* e *GPX1* no tecido ovaniano bovino cultivado *in vitro* na presença (800 ou400 μg/mL) do óleo essencial de *Lippia sidoides* ou timol.

- Analisar a atividade das enzimas SOD, CAT e GPX no tecido ovariano bovino cultivado *in vitro* na presença do óleo essencial de (3.200, 1.600, 800, 400 μg/mL) *Lippia sidoides* ou (800, 400 μg/mL) do timol.

6. CAPITULO 1

Lippia sidoides essential oil: chemical composition and antioxidant activity during _in vitro_ culture of bovine ovarian tissue

Lippia sidoides essential oil: chemical composition and antioxidant activity during *in vitro* culture of bovine ovarian tissue

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Abstract

This work aimed to analyze the chemical composition of *Lippia sidoides* essential oil (LSEO) and to investigate its effects on *in vitro* culture of bovine ovarian tissues.To do this constituents of *Lippia sidoides* essential oil were determined by Gas chromatography–mass spectrometry (GC/MS).To investigated its effects on bovine early follicles, fragments of ovarian cortical tissues were cultured for six days in control medium alone or supplemented with 400, 800, 1,600 and 3,200µg/mL of LSEO. The culture was carried out at 38.5°C in 5% CO2 in a humidified incubator and every two days 60% of the culture medium was replaced with fresh medium. At the end of the culture period, the fragments were destined for morphological analysis and determination of mRNA levels and activity antioxidant enzymessuperoxide dismutase (SOD), catalase (CAT), peroxiredoxin 6 (PRDX6) and glutathione peroxidase (GPX1). This experiment was repeated 10 times. Our results the presence of 400µg/mL LSEO in culture medium increased follicle growth and survival, as well as stromal cell density and collagen fibers in culture ovarian tissues. Interestingly, the tissues cultured with 400µg/mL LSEO had higher levels of mRNA for SOD, CAT and GPX1, when compared to tissues cultured in control medium. In addition, 400µg/mL LSEO increased the activity of GPX1 enzyme, but did not influence SOD and CAT activity. In conclusion, we found that 400µg/mL

LSEO increases follicle growth, survival, stromal cell density and collagen fibers in cultured ovarian tissues, as well as the levels of mRNA for SOD, CAT and GPX1, and the activity of GPX1 enzyme.

Keywords: *Lippia sidoides;* **E**ssential oil; **A**ntioxidant; **O**varian tissue; **B**ovine; ***I****n vitro* culture

1. Introduction

There is a consensus that activation and growth of primordial follicles can only be achieved by culturing the ovarian cortex containing follicles and stromal cells [Barbato et al, 2023; Yang et al., 2020]. Although the contribution of the ovarian stroma to regulating follicle growth is poorly defined, preserving the tissue structural integrity and the physical interactions between follicles and their surrounding environment is essential for *in vitro* follicle activation [Grosbois et al., 2023]. Collagen fibers provide physical support and mechanical properties for a more efficient *in vitro* culture microenvironment [Tang et al., 2020; Grosbois et al., 2023; Barbato et al., 2023]. Additionally, over the years, several studies have been proposed to analyze the nutritional requirements of ovarian tissue in bovine species with the purpose of establishing an ideal culture medium of ovarian cortical tissues [Telfer et al, 2008, McLaughlin et al, 2018]. However, some barriers have not yet been sufficiently overcome. For example, the occurrence of oxidative stress during the culture period remains a recurrent limitation. Indeed, oxidative stress has been strongly associated with decreased ability of follicles and somatic cells to neutralize reactive oxygen species (ROS), which are among the most prominent physiological inducers of cell damage and apoptosis [Cacciottola et al., 2018; Soto-Heras 2020]. Thus, the ideal conditions to promote *in vitro* follicle growths strongly depends on the supplementation of culture medium with antioxidants. In this sense, several studies have investigated the use of essential oils (EOs), which are important biotechnological products that can be used to control oxidative stress and, consequently, to improve *in vitro* ovarian tissue culture systems [Vasconcelos et al., 2020]

Several studies using free radical elimination method (DPPH) radical have shown that LSEO has antioxidant activity in various concentrations [Ziyatdinova and Kalmykova., 2023; Nascimento et al., 2021; Da Silva et al., 2017, Monteiro et al 2017].Carvacrol and thymol are a well-known strong natural antioxidants found LSEO [Parente et al, 2018; Aquino et al., 2014;

Mota et al, 2012, Ruberto e Baratta., 2000]. This substance increase glutathione concentrations [Reyes-Becerril et al; 2021 Saccol et al 2013] and reduces cell apoptosis rate during *in vitro* maturation of oocytes [Pereira, 2015]. Additionally, LSEO improved the development and quality of bovine blastocysts produced *in vitro* [Sollecito et al, 2019]. However, its effects on activation, survival, stromal cells density and antioxidant defense bovine ovarian tissue cultured *in vitro* have not yet been investigated.

The present study aims to investigates the chemical composition of LSEO, as well as the influence different concentrations (400, 800, 1,600 and 3,200µg/mL) of LSEO on primordial follicles activation and survival, ovarian stromal cell density and collagen distribution in ECM in bovine ovarian tissue after 6 days of *in vitro* culture. In addition, the effects of LSEO on levels of thiol and mRNA for CAT, SOD, GPX1 and PRDX1, as well as on activity of CAT, SOD and GPX1 enzymes were also investigated.

2 Material and methods

2.1 Chemicals

Unless otherwise stated, the culture media and other chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA). The essential oil of Lippia sidoides was purchased commercially, with 100% purity from the Brazilian aromatherapy company Laszlo (Belo Horizonte, MG, Brazil).

2.2 Analysis of Lippia sidoides essential oil composition

The chemical analysis of LSEO constituents was performed with a Shimadzu QP-2010 Ultra instrument employing the following conditions: Column: Rtx-5MS (Crossbond, 5% diphenyl/95 % dimethyl polysiloxane) measuring 30 m x0.25 mm x0.25 µm df; carrier gas: He (24.2 mL/min, in constant linear velocity mode); injector temperature of 250◦C, in split mode (1:100); and detector temperature of 250 ◦C. The column temperature was programmed for 35–18 0 ◦C at 4 ◦C/min, then180–280 ◦C at 17 ◦C/min, and 280 ◦C for 10 min. Mass spectra were obtained at electron impact of 70 eV. The volume of LSEO injected was 1 µL. The components were identified from its gas chromato-graphy–mass (GC)retention times, calculated by linear interpolation relative to retention times of main compounds and by comparison of its mass

spectra with those present in the computer data bank (NIST) and published literature [Adams, 2012].

2.2 In vitro effects of Lippia sidoides essential oil on ovarian tissues

Bovine ovaries (n = 24) of mixed breed cows were collected at a local slaughterhouse. Immediately after death, each pair of ovaries was washed in ethanol (70%) for approximately 10 s, followed by two washes in 0.9% saline solution supplemented with penicillin (100 µg/mL) and streptomycin (100 µg/mL). After washing, each pair of ovaries was individually transported to the laboratory in falcon tubes containing α-MEM supplemented with penicillin (100 µg/mL) and streptomycin (100 µg/mL) at 4ºC, within 1 hour. This study was approved by the Committee of Ethics and Animal Welfare of the Federal University of Ceará (Nº 09/2021).

In the laboratory, using a sterile scalpel n.22, ovarian cortical tissue from the same ovarian pair (n = 12 pairs) was cut in twenty-two slices (3 mm × 3 mm × 1 mm) in dissection medium composed of α-MEM supplemented with penicillin (100 µg/mL) and streptomycin (100 µg/mL). For each animal, two cortical slices were fixed in paraformaldehyde (4%) for 24 hours at 4ºC for histological analysis (uncultured control), as well as for analysis of collagen fibers in extracellular matrix. The remaining fragments were cultured in 24-well culture dishes for 6 days [Passos et al., 2016; Silva et al., 2017]. Culture was performed at 38.5°C in 5% CO2 in a humidified incubator. The basic culture medium consisted of α-MEM (pH 7.2–7.4) supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, and 5 ng/mL selenium), 2 mM glutamine, 2 mM hypoxanthine, antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin) and 1.25 mg/mL of bovine serum albumin (α-MEM+). The fragments were cultured in 0.5mL of control medium (α-MEM+) alone or in this control medium (α-MEM+) supplemented with dif-ferent concentrations of *Lippia sidoides* essential oil (400, 800, 1,600 or 3,200 µg/mL). The concentrations of *Lippia sidoides* essential oil were chosen according to Costa et al. [2021]. Every 2 days, 60% of the culture medium was replaced with fresh medium. Each treatment was repeated 10 times. After the end of the culture period, the fragments were destined to morphological and biochemical analysis.

2.4 Morphological analysis, evaluation of in vitro follicular growth

Histological analysis was performed according to the methodology of Bizarro-Silva et al. [2018]. Uncultured tissues (D0) and *in vitro* cultured tissues were fixed in paraformaldehyde (4%) for 24 hours, dehydrated in increasing concentrations of ethanol, cleared with xylene and embedded in paraffin. Serial sections (7 µm thick) were stained with hematoxylin and eosin. Only preantral follicles with an visible oocyte nucleus in the section were analyzed to avoid double counting. The coded and anonymized slides were examined under a microscope (Nikon, Tokyo, Japan) at magnifications of 100 and 400 times. The developmental stages of follicles were classified as primordial (one layer of flattened or flattened and cuboidal granulosa cells surrounding the oocyte) or growing follicles (primary: one layer of cuboidal granulosa cells and secondary: two or more layers of cuboidal granulosa cells surrounding the oocyte) [Figueiredo; Lima, 2017]. These follicles were further classified as morphologically normal when an intact oocyte was present, surrounded by well-organized granulosa cells in one or more layers and without pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, which has a pyknotic nucleus and/or is surrounded by disorganized granulosa cells, which are separated from the basement membrane [Telfer et al., 2008]. Overall, 100-160 follicles were evaluated for each treatment. The percentages of healthy primordial and developing follicles were calculated before (uncultured control) and after culture in a given medium.

2.5 Stromal cell density and collagen fibers analysis

To assess the density of ovarian stromal cells, serial sections (7 µm thick) were stained with hematoxylin and eosin before and after culture in the presence of *Lippia sidoides* essential oil. The analysis was performed by calculating the number of stromal cells in a 100 µm² area. For each treatment, ten fields from sections of five different animals were evaluated. The mean number of stromal cells per field was calculated as previously described [Cavalcante et al., 2019]. All assessments and measurements were performed by a single operator. The evaluation of the distribution of collagen fibers in the extracellular matrix was performed using Picrosirius Red (Abcam Kit) according to the methodology described by Rittié [2017]. Briefly, 6.0µm ovarian sections were deparaffinized in xylene and incubated in Sirius Red solution (0.1%) for 1 hour at room temperature. After removal of excess dye with an acetic acid solu-

tion (0.5%), the sections were dehydrated and evaluated under an optical microscope (Nikon, Eclipse, TS 100, Japan) at 400x magnification. For each treatment, the percentage of area occupied by collagen fibers in ten different fields was measured using a DS Cooled Camera Head DS-Ri1 coupled to a microscope (Nikon, Eclipse, TS 100, Japan) and the images were analyzed by Image J software (Version 1.51p, 2017). After staining, collagen fibers were stained in red by picrosirius, while follicles remained unstained. Image J software was used to quantify the percentage of collagen fibers in uncultured and cultured tissues. The intensity of collagen fiber staining was determined by measuring the average pixel intensity of the total photographed area after subtraction of the background.

2.5 Expression of mRNA for SOD, CAT, PRDX6 and GPX1 in ovarian tissue

For analysis of gene expression, ovarian tissues were stored at -80ºC immediately after the end of the culture period until the extraction of total RNA for further analysis of expression of SOD, CAT, PRDX6 and GPX1 mRNAs. Tissues cultured in control medium (α-MEM+) and those cultured in medium supplemented with 400 or 800 μg/mL *Lippia sidoides* essential oil, i.e., treatment that improved follicle growth and collagen fiber were selected to investigate mRNA expression. To this end, ovarian tissues were macerated using scalpels n.22 under sterile conditions and then subjected to extraction of total RNA, by using a Trizol® purification kit (Invitrogen, São Paulo, Brazil) in accordance with the manufacturer's instruc-tions. Quantification of mRNA was performed using SYBR Green. PCR reactions were com-posed of 1μL cDNA as a template in 7.5μL of SYBR Green Master Mix (PE Applied Biosys-tems, Foster City, CA, USA), 5.5μL of ultra-pure water, and 0.5μM of each primer. The primers were designed to perform amplification of SOD, CAT, PRDX6, GPX1 and GAPDH (Table 1). The specificity of each primer pairs was confirmed by melting curve analysis of PCR products. The thermal cycling profile for the first round of PCR was initial denaturation and activation of the polymerase for 10 min at 95°C, followed by 40 cycles of 15 sat 95°C, 30 sat 58°C, and 30 sat 72°C. The final extension was for 10 min at 72°C. All reactions were per-formed in a Step One Plus instrument (Applied Biosystems, Foster City, CA, USA). The 2^ΔΔCt method was used to transform the Ct values into normalized relative expression levels [Livak and Schmittgen, 2001].

Table 1 – Primers pairs used for real-time PCR.

Target gene	Primer sequence (5' → 3')	Sense (S), anti-sense (As	GenBank accession no.
GAPDH	TGTTTGTGATGGGCGTGAACCA	S	GI: 402744670
	ATGGCGCGTGGACAGTGGTCATAA	As	
PRDX6	GCACCTCCTCTTACTTCCCG	S	GI: 59858298
	GATGCGGCCGATGGTAGTAT	As	
GPX1	AACGTAGCATCGCTCTGAGG	S	GI:156602645
	GATGCCCAAACTGGTTGCAG	As	
SOD	GTGAACAACCTCAACGTCGC	S	GI: 31341527
	GGGTTCTCCACCACCGTTAG	As	
CAT	AAGTTCTGCATCGCCACTCA	S	GI:402693375
	GGGGCCCTACTGTCAGACTA	As	

2.6 Total proteins (Bradford method)

The protein concentration was determined by using the Bradford method. This method uses Coomassie blue (Quick start/Bradford; Catalogue No. 500-0205; Bio-Rad) to investigate the total concentration of proteins in each extract sample. In contact with proteins, the Coomassie blue stain forms a complex and emits a blue luminescence. The absorbance is directly related to the protein concentration of the sample and evaluated spectrophotometrically at a wavelength of 595 nm. The total protein concentration in samples of ovarian tissues was determined using a standard curve of bovine albumin (0, 2.5, 5, 10, 15, 25, 35 and 50 mg/mL), which was used to standardize the levels of pro-oxidants (thiol) and antioxidants (SOD, CAT, GPX), as described below.

2.6.1 Determination of pro-oxidant based on the thiol content.

In the samples of ovarian tissues, total thiol content was determined using 5,5`-dithiobis 2-nitrobenzoic acid (DTNB; D8130; Sigma-Aldrich Chem. Co, St Louis, USA) as an index of reduced thiol molecules. Thiol residues react with DTNB (10 mM), cleaving the di-sulfide bond

to form 2-nitro-5-thiobenzoate anion (NTB2–) at a neutral pH. NTB2– is quantified in a spectrophotometer by measuring absorbance at 412 nm, with results expressed as nMol of reduced DTNB per milligram of protein [Ellman, 1959]

2.6.2 Antioxidant activity of SOD, CAT and GPX1

Each 100 mg of ovarian tissue was homogenized 3 to 4 times, for 10 seconds at 4ºC, in 900 µL of buffer (10mM Tris-HCl, 0.9% NaCl (w/v), pH 7.4), containing inhibitors of protease (5mg/mL aprotinin and 34.8mg/mL PMSF) using an Ultraturrax homogenizer T25. The samples were centrifuged for 10 min at 4ºC and 720 G of speed. The supernatant was used to test the activity of the enzymes specified below, after determining the protein content of the sample using the Bradford method [Ellman, 1959]

The SOD activity was measured as the inhibition of adrenaline auto-oxidation [Bannister and Calabrese,1987]. Adrenaline oxidation, in the presence of CAT in basic medium, leads to the formation of O2– radical, which SOD reacts with, thus inhibiting the oxidation of adrenaline. The CAT solution (0.048 mg/mL; c9322; Sigma-Aldrich Chem. Co St Louis, USA) was performed adding (7:3) to glycine buffer, pH 10.2 (Dinâmica Química, São Paulo, Brazil). Three different volumes (10, 20 or 40 µL) of ovary homogenate were then added to the solution and then adrenaline (0.218 mg/mL; E4260; Sigma-Aldrich Chem. Co, St Louis, USA) was added to start oxidation. Oxidation was measured at 480 nm every 10 s for 180 s.

The CAT activity was measured as the consumption of H2O2 as a substrate at 240 nm [34]. A solution of H2O2 (152 µL/mL; PH09717RA; Êxodo Científica, Sumaré, Brazil) and phosphate-buffered saline (PBS; pH7.4) was mixed in a quartz cuvette at room temperature, and then 50 µL of the ovary homogenate were added. Every 30 seconds, the consumption of H2O2 was measured twice.

The GPX activity was measured by the oxidation of NADPH. NADPH is consumed by glutathione reductase (GR; G3664; Sigma-Aldrich) to convert oxidized glutathione (GSSG) to reduced glutathione (GSH). In the presence of H2O2, GPX oxidizes GSH to GSSG and reduces the peroxides to alcohols and water. The consumption of NADPH is di-rectly proportional to the consumption of H2O2 and, consequently, to the activity of GPX [Flohé, Guünzler, 1984].

The reactions were prepared by mixing 500 µL potassium phosphate buffer (100 mM), which is composed of 13.6 g/L monobasic potassium phosphate (P0662; Sigma-Aldrich) added to 1.86 g/L EDTA (5 mM; E4884; Sigma-Aldrich; pH 7.4) and 38 µg/mL GR, 3 µg/mL GSH,

and 100 µL homogenate of the ovarian tissue samples for 10 min at room temperature so that GPX cysteine can contact GR and GSH. Finally, 100 µL NADPH and 120 s later, 100 µL of H2O2 were added to the mixture, and NADPH oxidation was measured as the reduction in NAPDH with absorbance at 340 nm, being evaluated every 10 sec for 300 sec [Furtado el at, 2021]

2.7 Statistical analysis

Statistical analysis was carried out using GraphPad Prisma software (9.0). The percentages of normal follicles, primordial follicles and developing follicles in each treatment were evaluated using the Chi-squared test. Data on the distribution of collagen fibers was analyzed using the Kruskal-Wallis test, followed by Dunn's comparison. The levels of mRNA were analyzed and the results of total protein and antioxidant enzyme activity were analyzed by analysis of variance (ANOVA) and the Kruskal-Wallis test. Comparisons between groups were made using two-way analysis of variance (ANOVA). Differences were statistically significant when $P<0.05$.

3. Results

3.1 Chemical composition of Lippia sidoides essential oil.

The components of LSEO, their percentages and retention index (RI) are listed in table 2. The constituents were identified by GC/MS and represented as 100.0% LSEO. The oxygen monoterpenes represented 63.3%, while hydrogenated monoterpenes were found in lower percentages (36.7%). Thymol (54.5%) and para-Cymene (27.5%) were the major components identified in LSEO.

Table 2. Chemical constituents of *Lippia sidoides* essential oil identified by GC-MS and their respective contents**.**

Constituents	*K_{IC}	**K_{LIT}	Content (%)
Oxygen monoterpenes			**63.3**
1,8-Cineole	1031	1031	1.25
β-Linaloo	1101	1096	4.55
Thymol	1290	1290	54.51
Carvacrol	1298	1290	2.99
Hydrogenated monoterpenes			**36.7**
α-Pinene	940	939	1.97

α- Fenchene	953	952	0.99
Mircene	994	990	0.88
p – Cymene	1025	1024	27.52
Limonene	1028	1024	1.95
y-Terpinen	1059	1059	3.39
Total			100.0

*K_{IC} - calculated Kovats index; ** *K_{LIT} - Kovats index litetature

3.2. Effects of Lippia sidoides essential oil on follicular morphology after in vitro culture

After a 6-day culture period, a significant decrease in the percentage of normal follicles was observed in tissues cultured in all treatments compared to uncultured tissues ($P < 0.05$). The presence of 400 and 800 µg/mL LSEO in culture medium increased the percentage of morphologically normal follicles when compared to tissues cultured in the control medium. In addition, ovarian tissues cultured with 1.600 µg/mL or 3.200 µg/mL did not differ from those cultured in control mediun (Figure 1a).

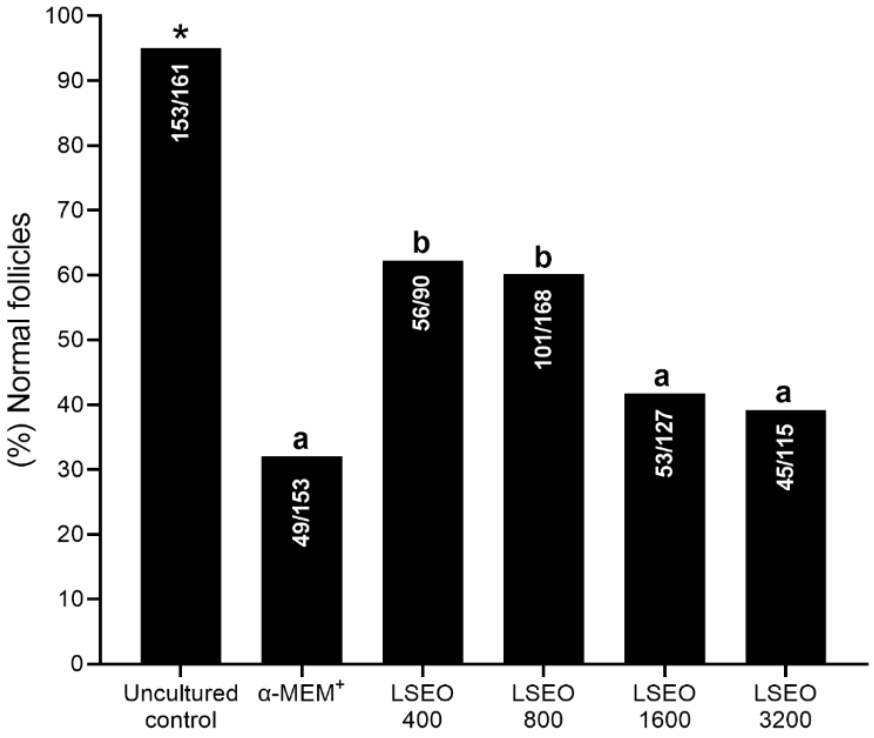

Figure 1. Percentages of normal follicles (A) inuncultured tissues and in tissues cultured for 6 days in control medium alone or supplemented with 400, 800, 1,600 or 3,200 µg/mL *Lippia sidoides* essential oil. a,b,c Lowercase letters represent statistically significant differences between treatments ($P < 0.05$).

3.3 Effects of Lippia sidoides essential oil on activation and development of primordial follicles

Ovarian tissues cultured in all treatments showed a reduction in the percentage of primordial follicles and increase of developing follicles when compared to uncultured tissues. However, the presence of 400µg/mL LSEO in the culture medium significantly increased the percentage of developing follicles when compared to tissues cultured in the control medium alone or supplemented with 800, 1,600 and 3,200 µg/mL LSEO. Additionally, tissues cultured with (800, 1,600 and 3,200 µg/mL LSEO also showed a higher percentage of developing follicles than thoseobserved in tissues cultured in control medium (Figure 2a).

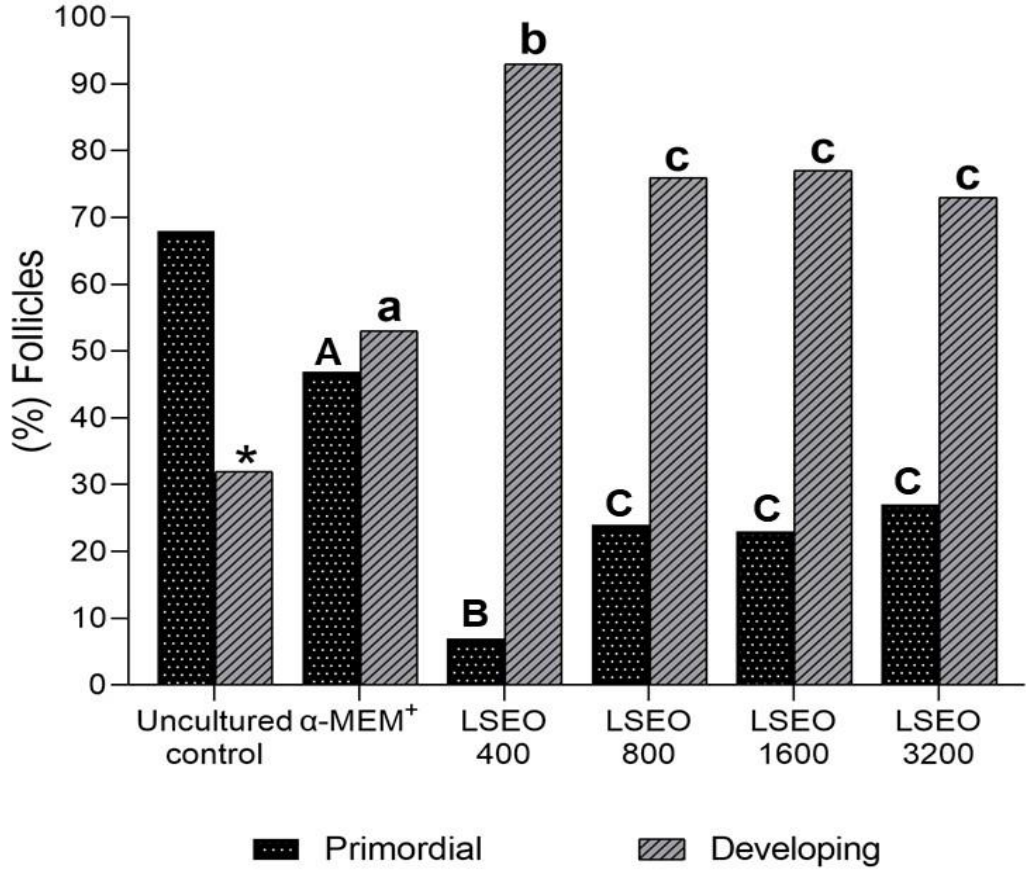

Figure 2. Percentages of primordial and developing follicles (A) in-uncultured tissues and in tissues cultured for 6 days in control medium alone or supplemented with 400,800, 1,600 or 3,200 µg/mL LSEO. aA,bB,cC Lowercase letters represent statistically significant differences between treatments ($P < 0.05$).

3.4 Effects of Lippia sidoides essential oil on stromal cell density after in vitro culture

After 6 days of culture, a significant reduction in the density of stromal cells ($P < 0.05$) was observed in the tissues cultured in all treatments when compared to the uncultured tissues

(Fig. 3a). However, the presence of 400 µg/mL of LSEO in the culture medium increased the density of stromal cells compared to the tissues cultured in the control medium ($P < 0.05$). In addition, 800 and 1,600 µg/mL of LSEO did not influence the density of stromal cells, but a reduction in the number of cells was observed in tissues cultured with 3,200 µg/mL of LSEO (Figure 3ab).

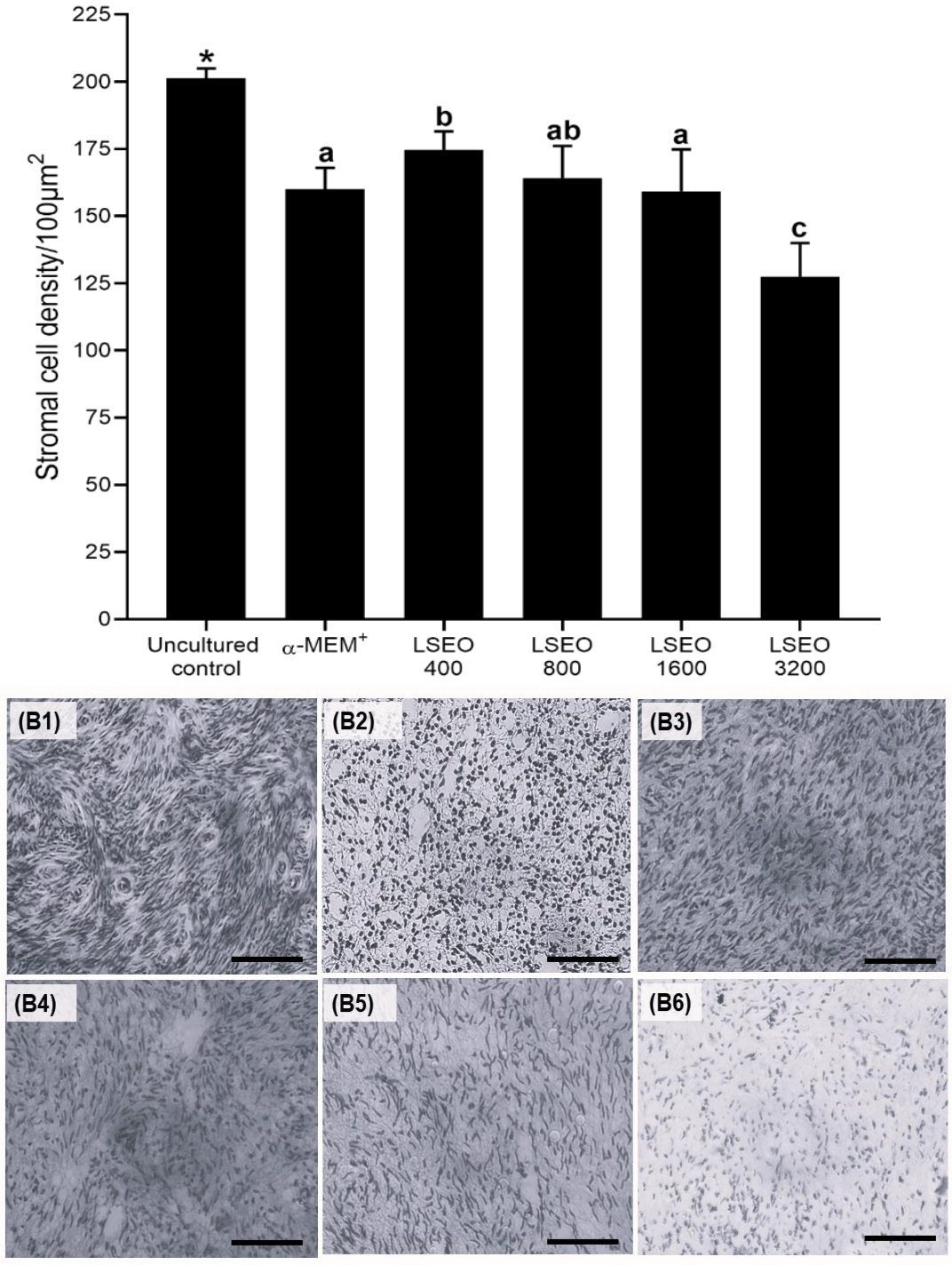

Figure 3. Stromal cell denity (cells/100 µ2) in (A) in uncultured tissues and in tissues cultured for 6 days in control medium alone or supplemented with 400,800, 1,600 or 3,200 µg/mL LSEO. a–c. Different lowercase letters indicate statistically significant differences between treatments.

(B) Representative images stromal ovarian in uncultured tissues (B1) and in tissues cultured for 6 days in control medium alone (B2) or supplemented with 400,800, 1600 or 3200 µg/mL LSEO (B3-B6). Scale bars: 100 µm

3.5. Assessment of collagen fibers in ovarian extracellular matrix

Figure 4 shows a significant reduction in collagen fibers in tissues cultured in all treatements ($P < 0.05$) when compared to non-cultured tissues. However, ovarian tissues cultured with 400µg/mL of *Lippia sidoides* essential oil had graeater percentages of collagen fibers than tissues cultured in control medium ($P < 0.05$). In addition, 800, 1,600 and 3,200 µg/mL *Lippia sidoides* essential oil did not influence collagen fibers (Figure 4a).

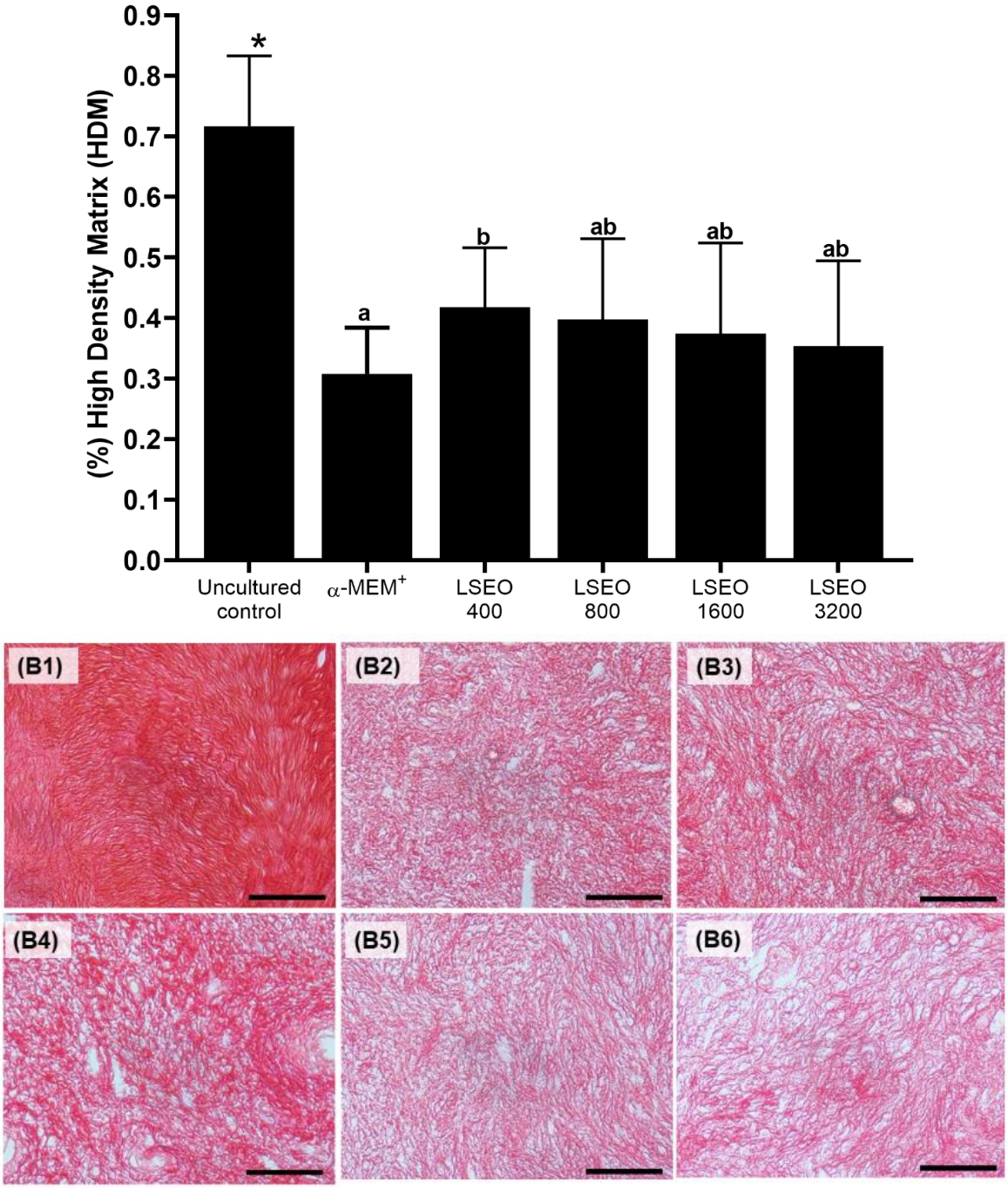

Figure 4. (A) Percentages of collagen fibers in uncultured tissues and in tissues cultured for 6 days in control medium alone or supplemented with 400, 800, 1,600 or 3,200 *Lippia sidoides* essential oil. a–c. Different lowercase letters indicate statistically significant differences between treatments ($P < 0.05$).(B) Representative images of collagen fibres in uncultured tissues (B1) and in tissues cultured for 6 days in control medium alone (B2) or supplemented with 400,800, 1,600 or 3,200 *Lippia sidoides* essential oil (B3-B6) Scale bars: 100 µm

3.*6 Levels of mRNA for SOD, CAT, PRDX6 and GPX1 in cultured tissues*

Figure 5 shows the mRNA levels for *SOD, CAT, PRDX6* and *GPX1* in bovine ovarian tissues after 6 days of culture. The presence of 400 µg/mL of LSEO in the culture medium increased the mRNA levels for SOD, CAT and GPX1 when compared to tissues cultured in control medium ($P < 0.05$). In addition, 800 µg/mL of LSEO also increased the mRNA levels for GPX1. On the other hand, LSEO did not influence the expression of mRNA for PRDX6 in cultured ovarian tissues.

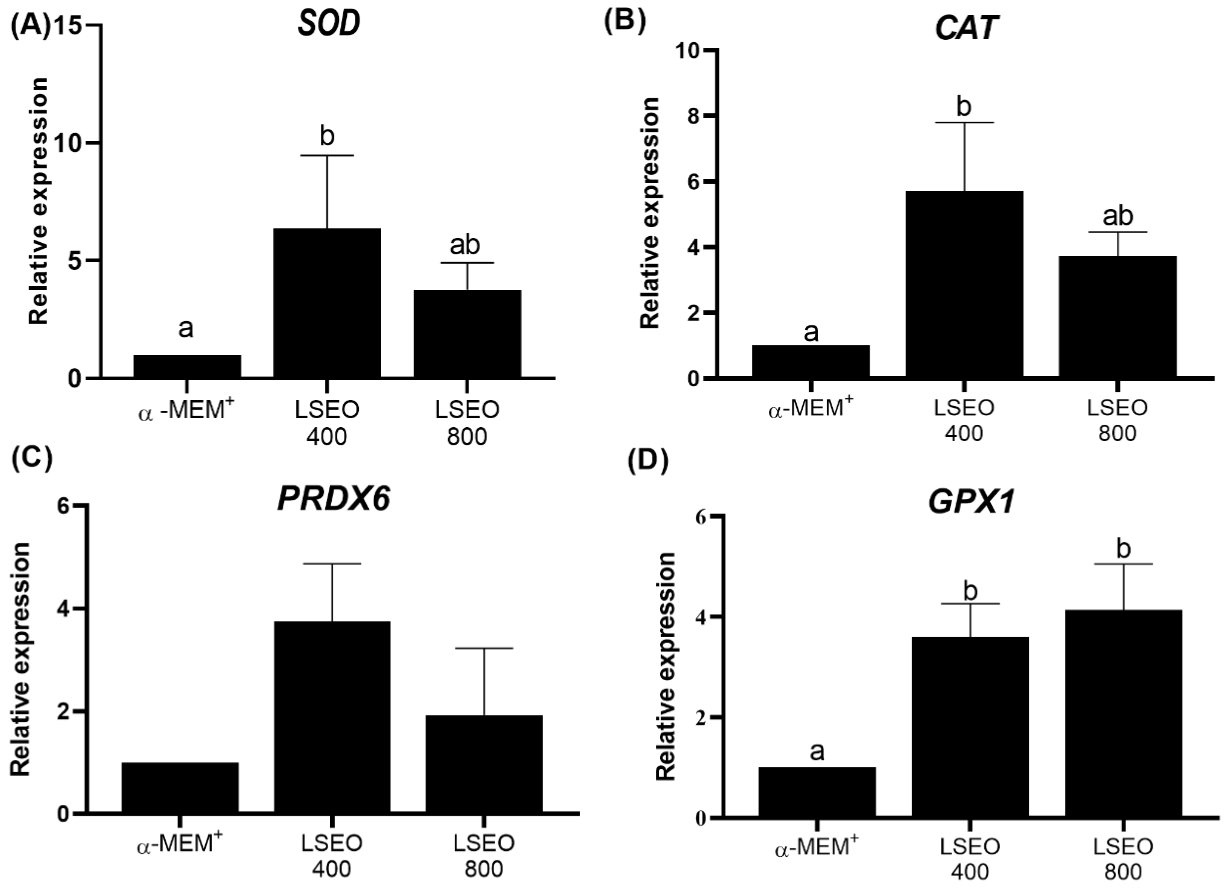

Figure 5. Levels de mRNA(means ± standard deviation (SD) for (A) *SOD*, (B) *CAT*, (C) *PRDX6* and (D) *GPX1* in tissue ovarian cultured *in vitro* for 6 days in control mediun alone or supplemented with 400,800,1.6000 0r 3.200 µg/mL of LSEO. a,b,c Lowercase letters represent statistically significant differences between treatments ($P < 0.05$)

3.7 Levels of thiol and activity of SOD, CAT, *and* GPX1 *enzymes*

Figure 6 shows the levels of thiol and activity of SOD, CAT, and GPX1 enzymes in bovine tissue ovarian after 6 days of culture in control medium alone or supplemented with LSEO. The presence of 400 µg/mL *Lippia sidoides* essential oil in culture medium significantly increased the activity for GPX1 in tissue ovarian when compared with those cultured in control medium ($P < 0.05$). However, the levels of thiol and activity of CAT and SOD were not influenced by LSEO.

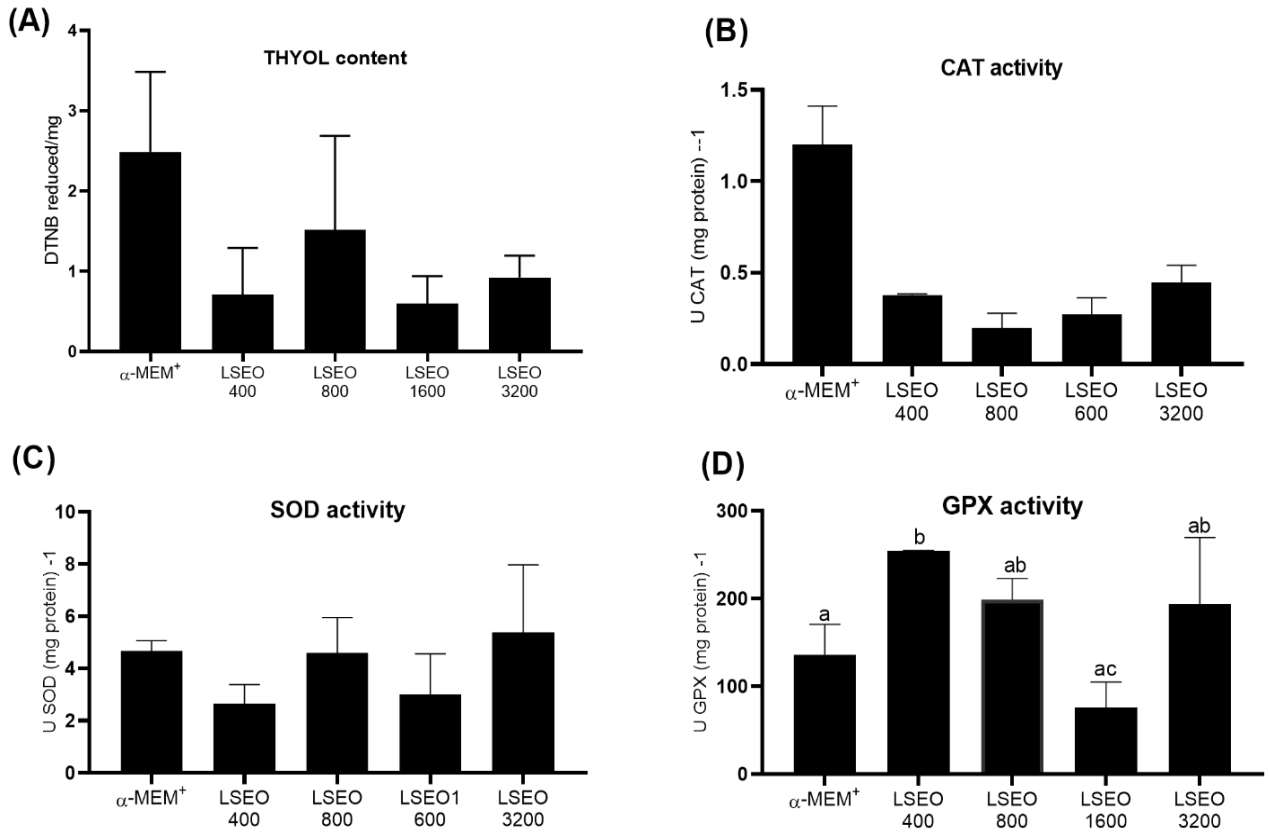

Figure 6. Levels of activity (means ± standard deviation (SD)) for (A)Thiol (B) CAT, (C) SOD and (D) GPX1 in tissue ovarian cultured *in vitro* for 06 days in control mediun alone or supplemented with 400, 800, 1,600 or 3,200 µg/mL *Lippia sidoides* essential oil(LSEO). a,b,c. Lowercase letters represent statistically significant differences between treatments ($P < 0.05$)

3. Discussion

Essential oils are important biotechnological products made up of a complex mixture of organic compounds that can be used to supplement culture media and, consequently, improve in vitro cell culture systems [Vasconcelos et al., 2020]. In this context, identifying the chemical

constituents of essential oils is an important step in evaluating a given biological response under in vivo or in vitro conditions [Caesar and Cech.,2019]. In our research, the composition of OELS indicated the presence of 63.3% oxygenated compounds. This complex mixture of organic compounds can act synergistically or antagonistically, modulating biological activity [Huang et al., 2021].

We demonstrated for the first time that, during the in vitro culture of ovarian tissue, LSEO increased the activation, development and survival rates of follicles (62 to 92%) compared to the control, but 400 and 800µg/mL had a better influence on morphology (+60%) and these results may be associated with an improvement in the maintenance of the redox balance of the cell and tissue culture system at low OELS concentrations [Sá et al., 2018]. Studies have shown the positive effect of extracts and essential oils from the Lippia genus at low concentrations on the in vitro viability of different cell cultures [Pereira, 2015; Sollecito et al., 2019]. Supplementing our findings, Yang et al. [2020] reported that the development of primordial follicles to the secondary stage can only be achieved by culturing intact ovarian cortex, which requires the optimization of in vitro culture conditions [Sá et al., 2018]. This protective activity of OELS may be strongly related to the presence of phenols, such as thymol (54.5%), which has been shown to be responsible for the antioxidant activity of many essential oils [Ruberto and Baratta, 2000].

This study shows that 400 µg/mL of LSEO increased the density of stromal cells in cultured ovarian tissues. This fact may have contributed to maintaining the percentage of morphologically normal follicles, since a good culture microenvironment must also maintain healthy stromal cells, allowing interaction between the stromal and follicular compartments [Barbato et al., 2023]. In fact, follicular development is strongly influenced by ovarian stromal cells, which support the tissue and perform several important functions [De Assis et al., 2022]. Mbemya et al. [2017] demonstrated the relationship between follicular morphology and stromal cell density during caprine ovarian tissue culture. Interestingly, 400 µg/mL of LSEO increased collagen fibers in the cultured tissues. The ECM is composed of a network of collagen-rich proteins, polysaccharides and water [Alamgeer et al., 2019] that together perform a variety of cellular functions in many tissues, including the ovary, regulating follicular development from signaling cascades strongly influenced by biochemical and biomechanical properties [Zhao et al., 2023]. In addition, maintaining the microarchitecture of the ECM provides a permissive microenvironment that regulates cell development, steroidogenesis, regulation of cell aggregation, morphology and cell-cell and cell-tissue communication [Grosbois et al., 2023;

Theocharis et al., 2016]. Thus, our results show that LSEO improves the relationship between stromal density and ECM remodeling during follicular development in bovine ovarian tissue cultured in vitro, which is in line with previous studies [Franchi et al., 2020; Grobois et al., 2023].

The present research indicates strong evidence that the positive effects of LSEO depend on its antioxidant activity. To this end, the thiol level was analyzed. In biological systems, the oxidation of the thiol group on protein residues is crucial for redox signaling, a process by which the biological system responds to changes in EROS levels [Jones, 2013]. In our study, no changes in thiol content were observed in the LSEO treatments. Therefore, it is unlikely that protein dysfunction caused by reversible thiol oxidation occurred during ovarian tissue culture. However, this result does not exclude the possibility that specific signal transduction proteins, transcription factors or compartmentalized proteins may have acted as second messengers in the EROS signalling pathways. Thus, LSEO contributed to a less oxidizing environment, which is corroborated by [Saccol et al., 2013; Reyes-Becerril et al., 2021], who analyzed the beneficial effects of essential oils from the Lippia genus in controlling oxidative stress.

We found that 400 µg/mL of LSOE increased the expression of GPX1. SOD catalyzes the dismutation of the extremely reactive superoxide anion into H2O2, which can subsequently be separated into water and oxygen by CAT or GPX1 [Kurutas, 2016] The last two enzymes act to prevent the accumulation of hydrogen peroxide. This integrated action is an important inhibitor of OH°, thus reducing levels of oxidative stress [Barbosa et al., 2010]. The influence of essential oils from plants of the Lippia genus and their main components on GPX concentrations has been reported previously [Reyes-Becerril et al., 2021; Teixeira et al., 2014; Costa et al., 2022, Güvenç et al., 2019]. These studies have shown its benefits for the in vitro maturation of bovine oocytes [Pereira, 2015] and for improving sperm quality parameters [Güvenç et al., 2019]. In addition, the higher expression of GPX1 in bovine ovarian tissue may be associated with follicle survival, since GPX1 is significantly increased in healthy follicles compared to atretic follicles [Ceko et al., 2015] which is in line with the results found in our morphological analysis.

In conclusion, 400µg/mL LSEO increases follicular growth, survival, stromal cell density and collagen fibers in cultured ovarian tissues, as well as mRNA levels for SOD, CAT and GPX1 and GPX1 enzyme activity

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Statement of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Ethical Standards

The authors declare that all procedures were performed according to national and institutional guides on the care and use of animals.

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7. CAPITULO II

Thymol increases primordial follicle survival and growth, stromal cells density and down-regulates expression of mRNA for superoxide dismutase, catalase and periredoxin 6 in cultured bovine ovarian tissues.

Thymol increases primordial follicle survival and growth, stromal cells density and down-regulates expression of mRNA for superoxide dismutase, catalase and periredoxin 6 in cultured bovine ovarian tissues.

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Abstract

This study aims to evaluate the effects of thymol on primordial follicle activation, development and survival, as well as on distribution of collagen fibers and stromal cells density in bovine ovarian tissues cultured *in vitro*. The activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), the thiol levels and the expression of mRNAs for superoxide dismutase (*SOD)*, catalase *(CAT)*, periredoxin 6 (*PRDX6*) and Glutathione peroxidase 1 (*GPX1*) were also investigated. Ovarian fragments were cultured in α-MEM$^+$ alone or supplemented with thymol (400, 800, 1.600 or 3.200 µg/mL) for a period of six days. The results show that, except for the concentration of 3.200 µg/mL, the presence of thymol in culture medium increased the percentage of morphologically normal follicles when compared to tissued cultured in control medium. Thymol, at all concentrations tested also increased primordial follicle activation rate. In addition, 400 µg/mL thymol increased collagen fibers and stromal density of collagen fibers when compared to tissues cultured in control medium. The presence of 800 µg/mL thymol in culture medium increased CAT activity, but reduced that of SOD. Thymol (400 or 800 µg/mL) reduced mRNA levels for *SOD, CAT* and *PRDX6* but did not alter *GPX1* expression in cultured ovarian tissue. In conclusion, thymol increases primordial follicle survival and growth, stromal cells density and down-regulates expression of mRNA for superoxide dismutase, catalase and periredoxin 6 in cultured bovine ovarian tissues.

Keywords: thymol; bovine species, ovarian tissue, antioxidant; *in vitro* culture

1. Introduction

In vitro culture of ovarian cortex rich in primordial follicles has been strategic for studying primordial follicle development, as well as to understand the factors that regulate early follicular development (Figueiredo et al., 2007; Araújo et al., 2014). Despite a massive growth of primordial follicles after a culture period of six days (Telfer et al., 2008), several studies have described an increase in degeneration rate during culture (Araújo et al., 2010; Araújo et al, 2021). This fact may be due to excessive formation of free radicals by the cellular oxidative metabolism by different cell types enclosed in ovarian tissues. Oxidative stress is characterized by an imbalance between the generation of oxidant and antioxidant compounds at the cellular level that results in the formation of free radicals and reactive oxygen species (ROS). This process leads to oxidation of biomolecules with consequent loss of their biological functions (Halliwel, Whiteman, 2004; Sies, Berndt, Jones, 2017). For these reasons, there is a need to improve *in vitro* culture systems for ovarian tissues, especially in domestic species, in which complete follicle development occurs over a period of approximately six months (Paulino et al., 2022).

Previous studies have shown that thymol scavenges free radicals by enhancing the activities of superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione-S-transferase (GST) and by reducing glutathione (GSH) in rat myocardial cells (Meeran and Prince, 2012). In addition, thymol has SOD-like activity in removing superoxide radicals in fibroblasts *in vitro* (Kruk et al., 2000; Undeger et al., 2009). Archana et al. (2011) showed that thymol has potent antioxidant activity, modulating enzymatic antioxidant activities and decreasing lipid peroxidation in gamma ray-induced Chinese hamster V79 cells. Furthermore, thymol attenuated H_2O_2-induced oxidative stress in Caco-2 cell line (Cabello et al., 2015) and in human neutrophils (Perez-Roses et al., 2016). In addition to these results, Costa et al. (2019) demonstrated that thymol improves wound healing by promoting the survival and growth of fibroblasts and keratinocytes. In parallel, it was revealed that thymol stimulates extracellular matrix (ECM) mineralization during bone formation (Trzaskowska et al.,2023). Furthermore, Mahran et al. (2019) reported that thymol improves total antioxidant capacity in ovaries of rats irradiated with whole-body gamma radiation. However, until now, the effects of adding thymol of ovarian tissue in the bovine species are still unknown.

So, the present study aimed to evaluate the effect of adding thymol as a supplement in the culture medium of bovine ovarian tissue on morphological parameters, of follicular viability,

activation, evaluation extracellular matrix, the thiol levels and activity of SOD, CAT and GPX, futhermore mRNA expression for antioxidant genes (*SOD*, *CAT*, *PRDX6* and *GPX1*) after culture *in vitro* for 6 day.

2. Material and methods

2.1 Chemicals

Unless otherwise stated, thymol and the culture media and other chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.2 Source of ovaries

Bovine ovaries (n = 24) of mixed breed cows were collected at a local slaughterhouse. Immediately after death, each pair of ovaries was washed in ethanol (70%) for approximately 10 s, followed by two washes in 0.9% saline solution supplemented with penicillin (100 µg/mL) and streptomycin (100 µg/mL). After washing, each pair of ovaries was individually transported to the laboratory in falcon tubes containing α-MEM supplemented with penicillin (100 µg/mL) and streptomycin (100 µg/mL) at 4ºC, within 1 hour. This study was approved by the Committee of Ethics and Animal Welfare of the Federal University of Ceará (Nº 09/2021).

2.3. In vitro culture of ovarian tissue

In the laboratory, ovarian cortical tissues from the same ovarian pair (n = 12 pairs) was cut in sixty-eight slices (3 mm × 3 mm × 1 mm) in dissection medium composed of α-MEM supplemented with penicillin (100 µg/mL) and streptomycin (10µg/mL). For each animal, two cortical slices were fixed in paraformaldehyde (4%) for 24 hours at 4ºC for histological analysis (uncultured control), as well as for analysis of the extracellular matrix and stromal cells. The remaining fragments were cultured in 24-well culture dishes for 6 days (Passos et al., 2016; Silva et al., 2017). Culture was performed at 38.5°C in 5% CO_2 in a humidified incubator. The basic culture medium consisted of α-MEM (pH 7.2–7.4) supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, and 5 ng/ml selenium), 2 mM glutamine, 2 mM hypoxanthine, antibiotics (100 IU/mL penicillin and 10 mg/mL streptomycin) and 1.25 mg/ml of bovine serum albumin (α-MEM$^+$). The fragments were cultured in 0.5 mL of control medium (α-MEM$^+$)

alone or supplemented with different concentrations of thymol (400, 800, 1.600 or 3.200 μg/mL). The concentrations of thymol were chosen according to Costa et al. (2018). Every 2 days, 60% of the culture medium was replaced with fresh medium. Each treatment was repeated 10 times. At the end of the culture period, the fragments were to evaluate follicle morphology, ECM, stromal cell density, as well as the activity of antioxidant enzymes, thyol levels and expression of mRNA by polymerase chain reaction (PCR).

2.4 Morphological analyses and assessment of in vitro follicular growth

Histological analysis was performed according to the methodology of Bizarro-Silva et al. (2018). Fresh control (D0) and cultured ovarian fragments were fixed in paraformaldehyde (4%) for 24 hours, dehydrated in increasing concentrations of ethanol, cleared with xylol and included in paraffin. Serial sections (7 μm thickness) were performed and, each 5th section, stained with hematoxylin and eosin. Only preantral follicles with oocytes nucleus in the section were analyzed to avoid double counting. Coded anonymized slides were examined under a microscope (Nikon, Tokyo, Japan) at ×100 and ×400 magnification. The developmental stages of follicles were classified as primordial or developing follicles (Figueiredo; Lima, 2017). These follicles were further classified as morphologically normal when an intact oocyte was present, surrounded by granulosa cells that were well organized in one or more layers, and had no pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, which has a pyknotic nucleus and/or is surrounded by disorganized granulosa cells, which are detached from the basement membrane (Telfer et al., 2008). The percentages of healthy primordial and developing follicles were calculated before (uncultured control) and after culture in a particular medium.

2.5 Analysis of collagen fibers in extracellular matriz

To evaluate collagen fibers distribution in ECM, ovarian cortical tissues were stained with Picrosirius Red (Abcam Kit), according to the methodology described by Rittié (2017). Briefly, 6.0μm ovarian sections were dewaxed in xylene and incubated in Sirius Red solution (0.1%) for 1 hour at room temperature. After removal of excess dye with acetic acid solution (0.5%), the sections were dehydrated and evaluated under an optical microscope (Nikon, Eclipse, TS 100, Japan) at 400x magnification. For each treatment, the percentage of area

occupied by collagen fibers in ten different fields was measured with the aid of a DS Cooled Camera Head DS-Ri1 coupled to a microscope (Nikon, Eclipse, TS 100, Japan) and the images were analyzed by Image J Software (Version 1.51p, 2017). After staining, the collagen fibers were marked in red by picrosirius, while the follicles remained uncolored. The Image J Software was used to quantify the percentage of collagen fiber in uncultured and cultured tissues. The staining intensity of collagen fibers was determined by measuring the average pixel intensity of the total area after background subtraction.

2.6 Ovarian stromal cell density

Ovarian stromal cell density before and after culture in the presence of thymol was evaluated by calculating the stromal cell number in an area of 100 μm2. For each treatment, ten fields of sections of five different animals were assessed. The mean number of stromal cells per field was calculated as described previously (Cavalcante et al., 2019). All evaluations and measurements were performed by a single operator.

2.7 Quantification of messenger RNAs for SOD, CAT, GPX1 and PRDX-6

For analysis of gene expression, ovarian tissues were stored at -80ºC immediately after the end of the culture period until the extraction of total RNA for further analysis of expression of *SOD, CAT, PRDX6* and *GPX1* mRNAs. Tissues cultured in control medium (α-MEM^{+}) and those cultured in medium supplemented with 400 or 800 μg/mL were selected to investigate mRNA expression. To this end, ovarian tissues were macerated and then subjected to extraction of total RNA, by using a Trizol® purification kit (Invitrogen, São Paulo, Brazil) in accordance with the manufacturer's instructions. Quantification of mRNA was performed using SYBR Green. PCR reactions were composed of 1 μL cDNA as a template in 7.5 μL of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA, USA), 5.5 μL of ultra-pure water, and 0.5 μM of each primer. The primers were designed to perform amplification of *SOD, CAT, PRDX6, GPX1* and *GAPDH* (Table 1). The specificity of each primer pairs was confirmed by melting curve analysis of PCR products. The thermal cycling profile for the first round of PCR was initial denaturation and activation of the polymerase for 10 min at 95°C, followed by 40 cycles of 15 sat 95°C, 30 sat 58°C, and 30 sat 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a Step One Plus instrument (Applied Biosystems, Foster City,

CA, USA). The 2^ΔΔCt method was used to transform the Ct values into normalized relative expression levels (Livak and Schmittgen, 2001).

Table 1 – Primers pairs used for real-time PCR.

Target gene	**Primer sequence (5' → 3')**	**Sense (S), anti-sense (As**	**GenBank accession no.**
GAPDH	TGTTTGTGATGGGCGTGAACCA ATGGCGCGTGGACAGTGGTCATAA	S As	GI: 402744670
PRDX6	GCACCTCCTCTTACTTCCCG GATGCGGCCGATGGTAGTAT	S As	GI: 59858298
GPX1	AACGTAGCATCGCTCTGAGG GATGCCCAAACTGGTTGCAG	S As	GI:156602645
SOD	GTGAACAACCTCAACGTCGC GGGTTCTCCACCACCGTTAG	S As	GI: 31341527
CAT	AAGTTCTGCATCGCCACTCA GGGGCCCTACTGTCAGACTA	S As	GI:402693375

2.8 Total proteins (Bradford method)

The protein concentration was determined using the Bradford method. This method uses Coomassie blue (Quick start/Bradford; Catalogue No. 500-0205; Bio-Rad) to determine the total concentration of proteins in each extract sample. In contact with proteins, the Coomassie blue stain forms a complex and emits a blue luminescence. The absorbance is directly related to the protein concentration of the sample and evaluated spectrophotometrically at a wavelength of 595 nm. The total protein concentration in samples was determined using a standard curve of bovine albumin (0, 2.5, 5, 10, 15, 25, 35 and 50 mg/mL), which was used to standardize the levels of pro-oxidants (thiol) and antioxidants (SOD, CAT and GPX), as described below.

2.9. Analysis of thiol levels and activity of SOD, CAT and GPX enzymes

Samples of ovarian tissue (100 mg/ mL) were macerated using potassium phosphate buffer (KH2PO4 and K2HPO4; P9791 and P3786; Sigma-Aldrich; 1:9), pH 7.5. The ovary homogenates were centrifuged at 1500g for 10 min at 48C and the supernatant collected for use in the spectrophotometric assays described below, using quartz cuvettes (Genesis 10s UV-vis; Thermo Scientific), as described previously (Ellman, 1959). Data are expressed as the mean s.e.m. enzyme unit per milligram of protein (U/mg protein).

The SOD activity was measured as the inhibition of adrenaline auto-oxidation (Bannister and Calabrese, 1987). Adrenaline oxidation, in the presence of CAT in basic medium, leads to the formation of O2– radical, which SOD reacts with, thus inhibiting the oxidation of adrenaline. The CAT solution (0.048 mg/mL; c9322; Sigma-Aldrich Chem. Co St Louis, USA) was performed by adding Catalase, Andelanine and of ovary homogenate (7:3) to glycine buffer, pH 10.2 (Dinâmica Química, São Paulo, Brazil). Three different volumes (10, 20 or 40 µL) of ovary homogenate were then added to the solution and then adrenaline (0.218 mg/mL; E4260;

Sigma-Aldrich Chem. Co, St Louis, USA) was added to start oxidation. Oxidation was measured at 480 nm every 10 s for 180 s. The CAT activity was measured as the consumption of H_2O_2 as a substrate at 240 nm (Aeibi, 1984). A solution of H_2O_2 (152 µL/mL; PH09717RA; Êxodo Científica, Sumaré, Brazil) and phosphate-buffered saline (PBS; pH7.4) was mixed in a quartz cuvette at room temperature, and then 50 µL of the ovary homogenate were added. Every 30 seconds, the consumption of H_2O_2 was measured twice.

The GPX activity has been measured by the oxidation of NADPH. NADPH is consumed by glutathione reductase (GR; G3664; Sigma-Aldrich) to convert oxidized glutathione (GSSG) to reduced glutathione (GSH). In the presence of H_2O_2, GPX oxidizes GSH to GSSG and reduces the peroxides to alcohols and water. The consumption of NADPH is directly proportional to the consumption of H_2O_2 and, consequently, to the activity of GPX (Flohé et al., 2011). The reactions were prepared by mixing 500 µL potassium phosphate buffer (100 mM), which is composed of 13.6 g/L monobasic potassium phosphate (P0662; Sigma-Aldrich) added to 1.86 g/L EDTA (5 mM; E4884; Sigma-Aldrich; pH 7.4) and 38 µg/mL GR, 3 µg/mL GSH, and 100 µL homogenate of the ovarian tissue samples for 10 min at room temperature so that GPX cysteine can contact GR and GSH. Finally, 100 µL NADPH and 120 s later, 100 µL of H_2O_2 were added to the mixture, and NADPH oxidation was measured as the reduction in

NAPDH with absorbance at 340 nm, being evaluated every 10 sec for 300 sec (Furtado et al., 2021).

Total thiol content was determined using 5,5`-dithiobis 2-nitrobenzoic acid (DTNB; D8130; Sigma-Aldrich Chem. Co, St Louis, USA) as an index of reduced thiol molecules. Thiol residues react with DTNB (10 mM), cleaving the disulfide bond to form 2-nitro-5-thiobenzoate anion (NTB2–) at a neutral pH. NTB2– is quantified in a spectrophotometer by measuring absorbance at 412 nm, with results expressed as nMol of reduced DTNB per milligram of protein (Takarashi et al., 1978)

2.10 Statistical analysis

The percentages of primordial and developing follicles, as well as of those of morphologically normal after 6 days of culture in the different treatments were compared by Chi-squared test (GraphPad Prism 9.0). Data on collagen fiber distribution, stromal cell density, thiol levels, activity of SOD, CAT and GPx enzymes, as well as the levels of mRNA for SOD, CAT, PRDX6 and GPX1 were analyzed by ANOVA and compared by Krukal-Wallis test. Results were expressed as mean ± S.E.M and the differences were considered significant when $P < 0.05$.and Kruskal-Wallis test, followed by Dunn's comparison. The differences were statistically significant when $P<0.05$.

3. Results

3.1 Effects of thymol on activation and development of primordial follicles

The ovarian tissues cultured in all treatments showed a reduction in the percentage of primordial follicles and increase of developing follicles when compared to uncultured tissues. However, the presence of 800µg/mL of thymol in culture medium significantly increased the percentage of developing follicles when compared to tissues cultured in control medium or in other treatments (Figure 1). The other concentrations of thymol (400, 1.600 and 3.200 µg/mL) also showed a higher percentage of developing follicles than those seen in tissues cultured in control medium, but they were similar to each other (Figure 1)

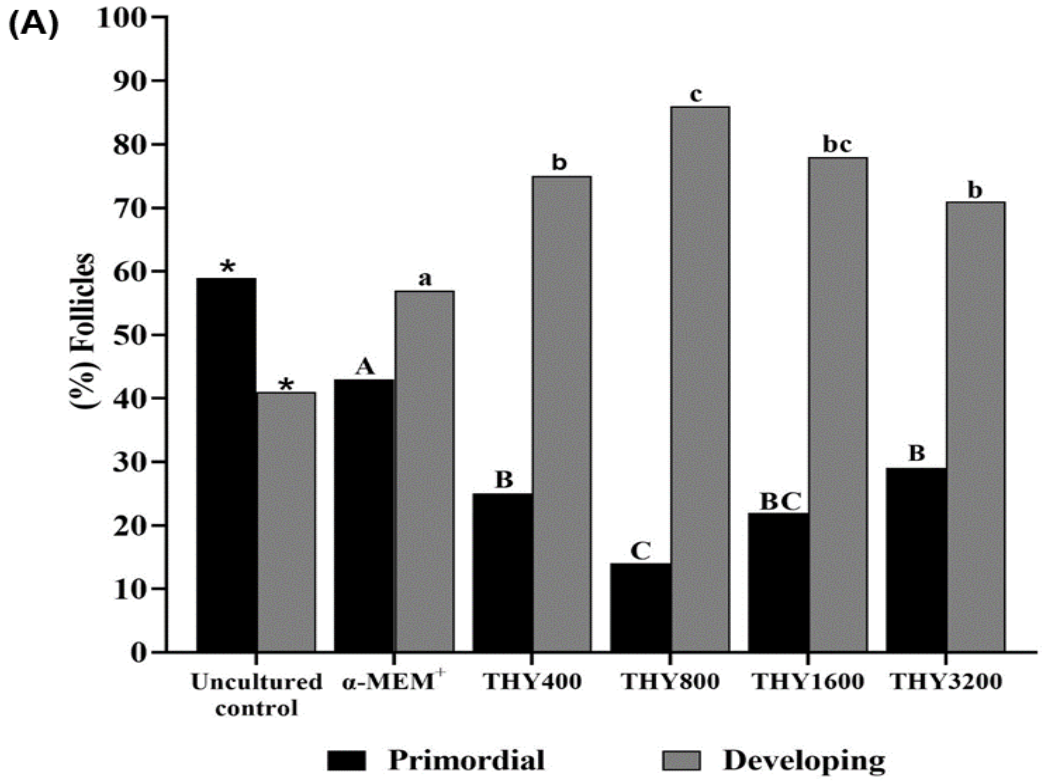

Figure 1. Percentages of primordial and developing follicles in uncultured tissues and in tissues cultured for 6 days in control medium alone or supplemented with 400, 800, 1,600 or 3,200 µg/mL thymol. a–c Different lowercase letters indicate statistically significant differences between treatments (P< 0,05)

3.2 Effects of thymol on percentages of normal follicles after in vitro culture

Figure 2B shows the morphology of normal and degenerated follicles after *in vitro* culture. After 6 days, cultured tissues showed a significant decrease in the percentage of normal follicles in all treatments, when compared with uncultured tissues ($P < 0.05$). The presence of 400 and 800 µg/mL thymol increased the percentage of morphologically normal follicles when compared to tissues cultured in control medium ($P < 0.05$) (Figure 2a).

Ovarian tissues cultured in all treatments showed a reduction in the percentage of normal follicles when compared to uncultured tissues. However, the presence of 400µg/mL thymol in the culture medium significantly increased the percentage of developing follicles when compared to tissues cultured in the control medium or the other treatments. The other concentrations of thymol (800, 1,600 and 3,200 µg/mL) also showed a higher percentage of developing follicles than that observed in tissues cultured in control medium, but were similar to each other (Figure 2a).

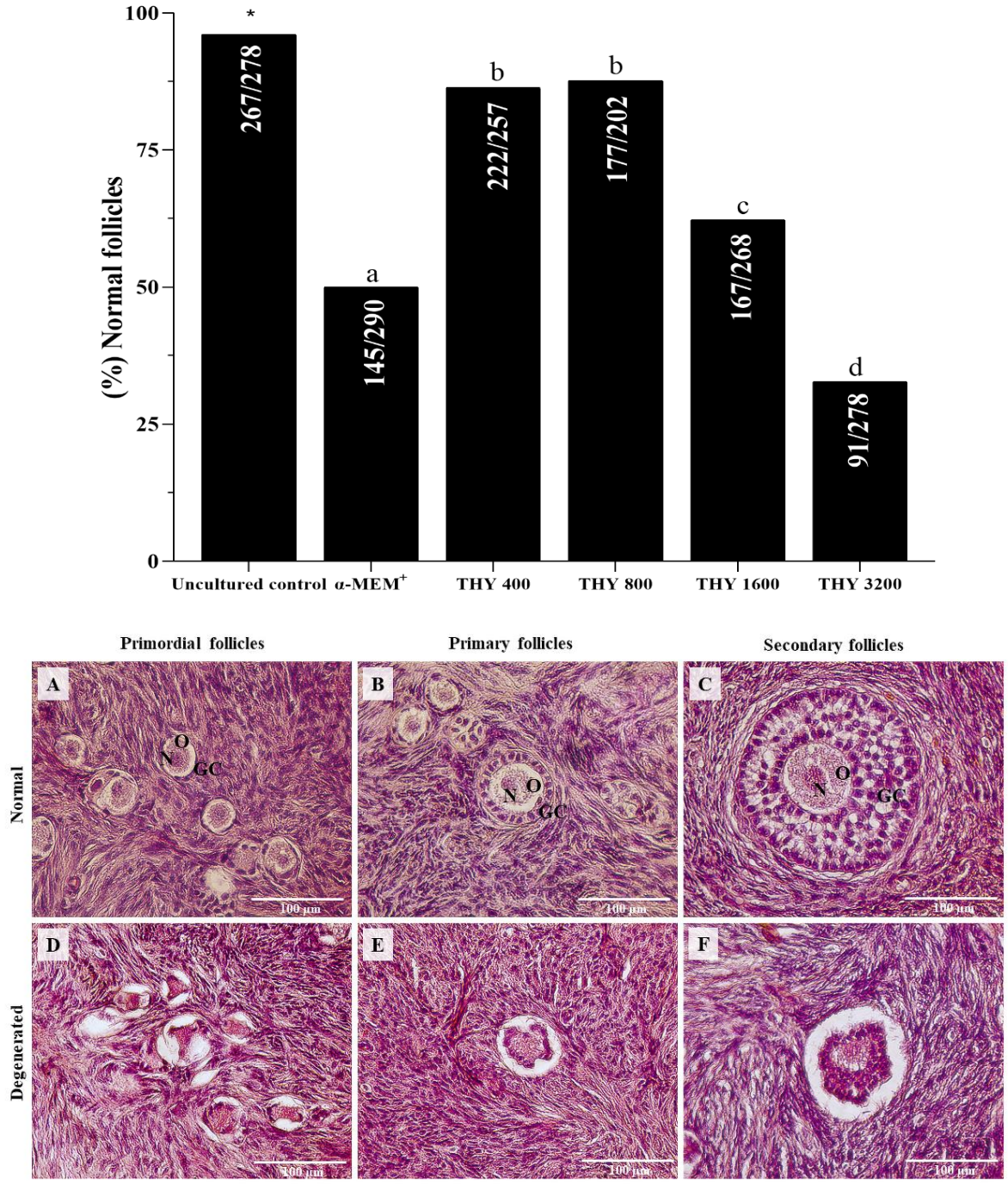

Figure 2. B) Representative images of bovine ovarian tissue sections showing morphologically normal (a-c) and degenerated (d-f) follicles of different categories stained with hematoxylin and eosin. Normal (a) and degenerated (d) primordial follicles; normal (b) and degenerated (e) primary follicles; normal (c) and degenerated (f) secondary follicles. Scale bar: 100 µm.

3.3 Assessment of collagen fibers in ovarian extracellular matrix

Ovarian tissues cultured in all treatments had reduced percentages of collagen fiber when compared with uncultured tissues. However, 400 µg/mL thymol increased the percentages of collagen fibers when compared to tissues cultured in control medium. The other concentrations of thymol (800, 1.600 and 3.200 µg/mL) did not influence collagen fiber distribution ($P < 0.05$) (Figure 3 ab).

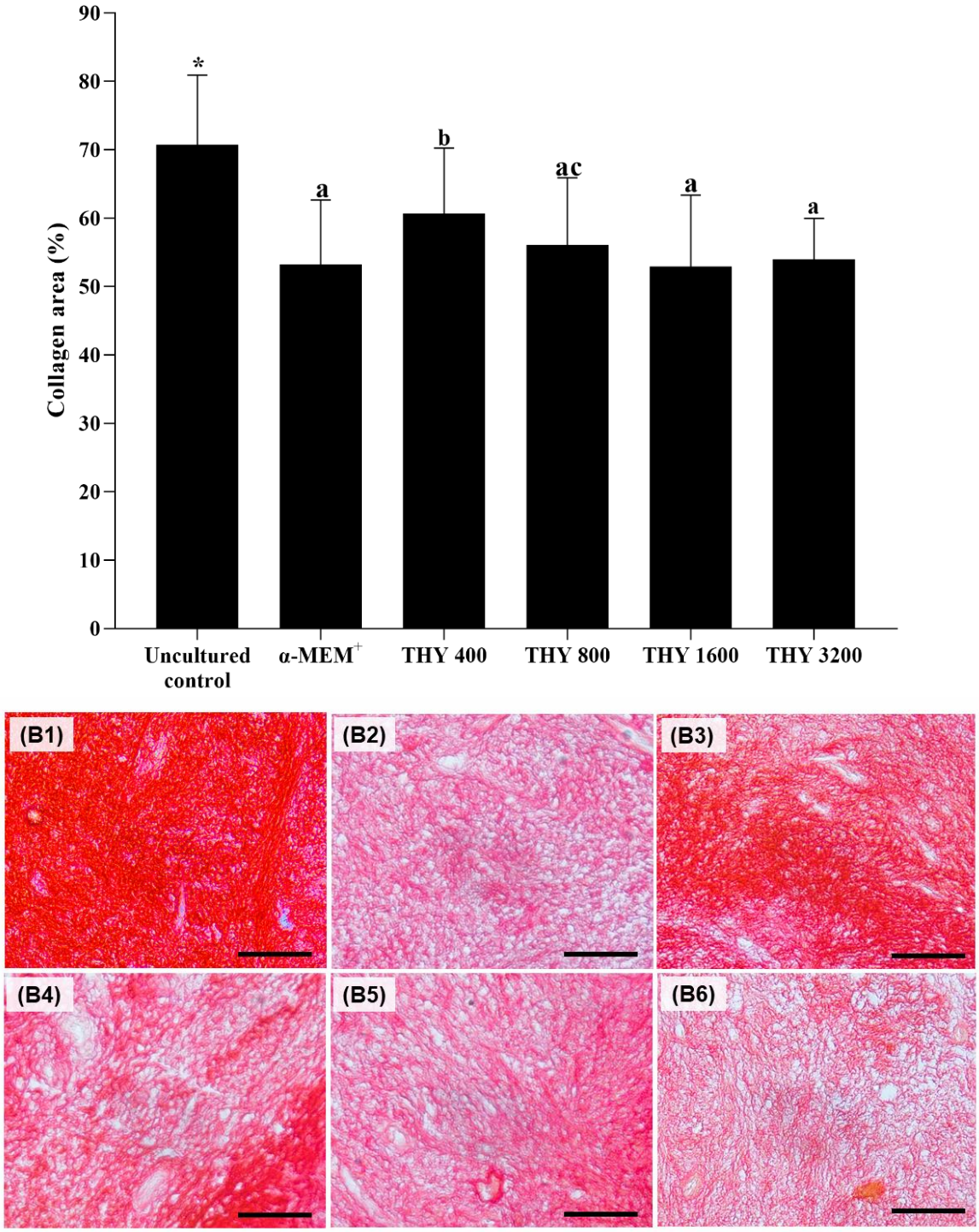

Figure 3. (A) Percentages of collagen in uncultured tissues and in tissues cultured for 6 days in control medium alone or suplemented with 400,800, 1600 or 3200 µg/mL of thymol. a–c. Different lowercase letters indicate statistically significant differ-ences between treatments. (B) Representative images of collagen fibres in uncultured tissues (B1) and in tissues cultured for 6 days in control medium alone (B2) or supplemented with 400,800, 1600 or 3200 Thymol (B3-B6).. Scale bars: 100 µ

3.4 Effects of thymol on stromal cell density after in vitro culture of ovarian tissue

Ovarian tissues cultured in all treatments reduced stromal cell density when compared to the uncultured tissues (Figure 4ab). However, the presence of 400 µg/mL thymol in culture

medium increased cellular density when compared to tissues cultured in control medium alone or supplemetned with other concentrations of thymol ($P < 0.05$).

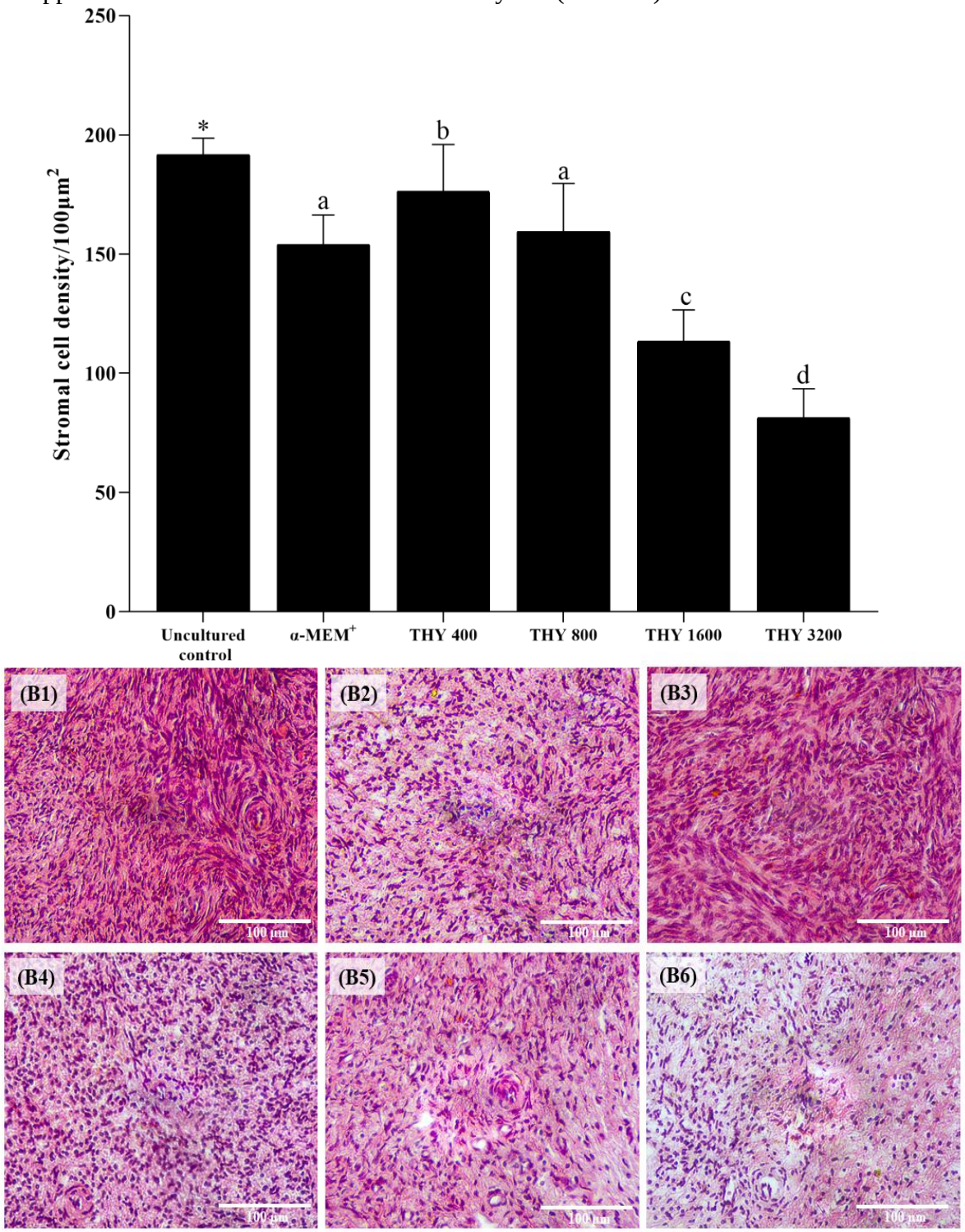

Figure 4. (A) Stromal cell density (cells/100 µm2) in uncultured tissues and in tissues cultured for 6 days in control medium alone or supplemented with 400,800, 1600 or 3200 µg/mL thymol. (B) Representative images stromal ovarian in uncultured tissues (B1) and in tissues cultured for 6 days in control medium alone (B2) or supplemented with 400,800, 1600 or 3200 Thymol (B3-B6) .Scale bars: 100 µm. a–c. Different lowercase letters indicate statistically significant differences between treat-ments.

3.5 Levels of mRNA for SOD, CAT, PRDX6 and GPX1 after in vitro culture

Figure 5 shows the levels of mRNA for *SOD*, *CAT*, *PRDX6* and *GPX1* in bovine ovarian tissues after 6 days of culture. The presence of 400 and 800µg/mL thymol in culture medium reduced levels of mRNA for *SOD*, *CAT* and *PRDX6* in ovarian tissue when compared with those cultured in control medium ($P < 0.05$), except for 400 µg/mL thymol that did not influence *CAT* expression. In addition, thymol did not change the expression of mRNA for *GPX1* in cultured ovarian tissue.

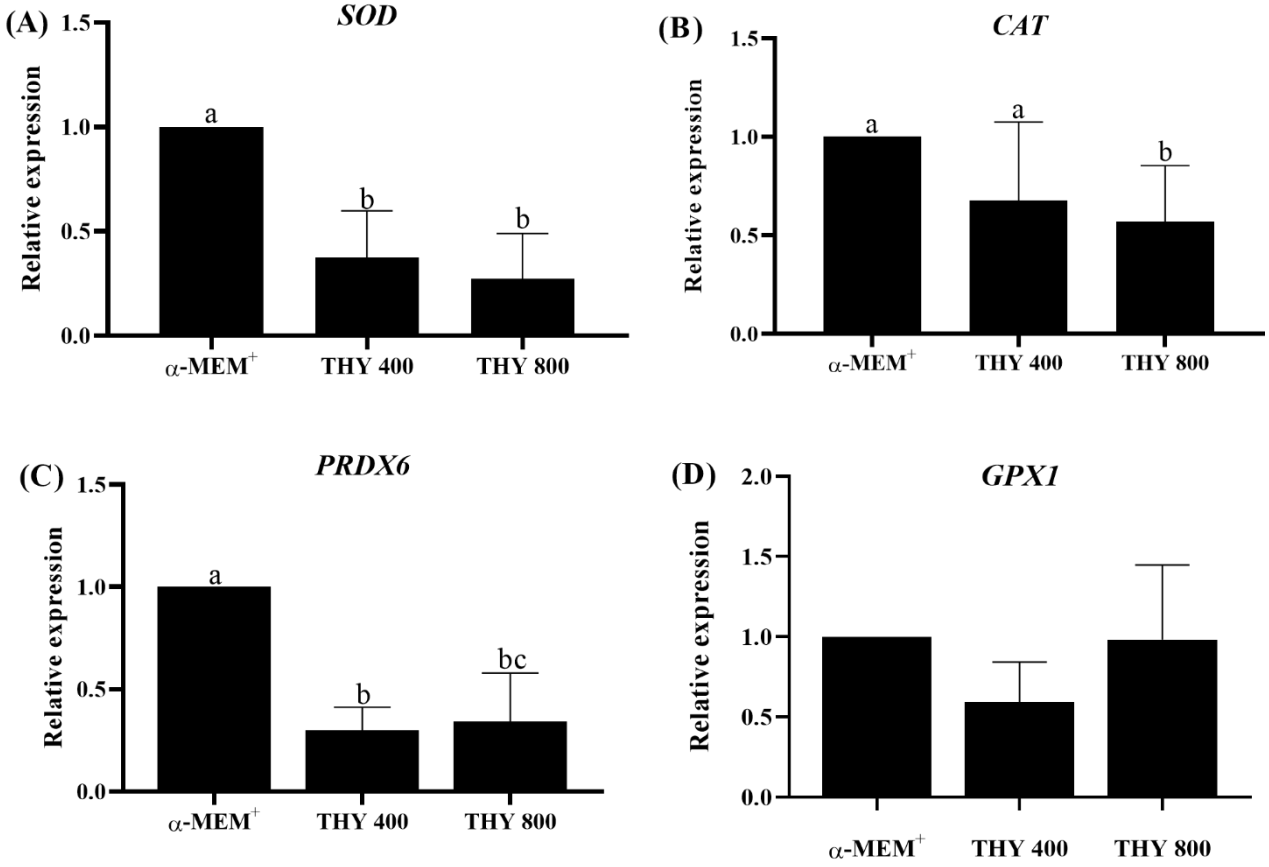

Figure 5. Levels of mRNA for *SOD* (A), *CAT* (B), *PRDX6* (C) and *GPX1* (D) in ovarian tissues cultured for 6 days in con-trol medium alone or supplemented 400,800,1,600 or 3,200 µg/mL of thimol .a,b,c Lowercase letters represent statistically significant differences between treatments ($P < 0.05$).

3.6. Activity of enzymes SOD, CAT and GPX1 and thiol levels after in vitro culture

Figure 6 shows the levels of thiol and activity for SOD, CAT, and GPX in bovine ovarian tissue after 6 days of culture. The presence of 800 µg/mL thymol in the culture medium significantly increased the activity for CAT in ovarian tissue when compared to those cultured

in control medium (P < 0.05). On the other hand, 800 µg/mL thymol reduced the activity of SOD. The thiol levels and the GPX1 activity was not influenced by thymol.

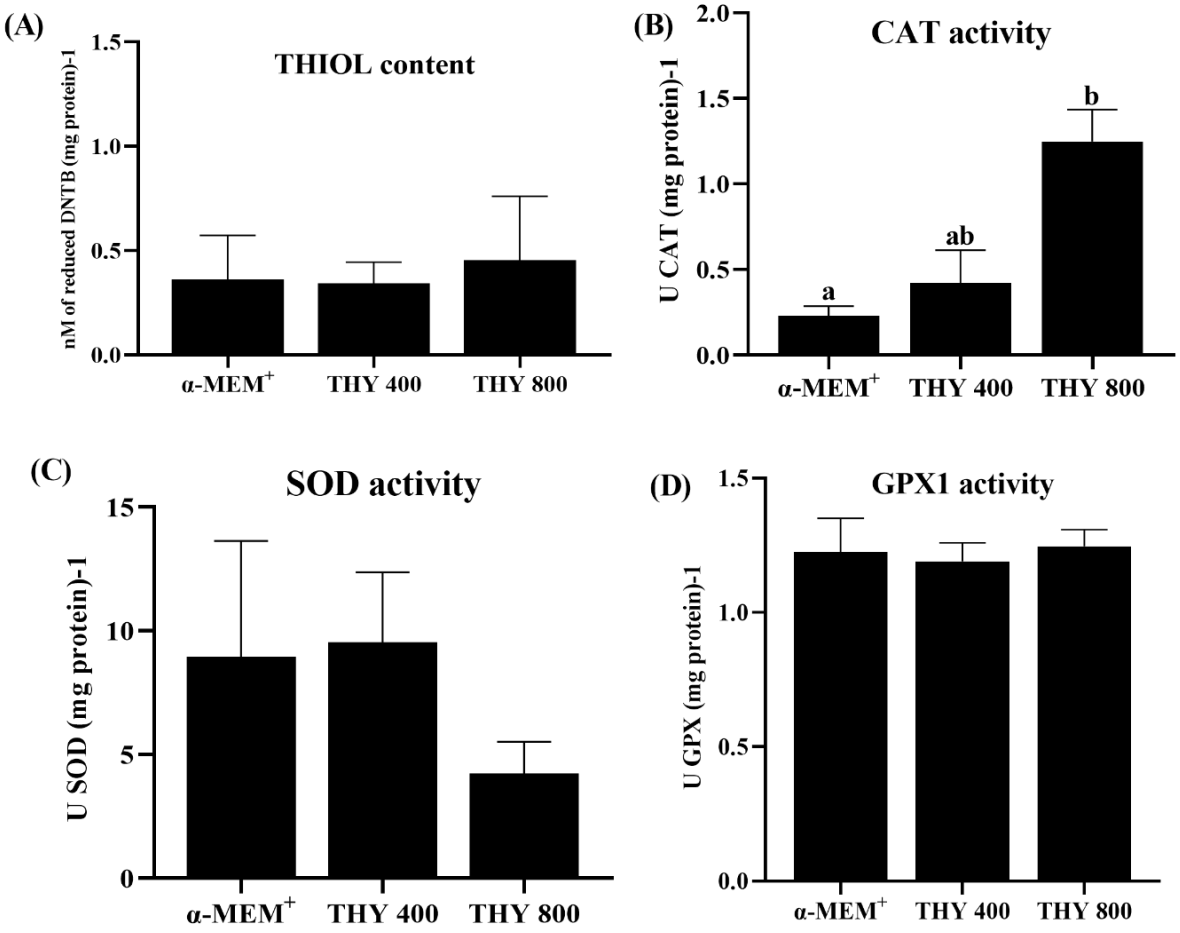

Figure 6. Levels of thiol for (A) and activity (means ± SD) (B) SOD, (C) CAT and (D) GPX in ovarian tissue cultured *in vitro* for 6 days in control medium alone or supplemented with 400,800,1,600 or 3.200 µg/mL of timol (THY). a,b,c Lowercase letters represent statistically significant differences between treatments (P < 0.05).

4. Discussion

This is the first study to demonstrate that thymol promotes the activation of bovine primordial follicles cultured *in vitro*. The activation of primordial follicles is known as the transition from the quiescent set of primordial follicles to the growth phase (Picton, 2001). This phase begins with the differentiation of flattened granulosa cells into cuboids and their proliferation, with a subsequent increase in oocyte size (Zheng et al, 2014). Brito et al (2018) showed that the presenceof thymol-rich essential oil (88.2%) into the culture medium increased

the proliferation of stem cells derived from human adipose tissue, indicating that this compound had an important effect on cell metabolism.

Thymol (400 and 800 µg/mL) helps to preserve the integrity of ovarian follicles in bovine ovarian tissue during *in vitro* culture. On the other hand, higher doses of thymol (1600 and 3200 µg/mL) negatively affect follicle morphology and the maintenance of the structural integrity of ovarian tissue. This effect of thymol at high doses may be associated with its pro-oxidant effect (Geyikoglu et al., 2018), while the protective effect of the lower concentrations of thymol are directly related to its antioxidant activity, since the maintenance of follicular morphology and tissue integrity depends on the redox balance of cells and tissues (Sá et al., 2018). In this regard, it is known that phenolic compounds act by donating the hydrogens of hydroxyl groups to free radicals, preventing the oxidation of other compounds (Jamali et al., 2018). Previous studies have shown that the use of thymol rescued the ovarian reserve mainly by neutralizing oxidative stress and the deregulated cross-talk between IGF-1 and TNF-α in Wister rats with radiotherapy-induced ovarian insufficiency (Mahran et al., 2019). In this same study, the authors highlighted that thymol significantly improved follicular development and levels of anti-Mullerian hormone (AMH), estradiol and FSH, regulating oxidative stress mediated by irradiation. In addition, in male reproductive cells, the use of essential oil rich in thymol (74.2%) improved the viability and preservation of spermatozoa in rats (Omidpanah et al., 2020).

In the present investigation, thymol (400 µg/mL) increased the density of stromal cells in culutred bovine ovarian cortex. A recent study with human cortical tissue revealed that activation of the primordial follicle occurs concomitantly with a loosening of the ovarian cortex during culture, characterized by an early decrease in stromal cell density and dynamic remodelling of the ECM (Grosbois et al 2023). In fact, ovarian stromal cells are responsible for the production of growth factors and peptides that are essential for follicular development (Picton et al, 2008). In this regard, Mbemya et al, (2017) reported a positive relationship between follicular morphology and stromal cell density after culturing goat ovarian tissue.It is well documented that ECM is an essential component for the survival of granulosa cells, stimulating cell differentiation and proliferation (Huet et al., 2001; Berkholtz et al., 2006; Mastrorocco et al., 2021).

In the present study, thymol at a concentration of 400 ug/mL increased the percentages of collagen fibers in the ECM. The ECM plays an active and essential role in folliculogenesis and the structural support of ovarian follicles (Kulus et al., 2021; Almeida et al., 2023) and its

components come together to form a structurally stable compound, providing tissues with physical support for cellular constituents, as well as biochemical and biomechanical properties that are necessary for morphogenesis, differentiation and tissue homeostasis (Grosbois et al., 2023). Thymol, which is known to be an oxygenated monoterpene, can act as a crucial modulator to affect fibroblast metabolism and collagen synthesis (Costa et al., 2019; Moghtaderi et al., 2023). In addition, Ahmady et al (2022) reported that microparticles loaded with thymol significantly increased epithelialization, collagen deposition and induced skin regeneration in full thickness excisions in rats. In other studies, Hussein et al (2023) pointed out that thymol acted positively in regulating the expression of miR-29 in a bleomycin-induced lung fibrosis model and that this same molecule significantly stimulated the mineralization of ECM during bone formation (Trzaskowska et al, 2023). Taken together, these findings suggest that thymol may act to regulate the mechanisms that control the integrity of the ECM, which is necessary for follicle growth from the primordial to the ovulatory phase (Franchi et al., 2020) by modulating the occurrence of oxidative stress during the *in vitro* culture of bovine ovarian follicles.

Our results showed that thymol did not influence thiol concentration in cultured tissues. On the other hand, a clinical trial (Saber et al, 2021) showed that testicular tissues from adult male rats treated with thymol (22.5 or 30 mg/kg) had a significant increase in CAT, SOD and GSH, indicating that thymol alleviates imidacloprid-induced testicular toxicity by modulating oxidative stress. Furthermore, in a study on the liver tissue of male Wistar rats with hepatotoxicity caused by nano-titanium dioxide, oral administration of thymol (30mg/kg) led to a notable increase in GSH levels value compared to the nano-TiO2-treated group (Jafari et al., 2018). In addition, the antioxidant activity of thymol may depend on tissue carachteristics and dose, as demonstrated by Al-Khrashi et al (2022).

In the present study, 800 µg/mL thymol increased CAT activity in ovarian tissue. The CAT has been considered the main antioxidant enzyme in reproductive system (Khan et al, 2020). Its enzymatic action depends on high concentrations of hydrogen peroxide (H_20_2) which, when catalyzed, results in the formation of water and oxygen. Our results are in line with previous reports, Saber et al. (2021) demonstrated that thymol attenuated oxidative stress caused by imidacloprid (IMI) in adult male rats. In addition, Jafari et al (2018) suggested that thymol attenuated hepatic oxidative stress induced by titanium dioxide nanoparticles and also promoted an increase in CAT or its gene expression (Javed et al, 2019). Therefore, we found that thymol did not influence the activity of SOD and GPX1 in bovine ovarian tissue cultured

in vitro. The GPX1 is considered a widely expressed and important member of ROS metabolism (Chen et al, 2019) and catalyzes the oxidation of glutathione to hydroperoxide, playing a role in repairing damage caused by lipid peroxidation (Kurutas, 2016). In this regard, Betancur and Rojano (2017) reported that thymol had no effect on the activity of the antioxidant enzymes SOD and GPX in research with equine semen diluted for freezing. In addition, Al-Malki (2010) also observed no effect of thymol on the activity of SOD and GPX in normal rat liver tissue. In contrast, the increase in CAT activity in our research can be attributed to the antioxidant activity of thymol (Jafari et al., 2020), since the existence of the phenolic hydroxyl group in its chemical structure can eliminate and neutralize free radicals (Nagoor Meeran et al., 2017), playing a fundamental role in acting on ROS in ovarian tissue, ensuring the maintenance of morphology, follicular viability and cell density observed in this report.

After a six-day culture period, we noticed a reduction in mRNA levels for *SOD, CAT* and *PRDX* in tissued cultured with 800 µg/mL thymol. This reduction may depend ot thymol concntration, since its protective effects on lipid peroxidation of rat intestinal mucosa induced by 5-Fluorouracil (5-FU) is dose-dependent (Al-Khrashi et al., 2022). In addition, the reduction in *SOD* mRNA levels in bovine tissues cultured in the presence of 400 µg/mL thymol may be associated with a decrease in intracellular levels of reactive superoxide anion *in vitro*. Thymol has a similar activity to *SOD* in removing superoxide radicals in fibroblasts *in vitro* (Undeger et al., 2009).

Previous studies have already reported that the increased expression of genes such as *SOD, CAT, PRDX* and *GPX* depends on the level of cellular exposure to oxidative stress (Ceko et al., 2015). It is already known that several factors in *in vitro* tissue culture, including collection of ovaries, fragmentation of ovarian tissue, manipulation during routine maintenance and especially the period of *in vitro* culture, can influence the expression results of genes involved in the regulation of oxidative stress (Abedpour et al., 2018; Behnam et al., 2023). Furthermore, studies report that the greater the exposure of ovarian tissue to *in vitro* culture conditions, the greater the probability of an increase in the expression levels of genes involved in the control of oxidative stress, a fact that can be explained by greater cellular exposure to oxidative stress (Cavalcante et al., 2019; Nimse and Pal, 2015). In the present study there was a reduction in the mRNA expression of evaluated genes, a result that may be associated with the short period of tissue culture, which suggests that the tissue cells were not yet exposed to the oxidative stress of the *in vitro* environment enough to recruit stress control genes, knowing that previous studies that evaluated the expression of the same genes *in vitro* culture, it was

only possible to verify the regulation of expression levels after 18 days of culture, when the cells have longer exposure time to the induction of oxidative stress (Paulino et al., 2020; Vasconcelos et al., 2022). Therefore, 6 days of culture are not enough for reproductive cells to require the mechanism of action of genes that are associated with oxidative control or for thymol concentrations to have a protective effect on this induction during a short period of *in vitro* culture.

5. Conclusions

Thymol at concentrations (400, 800, 1,600 µg/mL) increases the survival and growth of the primordial follicle. At (400 µg/mL) it increases the density of stromal cells, but negatively regulates the expression of mRNA for superoxide dismutase, catalase and periredoxin 6 in cultured bovine ovarian tissues at concentrations 400 or 800 µg/mL

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Author contribution statement

Pedro A.A Barroso prepared the media and carried out the *in vitro* culture. Venância A.N Azevedo prepared the histological sections of the cultured tissues and analyzed the growth percentages of the follicles and their morphology and cell density. Vitória S. Bezerra analyzed the enzymatic activity of the samples. Lais R.M.F Paulino. quantified mRNA levels by real-time PCR. Francisco F.C. Filho and Francisco C. Costa carried out the statistical analysis, computational interpretation of the data and wrote the original manuscript José R. V. Silva and Geovany G. Amorim designed the experiment and critically revised the manuscript. All the authors read, revised and approved the final version of the manuscript.

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Declaration of Competing Interest

The authors report no declarations of interest

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8. CONCLUSÕES GERAIS

- O óleo essencial de *Lippia sidoides* e seu constituinte majoritário Timol apresentam efeitos biológicos semelhantes após 06 dias de cultivo de tecido *in vitro* ovariano bovino, mas apresentam mecanismos de ação antioxidante distintos;
- Concentrações elevadas do óleo essencial de *Lippia sidoides* e do Timol apresentam efeitos insatisfatórios sobre a manutenção da densidade estromal e integridade da MEC, quando comparado a concentração de 400µg/mL testadas no sistema de cultivo de tecido ovariano bovino *in vitro*;
- O óleo essencial de *Lippia sidoides* e o Timol em baixas concentrações (400µg/mL ou 800µg/mL) exercem efeito protetivo durante o cultivo *in vitro* de tecido ovariano bovino, modulando de formas diferentes a ocorrência do estresse oxidativo;
- O óleo essencial de *Lippia sidoides* e o Timol constituem importantes produtos biotecnológicos para compor os meios de cultivo *in vitro* de tecido ovariano bovino.

9. PERSPECTIVAS

Tendo em vista a influência deletéria do estresse oxidativo no cultivo *in vitro* de folículos ovarianos bovinos, associados ao fato que promoção da ativação e sobrevivência folicular, atrelados à manutenção da MEC e da densidade das células do estroma, foram observados em nosso estudo. Estes achados podem contribuir para fazer do óleo essencial de *Lippia sidoides* e do timol importantes aliados na melhoria de protocolos dessa natureza. Além disso, pesquisas biotecnológicas envolvendo outras substâncias antioxidantes de origem vegetal, encontrada nos diversos biomas brasileiros, ou ainda, hormônios e fatores de crescimento durante o cultivo de folículos pré-antrais são necessários para uma melhor compreensão da foliculogênese na espécie bovina. Desta forma, investigações adicionais são necessárias para complementar os achados aqui relatados bem como para reduzir a escassez de estudos sobre os efeitos destas substâncias na dinâmica folicular e tecidual sob condições de cultivo *in vitro.*

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