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Impact of varying doses of Moringa leaf extract supplementation in the cryopreservation media on sperm quality, antioxidant capacity and antimicrobial activity of frozen-thawed buck spermatozoa

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ABSTRACT

The current investigation was planned to evaluate the effect of Moringa leaves also called *Moringa oleifera* (*M. oleifera*) aqueous extract on buck semen quality during cryopreservation, and its antimicrobial potential against the *Escherichia coli (E. coli)* isolated from the semen samples. Semen was collected from 8 Jakhrana bucks, and from each buck, 8 ejaculates were collected. Semen samples were subjected to bacteriological studies and pathogenic *E. coli* were isolated from semen samples. Then, various concentrations of *M. oleifera* extract were evaluated for its antimicrobial potential. Good quality semen samples were pooled during each collection. Pooled semen samples were then divided into 4 equal parts, and diluted in TRIS buffer containing different concentration of *M. oleifera* leaf aqueous extract (Different groups, i.e. T_1-50 mg, T_2-100 mg, T_3-200 mg, C-0 mg of *M. oleifera* aqueous extract in 100 ml TRIS Buffer). Semen samples were evaluated for various sperm characteristics after cryopreservation. *M. oleifera* aqueous extract supplemented groups showed significant enhancement in sperm viability, sperm motility, acrosomal integrity and plasma membrane integrity. The treatment significantly reduced the lipid peroxidation in supplemented groups and *M. oleifera* extract shows the potent antimicrobial activity against the *E. coli* isolated from buck semen.

Keywords: Antimicrobial potential, Antioxidant, Buck semen, Fertility, *Moringa oleifera*

Buck semen cryopreservation, commercialization, and artificial insemination (AI) are highly demanding fields within the livestock industry, where AI serves as a widely used tool for genetic improvement. However, semen freezing induces sublethal damage to sperm, leading to the deterioration of cellular characteristics. These damages result from the imbalance of the semen's redox potential during cryopreservation. Additionally, bacterial contamination in semen can contribute to poor quality in the cryopreserved semen. Various genera of bacteria have been identified in semen samples (Guedea Betancourt *et al*. 2022), and their presence triggers toxic effects through toxin production, nutrient competition, and the induction of oxidative stress.

Genital tract infections and inflammation have been linked to buck infertility cases, impacting spermatozoa throughout their development and maturation. Both acute and chronic infections can disrupt spermatogenesis, leading to declines in both quantity and quality. In fact, infections in the buck's genitourinary tract contribute to nearly half of the semen samples testing positive for bacteriospermia (Kumaresan *et al.* 2022). Studies have demonstrated the protective effects of adding herbal extracts to semen diluents. For instance, *M. oleifera* seed extract in ram semen diluter (Carrera-Chávez *et al.* 2020), green tea extract in bull semen (Susilowati *et al.* 2021), grape seed extract in buck semen diluter (Wen *et al.* 2019), and giloy extract in ram semen diluter (Bajia *et al.* 2022) have all shown promise in improving semen cryopreservation outcomes. By enhancing the antioxidant potential of the diluter, herbal extracts can improve post-thaw semen quality. Additionally, many of these herbal extracts possess antimicrobial properties, further enhancing semen quality. Using these herbs as antimicrobial agents in diluters can reduce the need for unnecessary antimicrobial use, thereby lowering the risk of antimicrobial resistance in inseminated females. However, studies investigating the use of herbal supplements in buck semen cryopreservation are scarce, despite the considerable antioxidant potential and potent antimicrobial activity they possess. In this study, the antimicrobial effect of *M. oleifera* leaf extract on the significant human-animal pathogen, Shiga-toxin producing *E. coli* was investigated. Therefore, the aim of this study was to assess the antimicrobial and beneficial effects of *M. oleifera* leaf extract on buck semen cryopreservation.

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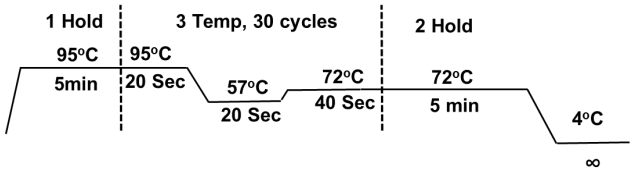
MATERIALS AND METHODS

Semen collection from bucks: For the study, 8 Jakhrana bucks having the age group of 1.5-3.5 years were used, and managed under semi-intensive system of rearing. Semen ejaculates from each buck were collected by using separate sterile artificial vagina in the morning hours.

Isolation and identification of E. coli bacteria from semen: Fresh semen samples collected from breeding bucks were subjected to bacteriological studies. About 100 µl of semen was used as inoculum, and streaked on brain heart infusion agar (BHIA) and MacConkey's agar (MA), and were incubated at 37°C for 24 h. After the incubation, the pink colored colonies on MA were subjected to gram's staining as per standard protocol (Kumaresan *et al*. 2022). Inoculation of pink colored colonies on EMB agar and biochemical tests like catalase, oxidase and IMViC were conducted to confirm the presence of *E. coli* (Supplementary Fig.1).

E. coli confirmation by PCR: Further molecular confirmation was done using multiplex conventional PCR (cPCR) using specific primer sets (Supplementary Table 1) for genes of *E. coli* as described previously (Modak *et al*. 2012) with the PCR set up and conditions as described earlier (Kumaresan *et al*. 2022)

Pathotyping multiplex PCR for E. coli virulence genes: To assess the presence of virulence among the *E. coli* strains obtained from the semen, we conducted pathotyping PCR which detects the virulence genes with primers (Supplementary Table 2) from previous studies (Gunzburg *et al*. 1995, Stacy Phipps *et al.* 1995, Paton and Paton 1998 and Lopez-saucedo 2003). The PCR is conducted in multiplex format using 25 µl total reaction volume with 12.5 µl of 2× EmeraldAmp® GT PCR Master (TaKaRa, Japan), 1 µl of unknown DNA template, 1 µl each of forward and reverse primer mix (10 pMol each). The thermal cycling was done as per the conditions below:



Antibiotic sensitivity of bacterial isolates: The *E. coli* isolates confirmed by cultural, morphological, biochemical and molecular characteristics were subjected to antimicrobial resistance studies. For this, antimicrobial sensitivity test (AST) as per the CLSI guidelines (CLSI VET -01-S-Ed-3) was performed according to Kirby-Bauer disc diffusion method on Mueller-Hinton agar (MHA). The antimicrobials used in the current study are as follows viz., Ampicillin (10µg), Amoxicillin-Clavulanic acid (20/10 µg), Ceftriaxone (30 µg), Cefpodoxime (10 µg), Aztreonam (30 µg), Cefotaxime (30 µg), Cefoxitin (30 µg), Imipenem (10 µg), Amikacin (30 µg), Tetracycline (30 µg), Enrofloxacin (5 µg), Nalidixic acid (30 µg), Trimethoprim-Sulfamethoxazole (1.23/23.75 µg) and Chloramphenicol (30 µg). After application of disks, the plates were incubated overnight at 37°C and the zone of inhibition for individual antimicrobial disks were measured and matched as per CLSI standards.

Preparation of the aqueous extract: Dried leaf powder (50 g) was weighed and soaked into 500 ml conical flask in which 400 ml of distilled water was added. The mixture was kept for 12 h with constant shaking at 30 min intervals. The extract was filtered using Whatman filter paper Grade 1. Extract (filtrate) was concentrated at 40°C under reduced pressure using evaporator, and then kept in small glass tubes/containers. The extract yield was 12.5% and the semisolid extract obtained was stored in a refrigerator for further use (Kumar *et al.* 2011).

Effect of M. oleifera extract on E. coli: Wells of 5 mm diameter were punched on MHA plates having lawn culture of STEC using sterilized cylindrical glass tube and the bottom were sealed using molten MHA to avoid loss of *M. oleifera* crude extract by capillary action/seepage between agar layer and glass surface. Once the bottom is sealed, various dilutions @ M1-100 mg/ml, M2-500 mg/ml and M3-1000 mg/ml of *Moringa* extracts (10 µl) were added to the punched wells carefully and the effective concentrations of *Moringa* extract per well was M1-1 mg, M2-5 mg and M3-10 mg respectively. Duplicates wells are kept for each dilution. Blank wells containing only the diluent (NWC1 and NWC2) in duplicates were also kept to rule out production of any non-specific zone of inhibition (ZoI). Similarly, positive disk controls (PDC1 and PDC2) as Gentamicin (10 µg) anti-microbial disk was applied on the plate as internal positive control. The plates were kept upright for 1-2 h in the incubator at 37°C (to allow complete diffusion of active ingredient), and later inverted and incubated for overnight at 37°C. The zone of inhibition was measured for each dilution in duplicates and average ZoI was measured.

Semen dilution and semen cryopreservation: The semen samples were diluted as described earlier by Gangwar *et al.* (2021). The various concentration of *M. oleifera* aqueous extract were added in the above extender (different groups i.e. T_1-50 mg, T_2-100 mg, T_3-200 mg, C-0 mg of *M. oleifera* aqueous extract in 100 ml TRIS Buffer). The semen samples were extended to maintain sperm concentration approximately 100–120 million/straw. All the diluted semen samples were kept at refrigerated temperature (5°C) for 4 h before being frozen. Semen was filled in French mini straws at 5°C after 4 h equilibration period and straws were sealed with polyvinyl alcohol powder. Straws were frozen in vapours of liquid nitrogen by keeping them 4 cm above the liquid nitrogen for 10 min. Finally, straws were plunged and stored into liquid nitrogen container, the semen samples were evaluated for various sperm characteristics and antioxidant status.

Evaluation of cryopreserved buck semen

Sperm motility: Semen samples were thawed at 37°C for

45 s in a water bath and a drop of 10 µl of semen samples were evaluated for motility under 40× magnification of phase contrast microscope.

Sperm viability was evaluated as per the procedure described by Gangwar *et al*. (2014). A total of 200 sperms were counted in different fields of the slide.

Effect on sperm acrosomal integrity: Giemsa stain was used to evaluate the acrosomal integrity of buck spermatozoa as per the method described by Gangwar *et al*. (2014). Around 200 sperms were assessed in various fields of every slide.

Effect on sperm plasma membrane: The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm plasma membrane as per the protocol used by Gangwar *et al*. (2021). Total of 200 spermatozoa were counted in at least five different microscopic fields.

Effect on lipid peroxidation (MDA estimation µM/ml): At post-thawing, lipid peroxidation level of buck spermatozoa was measured by estimating malondialdehyde (MDA) production using thiobarbituric acid (TBA) according to Gangwar *et al*. (2020).

Statistical analysis: The data were analysed by one-way analysis of variance (ANOVA) using SPSS computer software package (IBM SPSS, version 22; SPSS Inc., Chicago, IL, USA). Duncan multiple range test was used to test the significance of difference between the means within a treatment group. The pooled semen ejaculates were considered as experimental units and P value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Isolation, identification and pathotyping of E. coli: Isolation of *E.coli* was done from semen samples using standard bacteriological techniques, and was further confirmed by multiplex PCR (Fig. 1). To identify the virulence genes and the pathotypes amongst *E. coli* isolates, we did pathotyping multiplex PCR (Fig. 2). Among these, the semen *E. coli* isolate (No. 430) carrying the *stx*2 gene was included in the current study for assessing the sensitivity response to *M. oleifera* leaf extract at various concentrations (100 mg/ml, 500 mg/ml and 1000 mg/ml). The same isolate was also assayed for antimicrobial

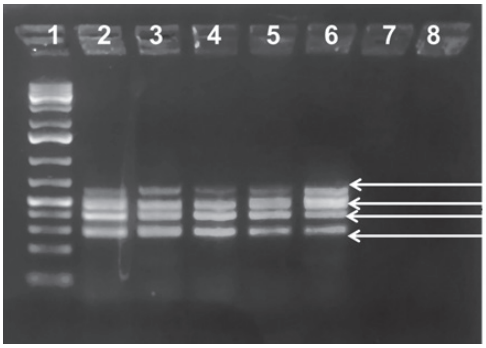


Fig. 1. Multiplex PCR for confirmation of *E. coli* isolates [Lane 1- 100 bp DNA ladder , Lane 2- Positive *E. coli* DNA, 3-6 unknown samples (positive for genes *uid*A, *Lac*Z, *cyd*A and *Lac*Y), 7- No template control].

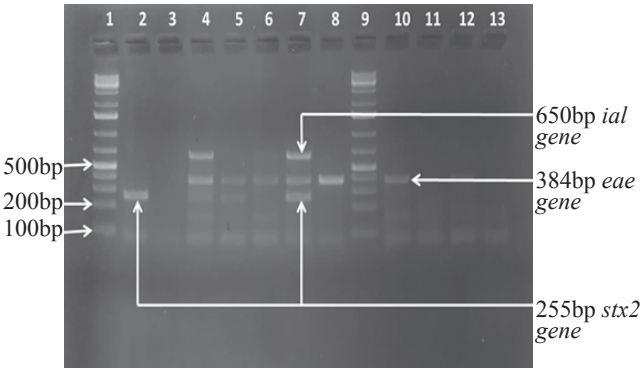


Fig. 2. *E. coli* Pathotyping PCR [Lane 1- DNA ladder, Lane 2- stx2 positive *E. coli*, Lane 3- No template control, Lane 4, 5 and 7 unknown *E. coli* samples positive for *stx2* (255 bp), Lane 4, 6 and 7 unknown *E. coli* samples positive for *ial* gene (650 bp), Lane 4, 5, 6, 7, 8, 10–unknown *E. coli* samples positive for *eae* gene (384 bp)].

sensitivity test (AST) as per CLSI standards (Table 1 and Fig. 3).

AST for the E. coli isolated from the buck semen samples: The AST for *stx2 E.coli* was conducted which was showing susceptibility to most of antimicrobials tested, but resistant to important antimicrobials, viz. cefotaxim (30 µg) and imipenem (10 µg), while showing intermediate sensitivity to enrofloxacin (5 µg). The results are tabulated below.

Effect of various concentration of M. oleifera leaf extract on stx2 positive E. coli isolate: The antimicrobial activity of *M. oleifera* leaf extract against the stx2 positive *E. coli* isolated from buck semen was assessed as per the protocol described in section 2.5. The results are displayed in the Fig. 4. The M1 treatment with 1000mg/ml of *M. oleifera* extract used @10 µl/well showed a higher ZoI of 13.0±0.70 mm followed by M2 (500 mg/ml) and M3 (100 mg/ml) which showed ZoI of 10.25±0.62 mm and 6.75±1.10 mm, respectively (Supplementary Fig. 2).

Sperm quality parameters: Semen quality significantly improved during semen cryopreservation containing different concentrations of *M. oleifera* leaf extract as compared to the control groups. Among the entire treatment groups, T_1 group (50 mg *M. oleifera* extract/100 ml TRIS) showed significantly higher sperm characteristics in terms of sperm motility, sperm viability, acrosomal integrity and plasma membrane integrity. Lipid peroxidation

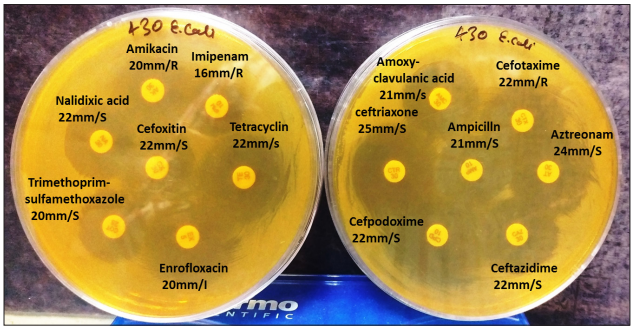


Fig. 3. Antimicrobial sensitivity test using Gram negative panel antimicrobial disks in MHA plates against the *E. coli* (*stx*2 positive, isolate no. 430) isolated from buck semen.

Table 1. Antimicrobial sensitivity profile of the *stx*2 gene positive semen *E. coli* isolate using the Gram-negative antimicrobial panel as per CLSI standards

Antimicrobial disk	Zone of inhibition (mm)	Sensitivity status (R/I/S)*
Amoxicillin-Clavulanic acid (AMC)	21	S
Sulfamethoxazole-Trimethoprim (COT)	20	S
Tetracycline (TE)	22	S
Amikacin (AK)	20	S
Chlormaphenicol (C)	23	S
Cefpodoxime (CPD)	22	S
Nalidixic acid (NA)	22	S
Ceftazidime (CAZ)	22	S
Ceftriaxone (CTR)	25	S
Cefotaxime (CTX)	22	R
Imipenem (IMP)	16	R
Aztreonam (AT)	24	S
Cefoxitin (CX)	22	S
Ampicillin (AMP)	21	S
Enrofloxacin (EX)	20	I

*R- Resistant; I- Intermediate; S- Susceptible.

was also significantly reduced in *M. oleifera* leaf extract supplemented group. The result shows that the *M. oleifera* leaf extract is having protective effect on buck spermatozoa during semen cryopreservation (Table 2).

Male urogenital tract infections significantly contribute to buck infertility, with recent attention focusing on the role of infections in semen quality degradation, particularly asymptomatic bacteriospermia (Gangwar *et al.* 2021). These infections can deteriorate spermatogenesis, impair sperm function, and cause seminal tract obstruction (Kumaresan *et al.* 2022). Therefore, improving semen cryopreservation protocols and monitoring bacterial contamination growth are crucial for enhancing artificial insemination efficiency in goats. Herbal extracts, such as *M. oleifera*, can improve semen quality during cryopreservation and act as antimicrobial agents when added to semen diluters (Gangwar *et al.* 2023). In our study, we evaluated various concentrations of *M. oleifera* extract on virulent bacterial agents isolated from buck semen.

We isolated Shiga toxin-producing *E. coli* (STEC) and *E. coli* carrying virulence genes like *ial* and *eae*, which are significant in neonatal diarrhea. However, their presence in buck semen may stem from environmental contamination or colonization in the bucks' genital tract, necessitating further investigation into potential pathological consequences. Previous research on pregnant and non-pregnant women revealed *E. coli* presence in the genital tract, particularly virulent strains in pregnant women (Forson *et al.* 2018). Chronic urethritis could lead to subclinical inflammation of the genital tract, affecting semen quality due to epididymitis (Rusz *et al.* 2011). While sterile artificial vaginas and clean semen collection methods mitigate pathogen contamination, in-situ colonization depends on factors like immunity, mucosal-associated microflora imbalances and other host-specific determinants.

STEC are key pathogens linked to severe infections like haemorrhagic colitis and haemolytic uremic syndrome (HUS) in humans, with ruminants playing a significant role in the infection cycle. These strains also cause enteritis and diarrhoea in ruminants (Ray and Singh, 2022), leading to substantial losses in the animal-based food industry and posing a public health concern (Hussein 2007). However, the current study does not explore the presence of STEC in semen or its impact on sperm survival or the female reproductive system. Additionally, personnel involved in semen collection and processing from STEC carriers/ infected animals may face similar risks over time.

Furthermore, in this study, stx2-producing *E. coli* isolated from semen exhibited Extended-Spectrum β-Lactamases (ESBL) production, indicated by its resistance to antimicrobial Cefotaxime following CLSI standards. This is significant as it signifies the presence of an ESBL-producing verotoxic *E. coli*, which was further evaluated for sensitivity to *Moringa* leaf aqueous extract. Past reports have highlighted ESBL-producing bacteria in the genital tract, particularly in women with asymptomatic genital infections, with occurrences as high as 69% (Jabbar 2013).

ESBL-producing *E. coli* was detected in rooster semen, indicating a correlation between their presence in the digestive and genital tracts (Mezhoud *et al.* 2015). Additionally, the pathogen showed resistance to Carbapenem based on primary AST results using the imipenem antimicrobial disk, although further tests such as phenotypic carbapenemase tests and MIC strip-based tests (not conducted in this study) are necessary for confirmation. Multi-drug-resistant *E. coli* have also been isolated from the seminal plasma of infertile men, highlighting colonization in the genital tract and its link to infertility (Enwuru *et al.* 2020).

In this study, the aqueous leaf extract of *M. oleifera* demonstrated promising results, with zone of inhibition

Table 2. Effect of different concentration of *Moringa* leaf extract on post thaw semen quality

Parameter	C (0 mg/100 ml)	T_1 (50 mg/100 ml)	T_2 (100 mg/100 ml)	T_3 (200 mg/100 ml)	P- value
Sperm motility	(48.75±2.95)^{b}	(59.75±1.38)^{a}	(58.50±2.13)^{a}	(51.87±2.09)^{b}	0.001
Sperm viability	(53.20±2.35)^{c}	(69.61±1.44)^{a}	(62.43±1.92)^{ab}	(60.93±3.49)^{b}	0.001
Acrosomal integrity	(66.75±2.08)^{b}	(74.62±1.08)^{a}	(69.50±2.05)^{ab}	(65.75±2.71)^{b}	0.007
Plasma membrane integrity	(58.65±2.55)^{ab}	(66.53±1.45)^{a}	(61.55±1.75)^{ab}	(53.97±3.94)^{b}	0.016
MDA estimation (μM/ml)	(460.50±30.24)^{a}	(420.42±25.45)^{ab}	(430.60±22.70)^{ab}	(405.85±16.80)^{b}	0.012

(ZoI) measurements of 13.0±0.70 mm, 10.25±0.62 mm, and 6.75±1.10 mm against concentrations of 10 mg/well, 5 mg/well, and 1mg/well, respectively. The inhibitory concentration ranged between 1-10 mg for 1.5×10^8 CFU/ml (McFarland tube no. 0.5). Previous research (Abalaka *et al.* 2012) indicated that *Moringa* aqueous leaf extract inhibited *E. coli* growth with a ZoI of 20.0±0.03 mm, while the chloroform leaf extract was more effective, with a ZoI of 30.0±0.01 mm. This inhibitory effect is attributed to various phytochemicals present in *Moringa*, including a short polypeptide named 4 (ά– L – rhamnosyloxy) benzyl-isothiocyanate, which inhibits cell wall synthesis and certain biochemical pathways (Ojiako 2014).

The antimicrobial effects of *Moringa* have been previously investigated (Moyo *et al.* 2012, Kalaiyan *et al.* 2021). While various researchers have used *M. oleifera* in semen diluters for different farm animals such as bulls (Hammad *et al.* 2019, Sokunbi *et al.* 2019) and bucks (Wahjuningsih *et al.* 2019, Dapawole and Sirappa, 2021), its antimicrobial activity in semen diluters specifically for buck semen has not been studied to date. In an earlier study, it was found that dietary Azolla supplementation improved buck semen quality and freezability (Gangwar *et al.* 2019). In the current study, promising results were obtained, with higher zone of inhibition recorded for *M. oleifera* aqueous extract compared to commonly used antibiotics. Similar work by (Swidan *et al.* 2020) isolated *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* from the semen of infertile men, noting *E. coli* as the most prevalent. They tested the antibacterial activity of ethanolic extracts of *Punica granatum* against all bacterial isolates, reporting its efficacy against the microbes.

Assessment of progressive motility is crucial for predicting the functionality of buck spermatozoa. In this study, sperm motility was significantly higher in extenders supplemented with 50 mg of *M. oleifera* extract compared to the control. Similar findings were reported by (Wahjuningsih *et al.* 2019) with 3% *M. oleifera* extract in buck semen diluter. Evaluating plasma membrane integrity is essential for assessing spermatozoa structural and functional integrity. Acrosomal integrity is crucial for the fertilization potential of buck spermatozoa, and successful fertilization requires intact acrosomes capable of undergoing the acrosome reaction. In this study, viable spermatozoa with intact acrosomes were significantly higher in *M. oleifera*-supplemented extenders at a dose of 50 mg/100 ml compared to the control. Consistent with our findings, El Seadawy *et al.* (2022) reported that adding 0.646 mg/ml *M. oleifera* extract to semen extenders resulted in improved acrosomal integrity in ram semen.

The beneficial effects of plant extracts during the freezing/thawing process have been documented in both bull (El-Sheshtawy *et al.* 2020) and ram spermatozoa (El Seadawy *et al.* 2022), indicating that fortifying extenders with *M. oleifera* (a natural antioxidant) improves plasma membrane integrity. Previously, Kumar *et al.* (2022) reported that extenders supplemented with herbal antioxidants enhance spermatozoa functionality. In comparison to the control, the addition of 0.646 mg/ml *M. oleifera* extract to semen extender resulted in reduced MDA production in ram semen (El Seadawy *et al.* 2022). Similarly, in rams, *M. oleifera* seed methanolic extract improved post-thaw semen motility and antioxidant capacity (Carrera-Chávez *et al.* 2020).

Earlier studies have identified various compounds in *M. oleifera*, including polyphenols, flavonoids, vitamins C and E, β-carotene, zinc, selenium, vitamins A and B, cryptochlorogenic acid, isoquercetin, and astragalin, all possessing strong antioxidant potential (Moyo *et al.* 2012, Jayawardana *et al.* 2015). Therefore, it can be inferred that the improved semen quality observed upon *M. oleifera* extract addition is likely due to reduced formation of reactive oxygen species (ROS) in sperm plasma membrane. This is supported by the fact that antioxidant compounds reduce spermatic lipid peroxidation resulting from ROS attack on the lipid matrix (Allai *et al.* 2016). However, the exact mechanism underlying sperm protection by *M. oleifera* extract is not fully understood, as the positive effects may result from the combined actions of its components. Thus, further research is needed to elucidate the precise mechanism of action protecting the sperm membrane and to identify the involved compound(s).

The treatment significantly reduced lipid peroxidation in supplemented groups, and *M. oleifera* extract exhibited potent antimicrobial activity against *E. coli* isolated from buck semen.

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