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COMPARATIVE EVALUATION OF PHYTOGENIC SEMEN EXTENDERS FOR PRESERVING CHILLED BALI BULL SPERM QUALITY AND KINEMATICS

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ABSTRACT

The present study aimed to evaluate the effectiveness of phytogenic extenders in preserving sperm quality and kinematic characteristics during chilled storage. Semen samples collected from Bali bull were diluted using a tris–egg yolk (TEY) extender supplemented with different phytogenic extracts and stored at 5°C for up to five days. Sperm quality parameters, including viability, motility, membrane integrity, acrosome integrity, and abnormality, as well as kinematic parameters assessed using a computer-assisted sperm analysis (CASA) system, were evaluated at daily intervals. The results showed a progressive decline in all parameters during storage; however, the extent of deterioration varied depending on the phytogenic extender used. The extender derived from *Kasumba Turatea* demonstrated superior preservation capacity, maintaining higher sperm quality and kinematic values compared to other treatments. In addition to conventional analysis, the area under the curve (AUC) and longevity threshold approaches were applied to provide a more comprehensive evaluation of sperm preservation. The AUC analysis confirmed the higher cumulative performance of the *K. Turatea*-based extender, while longevity threshold analysis indicated an extended functional lifespan of spermatozoa under this treatment. These findings suggest that the effectiveness of phytogenic extenders is closely related to their antioxidant properties and their ability to stabilize sperm membranes and support cellular metabolism during storage. Overall, the integration of conventional, cumulative, and longevity-based analyses highlights the potential of phytogenic extenders as sustainable and effective alternatives to improve semen preservation and enhance artificial insemination efficiency in livestock production systems.

***Keywords*:** Bali bull, Phytogenic extender, Chilled semen preservation, Sperm longevity, Area under the curve (AUC).

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1. INTRODUCTION

The preservation of semen quality during storage is a fundamental requirement for the success of artificial insemination programs and the efficient dissemination of superior genetics in livestock. In the Bali bull (*Bos javanicus*), a valuable indigenous genetic resource, maintaining sperm viability and function during storage is crucial for supporting reproductive efficiency and sustainable breeding programs (Rahmat et al., 2023). However, the application of chilled semen is still limited by the rapid decline in sperm quality during storage, which restricts its practical use in field conditions.

One of the major challenges in semen preservation is oxidative stress, which results from excessive production of reactive oxygen species (ROS) during storage (Shi et al., 2024). Spermatozoa are particularly vulnerable to oxidative damage due to the high content of polyunsaturated fatty acids in their plasma membrane (Gürler et al., 2016; Bollwein & Bittner, 2018). This leads to lipid peroxidation, reduced motility, impaired membrane integrity, and decreased fertilizing capacity (Correa et al., 2024). Consequently, improving the oxidative stability of spermatozoa during storage remains a critical concern in reproductive biotechnology.

Conventional semen extenders commonly rely on animal-derived components such as egg yolk, which, although effective, present several limitations, including variability in composition, risk of microbial contamination, and dependence on non-standardized biological materials (Shishova et al., 2017; Bustani & Baiee, 2024; Hameed et al., 2024). These limitations highlight the need for alternative strategies that are not only effective but also consistent, safe, and sustainable.

In this context, phytogenic compounds derived from plant materials have emerged as promising alternatives due to their natural antioxidant properties (Sapanidou et al., 2023; Shepherd et al., 2024). Bioactive compounds such as

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flavonoids, phenolics, and vitamins have been reported to enhance sperm resistance to oxidative stress and improve preservation outcomes (El-Sheshtawy & El-Nattat, 2020; Ros-Santaella & Pintus, 2021). Several plant-based extracts have been explored individually; however, the comparative effectiveness of different phytogenic sources and their potential for standardization in semen preservation systems remains inadequately understood.

Morenga oleifera, Kasumba turatea, and *Clitoria ternatea* are locally available plant resources known to possess strong antioxidant activity (Escher et al., 2020; Authaida et al., 2025; Bai et al., 2025). Despite their potential, there is limited information regarding their comparative performance as semen extender supplements, particularly in Bali bull under chilled storage conditions. Moreover, current studies often focus on single-parameter evaluation, lacking a comprehensive assessment of sperm quality dynamics during storage.

Therefore, this study was conducted to evaluate the comparative effectiveness of phytogenic semen extenders supplemented with *M. oleifera*, *K. turatea, and C. ternatea* in preserving the quality of chilled spermatozoa from Bali bulls. This study is expected to contribute to the development of more sustainable and locally adaptable semen preservation strategies, as well as provide a more comprehensive understanding of sperm quality dynamics during chilled storage.

2. MATERIALS AND METHODS

2.1. Experimental Design and Ethical Approval

This study was designed to evaluate the comparative effectiveness of three phytogenic semen extenders in preserving the quality of chilled Bali bull spermatozoa. Semen samples were collected from three clinically healthy Bali bulls aged 3–5 years with body weights ranging from 400 to 550 kg. The bulls were maintained in individual pens at the Faculty of Animal Science cattle facility, Hasanuddin University, Makassar, Indonesia, under standard management conditions. The animals were kept under ambient tropical conditions, with temperatures ranging from 25 to 32°C and relative humidity ranging from 70 to 90%. They were fed forage and commercial concentrate, with free access to clean drinking water. Semen was collected twice weekly in the morning using an artificial vagina, and five ejaculates were collected from each bull throughout the experimental period.

Three phytogenic semen extenders were evaluated: *M. oleifera* (T1), *K. turatea* (T2), and *C. ternatea* (T3). These phytogenic extracts were selected because they are rich in natural antioxidant compounds, including phenolics and flavonoids, which have been reported to reduce oxidative stress, protect sperm membrane integrity, and improve sperm survival during semen preservation (Escher et al., 2020; Iqbal et al., 2022; Firouzabadi et al., 2026).

Sperm quality was evaluated during a five-day chilled storage period at 5°C using functional, structural, and kinematic parameters. The analysis emphasized temporal changes in sperm quality and differences among extender types, as well as their interaction with storage duration.

All experimental procedures involving animals were conducted in accordance with institutional guidelines for animal care and use. Ethical approval for this study was obtained from the Animal Research Ethics Committee of Hasanuddin University prior to the commencement of the experiment (No.001/UN4.12/EC/II/2025).

2.2. Semen Collection

Semen collection was performed using an artificial vagina following standard procedures. Five ejaculates were collected from each bull in the morning to ensure optimal semen quality and consistency among ejaculates. Only ejaculates with progressive motility ≥70%, sperm concentration ≥800 × 10^6 sperm/mL, and <20% sperm abnormalities were included in the experiment, following the criteria described by Susilawati et al. (2020). Ejaculates that did not meet these criteria were excluded to ensure consistency and reliability of subsequent analyses.

2.3. Preparation of Phytogenic Extenders

Phytogenic extracts were prepared from *M. oleifera, K. turatea, and C. ternatea,* which were used as natural additives in semen extenders. These plant species were selected because they have been previously investigated by our research group for their antioxidant potential and application in semen preservation (Nirmala et al., 2025; Rajamuddin et al., 2026). Fresh plant materials were collected and selected based on their morphological characteristics and commonly recognized botanical identities. The plant species were identified based on standard botanical descriptions and taxonomic references available in the literature (Liu et al., 2022; Hasanah et al., 2023; Cheng et al., 2024). Fresh plant materials were washed, air-dried, and ground into fine powder using a laboratory grinder. The powdered samples were subjected to solvent-based maceration using ethanol as an extraction solvent (Nirmala et al., 2025; Rajamuddin et al., 2026).

The extracts were filtered using Whatman filter paper (Whatman No. 1, GE Healthcare, UK) and subsequently concentrated using a rotary evaporator (Buchi Rotavapor R-300, Switzerland) under reduced pressure to remove the solvent and obtain crude extracts rich in bioactive compounds, particularly antioxidants. The concentrated extracts were then stored at low temperature until further use.

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A basal semen extender was prepared using a Tris–egg yolk (TEY) extender, which is commonly applied in semen preservation studies to maintain osmotic balance and provide energy sources for spermatozoa (Anzar et al., 2019). The phytogenic extracts were incorporated into the TEY extender at a concentration of 1.5% (v/v) to formulate three treatments based on extracts of *M. oleifera* (T1), *K. turatea* (T2), and *C. ternatea* (T3). The 1.5% (v/v) concentration was selected based on our previous study, which demonstrated that it effectively maintained sperm motility, viability, plasma membrane integrity, and acrosome integrity during semen preservation (Nirmala et al., 2025). All extender preparations were performed under sterile conditions, and the extenders were equilibrated to the appropriate temperature prior to semen dilution.

2.4. Semen Dilution and Storage Conditions

Fresh semen samples that met the initial quality criteria were diluted with the respective phytogenic extenders, namely *M. oleifera* (T1), *K. tratea* (T2), *and C. ternatea* (T3). Dilution was performed under controlled laboratory conditions to ensure homogeneous mixing between semen and extender.

The diluted semen was gradually cooled and stored under chilled conditions at 5°C throughout the experimental period using a refrigerator (Midea, China). Sperm quality was evaluated over a 5-day storage period, with observations conducted at 24-hour intervals (Day 0, Day 1, Day 2, Day 3, Day 4, and Day 5) to assess the effectiveness of each extender in maintaining sperm quality during storage (Nirmal et al., 2025).

Before each evaluation, a small aliquot of the chilled semen sample was gently homogenized and warmed in a dry bath at 37°C for approximately 30 s before analysis. Only the aliquot designated for evaluation was warmed, while the remaining semen was kept at 5°C to maintain storage conditions. All handling procedures were applied uniformly across treatments to minimize variability and ensure consistency of the experimental conditions.

2.5. Sperm Quality Evaluation

Sperm quality was evaluated at predetermined storage intervals. All assessments were performed using standardized procedures under controlled laboratory conditions.

Sperm motility and kinematic parameters were analyzed using a computer-assisted sperm analysis (CASA) system (Minitube CASA System, Germany). A 5 µL semen sample was placed on a pre-warmed slide (37°C) and covered with a coverslip. At least five microscopic fields were recorded per sample, with a minimum of 200 spermatozoa analyzed for each observation.

Sperm viability and abnormality were assessed using 2% eosin staining. A drop of semen (10 µL) was mixed with an equal volume of eosin solution on a glass slide and smeared to form a thin layer. The smear was air-dried and examined under a light microscope (Olympus CX23, Japan) at 400× magnification. A minimum of 200 spermatozoa were counted per sample. Unstained spermatozoa were classified as viable, while stained cells were considered non-viable. Abnormality was evaluated based on morphological defects in the head, midpiece, and tail (Diansyah et al., 2025).

Plasma membrane integrity was evaluated using the hypo-osmotic swelling test (HOST). The HOST solution was prepared by dissolving 0.735 g sodium citrate and 1.351 g fructose in 100 mL of distilled water to achieve a hypo-osmotic solution. A 10 µL semen sample was mixed with 100 µL HOST solution and incubated at 37°C for 30 minutes. After incubation, a drop of the mixture was placed on a slide and observed under a microscope. A minimum of 200 spermatozoa were evaluated, and sperm showing tail swelling were considered to have intact plasma membranes.

Acrosome integrity was assessed using formol-saline solution. The solution was prepared by mixing 4 mL formaldehyde (37–40%) with 96 mL physiological saline (0.9% NaCl). A drop of semen (10 µL) was mixed with 100 µL formol-saline solution and placed on a slide. Observations were conducted under a light microscope at 400× magnification, and at least 200 spermatozoa were evaluated. Spermatozoa with intact acrosomes were identified based on the presence of a normal acrosomal cap.

2.6. Statistical Analysis

All data were analyzed using a two-factor experimental design with extender type (T1, T2, and T3) and storage time as fixed factors. A two-way analysis of variance (ANOVA) was used to evaluate the effects of extender and storage duration, and their interaction, on all sperm quality parameters. When significant differences were detected, mean values were compared using Tukey's post hoc test. Data were expressed as mean ± standard deviation (SD), and statistical significance was declared at $P<0.05$. All analyses were performed using SPSS software (IBM SPSS Statistics, version 25.0, USA).

In addition to primary measurements, several derived parameters were calculated to further evaluate sperm preservation dynamics during storage. The area under the curve (AUC) was calculated from the temporal changes in each parameter over the storage period using a trapezoidal approximation to provide an integrated measure of overall preservation performance (Walter, 2005).

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Longevity threshold analysis was conducted to determine the duration of sperm quality retention during chilled storage. This was defined as the last day on which each parameter remained above a specified proportion of its initial value. In this study, the threshold was set at 70% of the Day 0 value for parameters indicating positive sperm quality, whereas for abnormalities, the threshold was defined as the allowable increase relative to the initial level.

3. RESULTS

3.1. Sperm Quality and Kinematic Changes During Chilled Storage

Sperm quality parameters were significantly affected by storage time and its interaction with extender type. A significant interaction effect (P<0.001) was observed for viability, motility, membrane integrity, and acrosome integrity, indicating that the rate of decline differed among treatments during chilled storage (Table 1). In contrast, abnormality showed no significant interaction (P = 0.053), suggesting a similar increasing pattern across treatments.

Table 1: Sperm Quality Changes During Chilled Storage

Parameter	Day	Mean ± SD			Interaction (P)
		T1	T2	T3	
Viability	Day 0	86.0 ± 1.7aA	86.0 ± 1.9aA	86.1 ± 1.9aA	<0.001
	Day 1	82.5 ± 1.8aA	83.0 ± 1.6abA	81.8 ± 1.5bA	
	Day 2	78.1 ± 2.0bAB	80.3 ± 2.3bA	75.8 ± 1.6cB	
	Day 3	72.8 ± 2.3cAB	75.3 ± 1.9cA	70.1 ± 1.6dB	
	Day 4	67.2 ± 1.7dB	70.0 ± 1.7dA	63.3 ± 1.4eC	
	Day 5	60.6 ± 1.5eB	65.6 ± 1.2eA	56.7 ± 1.7fC	
Abnormality	Day 0	8.6 ± 0.5dA	8.5 ± 0.5dA	8.5 ± 0.5eA	0.053
	Day 1	9.6 ± 0.7dA	9.2 ± 0.7dA	9.8 ± 0.7deA	
	Day 2	10.9 ± 0.9cA	10.7 ± 0.7cA	11.2 ± 0.9dA	
	Day 3	12.5 ± 0.8cAB	11.8 ± 0.8cB	13.4 ± 0.9cA	
	Day 4	14.5 ± 1.0bAB	13.7 ± 0.8bB	15.8 ± 0.9bA	
	Day 5	16.8 ± 1.2aAB	15.5 ± 1.1aB	18.2 ± 1.3aA	
Motility	Day 0	77.9 ± 2.5aA	77.9 ± 2.6aA	77.7 ± 2.8aA	<0.001
	Day 1	72.3 ± 2.9bA	72.6 ± 3.2bA	70.0 ± 2.1bA	
	Day 2	63.9 ± 2.4cAB	66.7 ± 2.2cA	60.7 ± 1.7cB	
	Day 3	53.9 ± 2.1dB	58.0 ± 2.5dA	51.6 ± 1.6dB	
	Day 4	45.4 ± 1.6eB	51.2 ± 2.2eA	40.1 ± 1.0eC	
	Day 5	36.6 ± 2.1fB	43.9 ± 2.6fA	31.3 ± 0.6fC	
Membrane Integrity	Day 0	82.6 ± 1.7aA	82.8 ± 1.7aA	82.8 ± 1.8aA	<0.001
	Day 1	79.1 ± 1.9bA	79.4 ± 1.4bA	77.1 ± 1.3bA	
	Day 2	73.6 ± 1.4cAB	75.0 ± 1.8cA	71.9 ± 1.3cB	
	Day 3	67.3 ± 1.0dB	70.3 ± 1.6dA	65.5 ± 1.1dB	
	Day 4	60.8 ± 1.6eB	65.2 ± 1.0eA	57.8 ± 1.0eC	
	Day 5	53.3 ± 0.8fB	60.1 ± 1.3fA	49.8 ± 1.4fC	
Acrosome Integrity	Day 0	80.2 ± 2.8aA	80.2 ± 2.8aA	80.0 ± 2.8aA	<0.001
	Day 1	76.0 ± 2.3aA	75.2 ± 2.3aA	73.9 ± 2.9bA	
	Day 2	69.8 ± 2.8bA	71.0 ± 2.9bA	67.2 ± 1.6cA	
	Day 3	63.1 ± 1.4cAB	66.5 ± 2.7bA	60.0 ± 1.8dB	
	Day 4	55.9 ± 1.8dB	61.0 ± 2.0cA	51.8 ± 2.0eC	
	Day 5	48.6 ± 1.4eB	55.0 ± 2.6dA	44.3 ± 1.3fC	

Different lowercase letters within the same column (treatment) and a specific parameter indicate significant differences among storage times (P<0.05). Different uppercase letters within the same row (day) indicate significant differences among treatments (P<0.05). Data are presented as mean ± SD, where SD = standard deviation. Extenders: T1 = *M. oleifera*, T2 = *Kasumba turatea*, T3 = *Clitoria ternatea*.

Viability decreased significantly over time in all treatments (P<0.05), with differences among treatments becoming evident from Day 2 onward. T2 consistently maintained higher viability compared to T1 and T3 during the later storage period, while T3 showed the most /pronounced decline. A similar pattern was observed for motility, with T2 exhibiting significantly higher values than T1 and T3 from Day 3 to Day 5 (P<0.05), indicating better preservation of sperm motility.

Membrane integrity and acrosome integrity also declined significantly during storage (P<0.05), with T2 showing superior preservation compared to the other treatments, particularly at extended storage times. T3 consistently exhibited the lowest values, reflecting reduced resistance to storage-induced damage.

Sperm abnormality increased progressively during storage (P<0.05); however, differences among treatments were less pronounced compared to other parameters. Despite this, T3 tended to exhibit higher abnormality values at later storage times, whereas T2 maintained relatively lower levels. Overall, these results indicate that sperm quality

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deteriorated over time under chilled storage conditions, with T2 demonstrating superior ability to preserve sperm functional integrity compared to T1 and T3.

Sperm kinematic parameters were significantly influenced by storage time and its interaction with extender type. Significant interaction effects were observed for VCL, VAP, VSL, DCL, and DSL ($P<0.05$) (Table 2), indicating that the decline in sperm movement characteristics varied among treatments during storage. In contrast, no significant interaction effects were observed for DAP, LIN, STR, and WOB ($P>0.05$), suggesting a relatively similar pattern of change across treatments.

Velocity-related parameters (VCL, VAP, and VSL) showed a significant decline over time ($P<0.05$) in all treatments. Differences among treatments became evident from Day 2 onward, with T2 consistently exhibiting higher values compared to T1 and T3 during the later storage period. T3 generally showed the lowest values, indicating a greater reduction in sperm velocity.

A similar trend was observed in distance-related parameters (DCL and DSL), where T2 maintained significantly higher values compared to the other treatments at extended storage times ($P<0.05$). These findings suggest that T2 was more effective at preserving sperm trajectory and distance traveled. In contrast, DAP showed no significant interaction effect, although a general declining trend was observed across all treatments.

For motion pattern parameters (LIN, STR, and WOB), changes during storage were less pronounced. Although slight decreases were observed over time, differences among treatments were generally not significant, particularly for STR and WOB ($P>0.05$). This indicates that although overall sperm motility decreased, the movement pattern remained relatively stable during storage.

Overall, these results demonstrate that sperm kinematic characteristics deteriorated during chilled storage, with T2 showing superior performance in maintaining sperm velocity and movement dynamics compared to T1 and T3.

3.2. Area Under the Curve (AUC) Analysis of Sperm Quality and Kinematic Parameters

The AUC analysis demonstrated significant differences among phytogenic extenders in maintaining cumulative sperm quality during chilled storage ($P<0.05$) (Table 3). Overall, T2 consistently exhibited the highest AUC values across nearly all evaluated parameters, indicating superior preservation performance over time.

For primary sperm quality parameters, including viability, motility, membrane integrity, and acrosome integrity, T2 showed significantly higher AUC values compared to T1 and T3 ($P<0.05$). In contrast, T3 consistently presented the lowest AUC values, reflecting a greater decline in sperm functional quality throughout the storage period. Notably, sperm abnormality showed an opposite trend, where T3 exhibited the highest AUC value, indicating a greater accumulation of abnormal spermatozoa over time, while T2 maintained the lowest level ($P<0.05$).

A similar pattern was observed in sperm kinematic parameters. T2 demonstrated significantly higher AUC values for velocity-related parameters (VCL, VAP, and VSL) compared to T1 and T3 ($P<0.05$), suggesting improved maintenance of sperm motility dynamics. Likewise, distance-related parameters (DCL, DAP, and DSL) were also significantly higher in T2, followed by T1 and T3.

For motion pattern parameters, including LIN, STR, and WOB, significant differences among treatments were still observed ($P<0.05$). T2 showed the highest AUC value for STR, while T1 and T2 exhibited comparable values for WOB, both higher than T3. These findings indicate that T2 not only preserved sperm velocity but also maintained movement characteristics more effectively.

Overall, the AUC analysis confirms that the phytogenic extender used in T2 provided the most stable preservation environment, as evidenced by its ability to maintain higher cumulative sperm quality across both functional and kinematic parameters during chilled storage.

3.3. Longevity Threshold of Sperm Quality and Kinematic Parameters

The longevity threshold analysis revealed clear differences in the duration of sperm quality preservation among treatments (Table 4). The time required for each parameter to remain within acceptable functional limits varied depending on the phytogenic extender used.

For viability, both T1 and T2 maintained values above the defined threshold up to Day 5, whereas T3 declined below the threshold earlier, indicating a shorter functional lifespan. A similar pattern was observed for membrane integrity, where T2 demonstrated the longest preservation period, followed by T1 and T3.

In terms of motility, a more rapid decline was observed across all treatments; however, T2 maintained motility above the threshold for a longer duration compared to T1 and T3, which dropped below the threshold at earlier time points. This indicates that T2 was more effective in preserving sperm movement during storage.

For acrosome integrity, T2 again showed greater longevity than the other treatments, while T1 and T3 declined below the threshold earlier. In contrast, sperm abnormality increased over time, with T2 maintaining values within the acceptable threshold for a longer duration, whereas T3 exceeded the threshold earlier, indicating a higher accumulation of abnormal spermatozoa.

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Overall, T2 consistently demonstrated the longest longevity across most evaluated parameters, while T3 showed the shortest duration of functional preservation. These findings indicate that the phytogenic extender in T2 not only maintained higher sperm quality but also prolonged the functional lifespan of spermatozoa during chilled storage. The longevity threshold approach provides practical insight into the usable storage duration, complementing conventional quality and cumulative analyses.

Table 2: Sperm Kinematic Changes during Chilled Storage

Parameter	Day	Mean ± SD			Interaction (P)
		T1	T2	T3	
VCL	Day 0	119.2 ± 4.0aA	119.8 ± 3.1aA	119.8 ± 1.9aA	0.006
	Day 1	114.3 ± 4.3abA	115.3 ± 3.6abA	113.2 ± 3.2bA	
	Day 2	108.5 ± 2.8bAB	111.5 ± 3.1bA	105.9 ± 4.0cB	
	Day 3	100.3 ± 2.7cAB	104.3 ± 2.6cA	98.2 ± 2.0dB	
	Day 4	94.9 ± 2.8cB	99.5 ± 2.2cA	89.4 ± 2.4eC	
	Day 5	86.9 ± 3.0dB	93.1 ± 1.6dA	81.9 ± 2.3fC	
VAP	Day 0	84.5 ± 3.4aA	85.0 ± 2.6aA	85.3 ± 3.7aA	0.045
	Day 1	81.1 ± 4.0aA	81.0 ± 3.0aA	79.1 ± 3.0bA	
	Day 2	75.5 ± 2.0bA	77.4 ± 2.7bA	73.5 ± 3.6bA	
	Day 3	69.3 ± 2.5cAB	72.9 ± 2.8bA	66.9 ± 3.1cB	
	Day 4	63.8 ± 1.9dA	67.6 ± 2.5cA	59.4 ± 2.6dB	
	Day 5	57.2 ± 1.2eB	62.5 ± 2.9cA	54.3 ± 1.6dB	
VSL	Day 0	61.8 ± 1.6aA	61.8 ± 1.3aA	62.3 ± 1.8aA	<0.001
	Day 1	58.7 ± 2.1aA	59.5 ± 2.2aA	57.7 ± 2.2bA	
	Day 2	54.0 ± 1.9bAB	55.5 ± 1.4bA	52.8 ± 1.5cB	
	Day 3	48.2 ± 2.1cB	52.1 ± 2.3bA	47.0 ± 1.2dB	
	Day 4	43.9 ± 1.8dB	47.4 ± 1.3cA	40.7 ± 2.1eC	
	Day 5	38.3 ± 2.1eB	43.2 ± 2.2dA	34.8 ± 2.2fB	
DCL	Day 0	71.5 ± 2.5aA	72.2 ± 1.2aA	71.8 ± 1.8aA	0.039
	Day 1	68.4 ± 2.7abA	69.1 ± 1.8bA	67.1 ± 1.9bA	
	Day 2	65.8 ± 1.5bA	66.9 ± 1.5bA	63.5 ± 3.1bA	
	Day 3	59.9 ± 1.4cAB	61.9 ± 1.2cA	58.3 ± 1.7cB	
	Day 4	56.9 ± 1.3cA	59.6 ± 1.7cA	53.8 ± 2.0dB	
	Day 5	52.2 ± 1.8dB	56.2 ± 1.5dA	49.6 ± 1.7eB	
DAP	Day 0	56.3 ± 2.6aA	56.7 ± 1.4aA	56.9 ± 2.9aA	0.070
	Day 1	54.4 ± 3.4aA	54.1 ± 2.3aA	53.2 ± 2.4aA	
	Day 2	50.7 ± 2.1bA	51.4 ± 1.9bA	49.2 ± 1.7bA	
	Day 3	46.4 ± 2.2bcA	48.6 ± 2.3bA	45.2 ± 2.0bA	
	Day 4	42.4 ± 1.6cB	45.8 ± 2.0cA	40.0 ± 1.9cB	
	Day 5	37.9 ± 1.0dB	42.0 ± 1.6cA	36.7 ± 1.4cB	
DSL	Day 0	44.3 ± 0.7aA	44.2 ± 1.1aA	45.3 ± 1.5aA	<0.001
	Day 1	41.9 ± 1.6aA	43.4 ± 1.9aA	41.3 ± 1.9bA	
	Day 2	38.9 ± 1.4bAB	40.1 ± 0.9bA	38.0 ± 1.1cB	
	Day 3	34.7 ± 1.4cB	37.6 ± 1.5bA	33.8 ± 1.1dB	
	Day 4	31.7 ± 1.6dB	34.3 ± 1.2cA	29.1 ± 1.5eC	
	Day 5	27.4 ± 2.1eB	31.3 ± 1.8dA	24.9 ± 1.9fB	
LIN	Day 0	51.8 ± 1.0aA	51.5 ± 0.5aA	52.1 ± 1.1aA	0.068
	Day 1	51.3 ± 1.1aA	51.7 ± 1.1aA	51.0 ± 1.2aA	
	Day 2	49.7 ± 1.7aA	49.7 ± 0.6aA	49.9 ± 1.5aA	
	Day 3	48.1 ± 1.9bA	50.0 ± 1.4aA	47.9 ± 0.4bA	
	Day 4	46.2 ± 1.2bcA	47.6 ± 0.8bA	45.5 ± 1.9bA	
	Day 5	44.1 ± 1.7cAB	46.4 ± 2.3bA	42.5 ± 2.2cB	
STR	Day 0	73.1 ± 2.5aA	72.7 ± 2.1aA	73.1 ± 1.4aA	0.621
	Day 1	72.4 ± 3.6abA	73.6 ± 2.8aA	72.9 ± 2.5aA	
	Day 2	71.4 ± 1.5abA	71.7 ± 1.8aA	71.8 ± 1.6aA	
	Day 3	69.6 ± 3.6abA	71.5 ± 1.8aA	70.4 ± 2.5aA	
	Day 4	68.8 ± 2.8abA	70.0 ± 2.5aA	68.5 ± 3.9abA	
	Day 5	66.9 ± 2.8bA	69.3 ± 3.5aA	64.1 ± 3.0bA	
WOB	Day 0	70.9 ± 2.0aA	71.0 ± 2.1aA	71.2 ± 2.3aA	0.969
	Day 1	71.0 ± 2.0aA	70.2 ± 1.3aA	69.9 ± 2.1abA	
	Day 2	69.7 ± 2.4aA	69.4 ± 2.0aA	69.4 ± 2.9abA	
	Day 3	69.1 ± 1.6abA	69.9 ± 1.3aA	68.1 ± 2.7abA	
	Day 4	67.2 ± 1.1bA	68.0 ± 2.0aA	66.5 ± 2.3bA	
	Day 5	65.9 ± 1.1bA	67.1 ± 3.0aA	66.3 ± 0.7bA	

Different lowercase letters within the same column (treatment) and a specific parameter indicate significant differences among storage times (P<0.05). Different uppercase letters within the same row (day) indicate significant differences among treatments (P<0.05). Data are presented as mean ± SD, where SD = standard deviation. Extenders: T1 = *M. oleifera*, T2 = *Kasumba turatea*, T3 = *Clitoria ternatea*. VCL = curvilinear velocity (μm/s); VAP = average path velocity (μm/s); VSL = straight-line velocity (μm/s); DCL = distance along the curvilinear path (μm); DAP = distance along the average path (μm); DSL = straight-line distance (μm); LIN = linearity (%); STR = straightness (%); WOB = wobble (%).

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Table 3: Area under the curve (AUC) values of sperm quality and kinematic parameters during chilled storage

Parameter	T1	T2	T3
Viability	372.0b	381.9a	359.9c
Abnormality	60.0b	57.1c	63.0a
Motility	289.4b	306.8a	275.6c
Membrane Integrity	346.2b	359.6a	336.0c
Acrosome Integrity	327.6b	339.5a	312.9c
VCL	521.0b	537.2a	509.5c
VAP	360.5b	371.3a	344.4c
VSL	252.1b	267.0a	246.2c
DCL	312.8b	321.2a	304.4c
DAP	241.6b	248.1a	232.2c
DSL	181.5b	193.1a	176.9c
LIN	240.9b	247.9a	240.0c
STR	349.0c	359.2a	355.5b
WOB	345.1a	345.1b	337.4c

Different lowercase letters within the same row indicate significant differences among treatments (P<0.05). AUC values were calculated using the trapezoidal method to represent cumulative sperm quality during the five-day storage period. Extenders: T1 = *M. oleifera*, T2 = *Kasumba turatea*, T3 = *Clitoria ternatea*.

The longevity threshold analysis of sperm kinematic parameters revealed distinct differences in the duration of movement preservation among treatments (Table 5). Overall, T2 consistently maintained kinematic parameters above the defined threshold for a longer period compared to T1 and T3, indicating superior stability of sperm motion during chilled storage.

Velocity-related parameters (VCL, VAP, and VSL) showed a progressive decline over time, with T2 maintaining values above the threshold for the longest duration. In contrast, T3 exhibited an earlier decline below the threshold, indicating reduced capacity to sustain sperm velocity. Among these parameters, VSL showed the most rapid decrease, reflecting its sensitivity to storage-induced deterioration.

Table 4: Longevity threshold of sperm quality parameters during chilled storage

Parameter	Threshold criterion	T1 threshold	T1 longevity	T2 threshold	T2 longevity	T3 threshold	T3 longevity
Viability	70% of Day 0 (min)	60.2	Day 5	60.23	Day 5	60.27	Day 4
Abnormality	130% of Day 0 (max)	11.23	Day 2	11.08	Day 2	11.1	Day 1
Motility	70% of Day 0 (min)	54.52	Day 2	54.5	Day 3	54.38	Day 2
Membrane Integrity	70% of Day 0 (min)	57.81	Day 4	57.95	Day 5	57.99	Day 3
Acrosome Integrity	70% of Day 0 (min)	56.13	Day 3	56.15	Day 4	56	Day 3

Longevity threshold was defined as the last storage day at which each parameter remained ≥70% (for positive parameters) or ≤130% (for abnormality) of its Day 0 value. T1 = *M. oleifera*, T2 = *Kasumba turatea*, T3 = *Clitoria ternatea*.

Table 5: Longevity threshold of sperm kinematic parameters during chilled storage

Parameter	T1 threshold	T1 longevity	T2 threshold	T2 longevity	T3 threshold	T3 longevity
VCL	83.43	Day 5	83.89	Day 5	83.85	Day 4
VAP	59.18	Day 4	59.51	Day 5	59.72	Day 3
VSL	43.23	Day 4	43.23	Day 4	43.64	Day 3
DCL	50.05	Day 5	50.54	Day 5	50.26	Day 4
DAP	39.4	Day 4	39.7	Day 5	39.86	Day 4
DSL	31.02	Day 4	30.95	Day 5	31.68	Day 3
LIN	36.27	Day 5	36.08	Day 5	36.44	Day 5
STR	51.18	Day 5	50.88	Day 5	51.18	Day 5
WOB	49.66	Day 5	49.69	Day 5	49.85	Day 5

The longevity threshold was defined as the last storage day on which each parameter remained ≥70% of its Day 0 value. T1 = *M. oleifera*, T2 = *Kasumba turatea*, T3 = *Clitoria ternatea*. VCL = curvilinear velocity; VAP = average path velocity; VSL = straight-line velocity; DCL = distance along the curvilinear path; DAP = distance along the average path; DSL = straight-line distance; LIN = linearity; STR = straightness; WOB = wobble.

A similar trend was observed in distance-related parameters (DCL, DAP, and DSL), where T2 demonstrated extended longevity compared to T1 and T3. These results suggest that sperm trajectory and movement distance were better preserved in T2, while T3 showed a faster loss of movement efficiency.

In contrast, motion pattern parameters (LIN, STR, and WOB) exhibited relatively greater stability throughout the storage period. Although a gradual decline was observed, the longevity differences among treatments were less pronounced compared to velocity and distance parameters. Nevertheless, T2 generally maintained these parameters above the threshold for a slightly longer duration, while T3 tended to decline earlier.

Overall, the longevity threshold analysis of kinematic parameters confirms that T2 not only preserved sperm quality but also prolonged the functional movement capacity of spermatozoa. These findings highlight the effectiveness of the phytogenic extender in T2 in maintaining both sperm viability and motility dynamics during chilled storage.

4. DISCUSSION

The present study highlights the critical role of phytogenic extender composition in modulating sperm preservation during chilled storage. The differences observed among treatments can be attributed to the distinct bioactive compounds present in each plant-based extender, which influence oxidative balance, membrane stability, and cellular metabolism.

The decline in sperm quality parameters during storage is primarily associated with oxidative stress and

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membrane destabilization. It is well established that chilled storage induces excessive production of reactive oxygen species (ROS), which initiate lipid peroxidation in sperm plasma membranes, leading to loss of membrane integrity, reduced motility, and decreased viability (Sapanidou et al., 2016; Loetjettanarom et al., 2025). Spermatozoa are particularly vulnerable to oxidative damage due to the high content of polyunsaturated fatty acids in their membranes and their limited intrinsic antioxidant defense systems (Aitken et al., 2016; Aitken 2020; O'Flaherty & Scarlata 2022). Consequently, maintaining oxidative balance is a key factor in preserving sperm functionality during storage.

In this context, extenders enriched with phytogenic compounds possessing strong antioxidant activity are expected to provide enhanced protection. The superior preservation observed in the *K. turatea*-based extender suggests a higher efficacy in mitigating oxidative stress, potentially due to the presence of phenolic compounds, flavonoids, and carotenoids, which are known to act as free radical scavengers and inhibitors of lipid peroxidation (Rahman et al., 2006; Nirmala et al., 2025; Tüğen & Buruleanu 2026). These compounds may contribute to maintaining membrane fluidity, preventing structural damage and stabilizing sperm cells under cold storage conditions.

In contrast, extenders derived from *M. oleifera* and *C. ternatea* exhibited comparatively lower preservation capacity. Although both plants are known to contain antioxidant compounds, their effectiveness may depend on extraction efficiency, compound stability, and bioavailability. Previous studies have shown that the protective efficiency of plant-derived antioxidants varies depending on their interaction with sperm membranes and their resistance to degradation during storage (El-Sheshtawy & El-Nattat, 2020; Firouzabadi et al., 2026; Kiran et al., 2020). This suggests that not only the presence but also the functional activity of phytochemicals determines their effectiveness in semen preservation systems.

The deterioration of sperm kinematic parameters further supports the involvement of metabolic and mitochondrial dysfunction. Sperm motility is highly dependent on ATP generated through mitochondrial oxidative phosphorylation, and chilled storage has been shown to impair mitochondrial membrane potential, leading to reduced energy production (Iqbal et al., 2022; Shehata et al., 2025; Firouzabadi et al., 2026; Alam et al., 2025). The better preservation of velocity-related parameters indicates that certain phytogenic compounds may help protect mitochondrial integrity or reduce oxidative damage to mitochondrial structures, thereby sustaining energy availability for sperm movement. This is particularly relevant for parameters such as VSL, which are closely associated with progressive motility and fertilizing potential.

Interestingly, motion pattern parameters such as LIN, STR, and WOB were less affected by storage conditions. This observation is consistent with previous findings indicating that the coordination of sperm movement is relatively more stable than velocity magnitude, as it is largely governed by the structural integrity of the flagellar apparatus rather than solely by energy availability (Van de Hoek et al., 2024; Xu et al., 2025). Therefore, reductions in motility during storage are more likely attributed to decreased ATP availability than to alterations in movement coordination.

The AUC analysis provides an integrative assessment of sperm preservation by reflecting cumulative performance over time. Unlike single time-point measurements, AUC incorporates both the magnitude and duration of sperm quality, making it a more comprehensive indicator of extender effectiveness (Fernández-Novo et al., 2021; Neubert et al., 2026). The higher AUC values observed in the *K. turatea*-based extender indicate that its protective effects are sustained throughout the storage period, rather than being limited to early stages.

Furthermore, the longevity threshold analysis offers a practical evaluation of sperm usability by identifying the duration for which sperm parameters remain within acceptable functional limits. This approach is particularly relevant in applied reproductive technologies, where the timing of insemination is critical for achieving optimal fertility outcomes (Diskin, 2018). The extended longevity observed in the *K. turatea*-based extender suggests its potential to prolong the effective storage period of chilled semen, thereby enhancing its applicability in field conditions.

Despite these promising findings, several limitations should be acknowledged. First, the study was conducted under controlled laboratory conditions using a limited number of ejaculates, which may not fully represent field variability. Differences in animal physiology, environmental conditions, and semen handling practices could influence extender performance under practical conditions. Second, the specific bioactive compounds responsible for the observed effects were not directly identified or quantified. Further studies involving phytochemical characterization and dose optimization are therefore required to elucidate the mechanisms underlying their protective roles. Additionally, this study focused on in vitro parameters without directly evaluating fertility outcomes. Although sperm quality and kinematic parameters are widely used as indicators of fertilizing ability, confirmation through in vivo fertility trials is necessary.

From an applied perspective, these findings have important implications for the livestock sector, particularly in artificial insemination programs. The use of phytogenic extenders with superior preservation capacity may enhance the efficiency of semen storage and distribution by extending the usable lifespan of chilled semen. This could reduce the frequency of semen collection, improve logistical flexibility, and increase access to high-quality genetic material, especially in regions with limited cryopreservation infrastructure.

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Moreover, the adoption of plant-based extenders represents a sustainable and potentially cost-effective alternative to synthetic additives, aligning with current trends toward environmentally friendly livestock production systems. The ability of such extenders to maintain both sperm quality and functional longevity may contribute to improved reproductive efficiency, supporting genetic improvement programs and overall productivity in cattle breeding systems.

5. CONCLUSION

This study demonstrates that phytogenic extenders play a crucial role in improving sperm preservation during chilled storage, with effectiveness largely determined by their bioactive composition. The extender derived from *Kasumba Turatea* showed superior ability to maintain sperm quality, kinematic performance, and overall functional stability, as evidenced by consistently higher cumulative values and greater longevity than other treatments. Importantly, integrating area under the curve (AUC) and longevity threshold analyses provides a more comprehensive framework for evaluating semen preservation by capturing both cumulative performance and practical usability duration. These findings highlight the potential of phytogenically based extenders as effective, sustainable alternatives to enhance semen storage efficiency and optimize artificial insemination practices in livestock production systems.

Declarations

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Data Availability: All data supporting the study's findings are available in the manuscript.

Ethics Statement: All experimental procedures involving animals were conducted in accordance with institutional guidelines for animal care and use. Ethical approval for this study was obtained from the Animal Research Ethics Committee of Hasanuddin University prior to the commencement of the experiment (No.001/UN4.12/EC/II/2025).

Author's Contributions: MY contributed to supervising the experiment and conception and design of the study; AMD and S. contributed to conception and design of the study and improvement of the manuscript; AMD MM, collected data, data analysis, and drafted the manuscript.

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