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The Addition of Indian Almond Leaf Extract to Caudal Epididymal Plasma Diluents Effectively Maintains The Quality of Frozen Simmental Bull Sperm

(*PENAMBAHAN EKSTRAK DAUN KETAPANG DALAM PENGENCER PLASMA EPIDIDIMIS KAUDAL EFEKTIF MEMPERTAHANKAN KUALITAS SPERMA BEKU SAPI SIMMENTAL*)

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ABSTRACT

The decline in sperm quality during the freezing process is generally attributed to cold shock and the accumulation of Reactive Oxygen Species (ROS), which damage cell membranes, proteins and DNA, thereby reducing sperm motility, viability and potential fertility. Natural antioxidants such as Indian almond or Ketapang leaf extract (*Terminalia catappa*) have the potential to neutralize free radicals due to their flavonoid and phenolic content. This study was aimed to evaluate the effect of adding Indian almond or Ketapang leaf extract to Cauda Epididymis Plasma (CEP) diluent on the quality of frozen semen from Simmental bull. The study employed a completely randomized design with four Indian almond or Ketapang leaf extract doses (0, 0.013, 0.025, and 0.038 mg/mL) and six replicates. Sperm quality evaluation included motility, viability and plasma membrane integrity. Data were analyzed using Analysis of variance and a significantly difference between the treatments, was continued with the Duncan's multiple range test. Results showed that the addition of 0.038 mg/m extract yielded the highest values, namely sperm motility of 56.7 ± 2.9% (equilibrated) and 43.3 ± 2.9% (post-thawed), viability of 87.3 ± 1.2% (equilibrated) and 79 ± 0% (post-thawed) and membrane integrity of 84.7 ± 0.6% (equilibrated) and 79.7 ± 1.2% (post-thawed). These differences were significant compared to other treatments ($P<0.05$). This study concludes that a dose of 0.038 mg/mL of Indian almond or Ketapang leaf extract is effective in maintaining spermatozoa quality during freezing through its antioxidant activity, which prevents oxidative damage. This finding is potentially applicable in reproductive technology to improve the success of artificial insemination.

Keywords: CEP diluent; Indian almond leaves; natural antioxidants; sperm quality

ABSTRAK

Penurunan kualitas spermatozoa selama proses pembekuan umumnya disebabkan oleh kejutan dingin (*cold shock*) dan akumulasi *Reactive Oxygen Species* (ROS) yang merusak membran sel, protein dan DNA, sehingga menurunkan motilitas spermatozoa, viabilitas dan potensi fertilitas. Antioksidan alami seperti ekstrak daun ketapang (*Terminalia catappa*) berpotensi menetralkan radikal bebas karena kandungan flavonoid dan fenolik. Penelitian ini bertujuan mengevaluasi pengaruh penambahan ekstrak daun ketapang dalam pengencer *Cauda Epididymis Plasma* (CEP) terhadap kualitas semen beku sapi simmental. Penelitian ini menggunakan rancangan acak lengkap dengan empat dosis ekstrak daun ketapang (0; 0,013; 0,025; 0,038 mg/mL) dan enam ulangan. Evaluasi kualitas sperma meliputi motilitas, viabilitas, serta integritas membran plasma. Data dianalisis dengan sidik ragam dan perbedaan yang nyata antar perlakuan diuji lanjut dengan uji jarak berganda Duncan. Hasil menunjukkan bahwa penambahan 0,038 mg/mL ekstrak daun ketapang menghasilkan nilai tertinggi, yaitu motilitas spermatozoa 56,7 ± 2,9% (sebelum pembekuan) dan 43,3 ± 2,9% (setelah pencairan), viabilitas 87,3 ± 1,2% (sebelum pembekuan) dan 79 ± 0% (setelah pencairan), serta integritas membran 84,7 ± 0,6% (sebelum pembekuan) dan 79,7 ± 1,2% (setelah pencairan). Perbedaan tersebut signifikan dibandingkan perlakuan lain ($P<0.05$). Penilitian ini menyimpulkan bahwa dosis 0,038 mg/mL ekstrak daun ketapang efektif mempertahankan kualitas spermatozoa selama pembekuan melalui aktivitas antioksidan yang mencegah kerusakan oksidatif, sehingga berpotensi diaplikasikan pada teknologi reproduksi untuk meningkatkan keberhasilan inseminasi buatan.

Kata-kata kunci: pengencer CEP; daun ketapang; antioksidan alami; kualitas spermatozoa

INTRODUCTION

The Artificial insemination (AI) program can help farmers select high-quality bull genetics that are suitable for the conditions and demand (Mahyun *et al*., 2021). However, the success of AI is highly dependent on the quality of the semen used. Storing semen at low temperatures often causes sperm damage due to cold shock. Spermatozoa have high levels of polyunsaturated fatty acids (PUFAs) in their plasma membranes, making them sensitive to damage from cold shock and lipid peroxidation by free radicals, which affect motility, metabolism, ultrastructure and fertility (Ducha, 2018). Additionally, long-term storage can lead to the excessive production of Reactive Oxygen Species (ROS), which negatively impacts sperm quality (Len *et al*., 2019).

Spermatozoa can undergo damage during storage due to cold shock, which causes damage to the plasma membrane at low temperatures (Ducha, 2018). Maintaining the stability of spermatozoa during storage requires an effective semen diluent. An ideal diluent should be non-toxic, contain appropriate buffers, maintain pH balance and protect sperm from rapid cooling (Effendi *et al*., 2015). One commonly used diluent is Cauda Epididymis Plasma (CEP), which has an ion composition and osmolarity similar to seminal plasma in the cauda epididymis and contains fructose and essential minerals for spermatozoa (Susilawati and Yekti, 2018; Verberckmoes *et al.*, 2004). The addition of egg yolk further enhances sperm protection, particularly against temperature-induced damage (Tethool *et al*., 2022).

Free radicals are atoms or molecules that have one or more unpaired electrons, making them highly reactive and unstable. The ROS and Reactive Nitrogen Species (RNS) are produced both endogenously and exogenously (Mandal *et al*., 2022). Endogenously, free radicals are produced as natural by products of metabolic processes, such as mitochondrial electron transport, enzy-

matic reactions and immune responses. Exogenously, they arise from exposure to environmental factors such as ultraviolet (UV) radiation, pollution, tobacco smoke and industrial chemicals (Chandimali *et al.*, 2025). Changes in membrane structure occur due to the loss of some phospholipid and cholesterol components in the membrane. Another factor causing a decrease in spermatozoa is the presence of free radicals or ROS (Priyanto *et al.*, 2015).

In semen cryopreservation, oxidative stress develops as the freezing and thawing processes increase ROS production beyond the neutralizing capacity of sperm antioxidant defenses (Daenen *et al*., 2019). When ROS are produced in excess, these molecules can damage important structures in sperm, such as cell membranes, proteins and DNA. As a result, sperm motility, viability and fertilization capacity are impaired (Lira *et al*., 2024). Furthermore, oxidative stress can also cause high DNA fragmentation, which has profound implications for sperm genetic quality (Dorostghoal *et al*., 2017). To maintain optimal sperm cell function, a balance of redox potential is required, achieved by balancing ROS with antioxidants. Semen contains antioxidants and simultaneously performs nutritional and protective functions for sperm. However, the presence of these antioxidants is still insufficient to counteract the free radicals that spermatozoa encounter. Antioxidants are broadly categorized into enzymatic and non-enzymatic types (Kowalczyk, 2022). The primary enzymatic antioxidants in semen are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). They act synergistically to convert reactive oxygen species (ROS) into less harmful molecules such as water and oxygen. Meanwhile, non-enzymatic antioxidants function as radical scavengers that prevent oxidative damage (Wang *et al*., 2025).

Oxidative stress can impair spermatozoa quality, and the use of antioxidants both natural and synthetic has been shown to mitigate this effect (Kamoda *et al*., 2021). Natural antioxidants such as vitamins E and C, as well as glutathione are effective in neutral\izing free radicals (Zalukhu *et al*., 2016), while synthetic compounds like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are also widely used in reproductive studies (Sanger *et al*., 2018). Among natural sources, Indian almond or Ketapang (*Terminalia catappa L.*) is particularly attractive due to its rich con-tent of flavonoids and phenolic compounds, which possess strong antioxidant activity (Ningsih *et al.*, 2023). These phenolics effecttively scavenge free radicals and protect spermatozoa from oxidative damage (Kumar *et al*., 2019).

Based on this potential, the study was aimed to investigate the use of *T. catappa* leaf extract as a natural antioxidant in Simmental bull semen diluents. To date, the application of *T. catappa* in improving semen quality of Simmental bulls has not been extensively studied. This study therefore provides a novel approach to enhance frozen semen quality and improve the efficiency of artificial insemination in the livestock industry.

RESEARCH METHODS

Research Design

This study employed a Completely Randomised Design (CRD). The study consisted of four treatments of Indian almond or Ketapang leaf extract doses in CEP dilutions with six replicates sample tubes: P1 (0 mg/mL Indian almond or Ketapang leaf extract); P2 (0.013 mg/mL Indian almond or Ketapang leaf extract); P3 (0.025 mg/mL Indian almond or Ketapang leaf extract); P4 (0.038 mg/mL Indian almond or Ketapang leaf extract).

Preparation of Indian almond or Ketapang Leaf Extract

Indian almond or Ketapang leaf extract (*T. catappa*) was prepared using the maceration method with 70% ethanol (Maharadingga *et al*., 2021). A total of 600 g of washed leaves, dried for a week and ground

were soaked in 70% ethanol for 72 hours, with a solvent ratio of 1:3 on the first day and 1:2 on the subsequent days. The filtrate was filtered and the residue was remacerated and the final macerate was evaporated using a vacuum rotary evaporator. Evaporation is carried out for 2–3 hours at a water-bath temperature of 60°C until a viscous, concentrated extract is obtained.

Preparation of CEP Dilution

The preparation of CEP diluent followed the method of Ducha (2018), with the composition per liter including: 0,88 g NaCl; 0,52 g KCl; 0,44 g $CaCl_2 \cdot 2H_2O$; 0,6 g mmol $MgCl_2 \cdot 6H_2O$; 0,99 g $NaHCO_3$; 0,95 g NaH_2PO_4; 2,72 g KH_2PO_4; 9,9 g fructose; 1.0 g sorbitol; 2.0 g bovine serum albumin (BSA); 16,2 g Tris; 1 g penicillin, 1 g streptomycin, and 8,2 g citric acid. All ingredients were dissolved in sterile distilled water using the aliquot technique, sterilised using a 0.2 µm Millipore membrane, and then 20% egg yolk was added and homogenised. The mixture was stored in a refrigerator for 2–4 days until two layers formed, and the supernatant was used as a diluent. All diluents were stored at 4–5°C.

Freezing Process

Fresh semen from Simmental bull was collected at Artificial Insemination Center of Singosari, Malang, using an artificial vagina (AV). Macroscopic evaluation included volume, colour, pH, odour and consistency, while microscopic evaluation included sperm concentration, motility of the mass and individuals, viability, abnormalities, and integrity of the spermatozoa membrane.

According to Rahayu and Ducha (2022), the dilution process should be carried out in three stages: A1, A2 and B. The A1 dilution is performed in a water bath at 37°C with a 1:1 ratio. A2 dilution is performed on liquid semen or the result of A1 dilution stored at 4–5°C. The volume of diluent added in dilution A2 is calculated using the following formula, V total = V semen + V A1 + V A2 + V B. The liquid semen is then stored in a refrigerator at 4-5℃ for approximately 18 hours. Dilution B is then performed with the following total diluent volume $V.B = V_{total} \times 0.5$.

The pre-freezing stage is performed to gradually lower the temperature from 5°C to -140°C over 7 minutes using an automatic freezer with controlled rate specifically designed for cryopreservation processes (Digit-Cool machine®, IMV Technologies, Aras, France). Subsequently, the freezing process is carried out using liquid nitrogen at -196°C.

Sperm Motility Observation

Sperm motility was evaluated under a microscope at 400× magnification and 37°C. Spermatozoa displaying progressive forward movement were classified as motile, while those showing only circular or backward movement were considered non-progressive. Samples were considered acceptable when the percentage of motile spermatozoa was ≥ 40% (SNI, 2021).

Sperm Viability Observation

Sperm viability was evaluated using the eosin-nigrosin staining method, which involved mixing semen and stain in a 1:2 ratio, preparing a thin smear, and observing 200 cell spermatozoa under a 400x microscope. (Figure 1) spermatozoa were identified by red or purple-stained heads, while

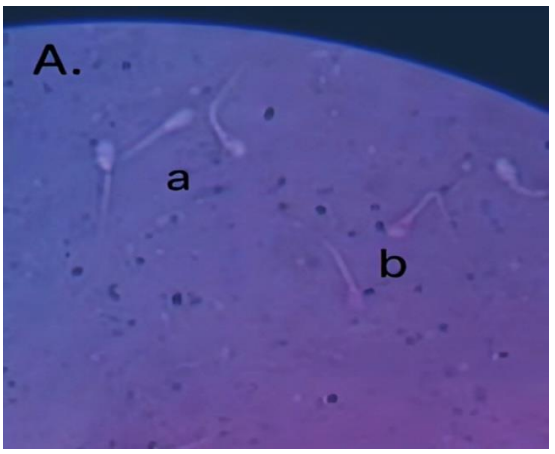

Figure 1. Microscopic apprearance of post-thawed Simmental spermatozoa stain with eosin-negrosin Note: a. Live spermatozoa with unstained heads; b. Dead sperm with red-stained heads

live spermatozoa had an unstained head (Ducha *et al*., 2025).

Observation of Sperm Membrane Integrity

Membrane integrity was tested using the hypoosmotic swelling test, which involved incubating 100 µL of semen with 1 mL of 125 mOsm/kg hypoosmotic solution at 37°C for 30 minutes. A 0.2 mL sample of the mixture was smeared onto a microscope slide, and 200 spermatozoa were observed under a microscope with a magnification of 400 times. Spermatozoa showing coiled or (Figure 2) swollen tails were considered to have intact plasma membranes (Mujahidurrohman *et al*., 2023).

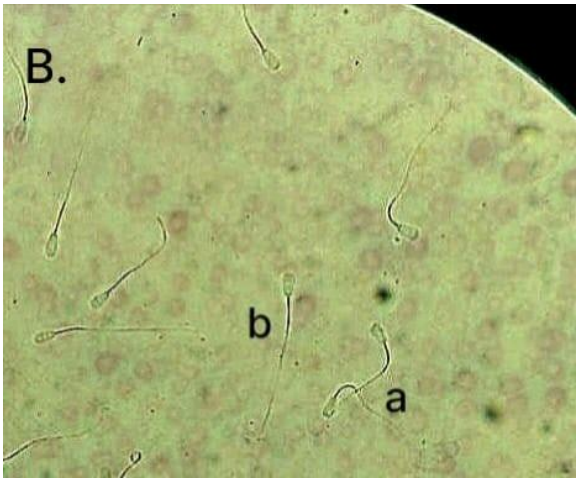

Figure 2. Microscopic appearance of immenal sperm after thawing mixed with HOST solution. Note: a. Live spermatozoa with coiled tail; b. Dead spermatozoa with straight tail

Data Analysis

Data on sperm motility, viability and membrane integrity percentages were transformed using the arcsin function, tested for normality and homogeneity, and then analyzed using Analysis of variance. When significant differences were detected (α = 0.05), Duncan's multiple range test was performed to determine the best treatment. All statistical analyses were conducted using SPSS version 25.

RESULTS AND DISCUSSION

Fresh Simmental bull semen was evaluated before the dilution process was carried out. The evaluation of fresh semen was conducted macroscopically and microscopically. The results showed that the fresh semen met the set requirements, with a motility rate of ≥ 70% and an abnormality rate of ≤ 20%. The results of the evaluation of fresh Simmental bull semen are presented in Table 1. In Table 1 it is shows that the fresh semen evaluation results indicate good quality. The milky white color and pH of 6.5 ± 0.1 are within the normal range, following the SNI standard for fresh bull semen of 6.4–6.8, and the volume of 9.45 ± 1.7 mL reflects optimal conditions. The sperm concentration of 1.67 ± 0,57 x 10^9 sperm/mL with individual motility of 73.2% ± 4.0%, indicates high fertility potential.

Table 1. Results of fresh semen evaluation Tests

Parameter	Mean ± Standard Deviation
Colour	Milky white
pH	6.5 ± 0.1
Consistency	Moderate
Volume (ml)	9.45 ± 1.7
Concentration (million/ml)	1,670 ± 570.6
Individual motility (%)	73.2 ± 4.0
Abnormalities (%)	10.7 ± 2.0
Viability (%)	95.1 ± 0.3
Membrane Integrity (%)	90.2 ± 3.9

Abnormalities of 10.7% ± 2.0% are within acceptable limits (<20%). Via-bility of 95.1% ± 0.3% and membrane inte-grity of 90.2% ± 3.9 indicate excellent spermatozoa quality.

Spermatozoa motility observations were conducted to assess the effects of various doses of Indian almond or Ketapang leaf extract in CEP diluent on the semen quality of Simmental bull. The results of the motility evaluation are presented in Table 2.

Table 2. Mean ± Standard deviation of sperm motility in Simmental bull with the addition of various doses of Indian almond or Ketapang leaf extract in Cauda Epididymis Plasma diluent solvent

Observation Time	Percentage of Motility (%) ± Standard Deviation			
	P1	P2	P3	P4
	0 mg/mL	0,013 mg/mL	0,025 mg/mL	0,038 mg/mL
Equilibrated	48,3 ± 2,9[a]	53,3 ± 2,9[ab]	51,7 ± 2,9[ab]	56,7 ± 2,9[b]
Post-thawed	31,7 ± 2,9[a]	36,7 ± 2,9[a]	36,7 ± 2,9[a]	43,3 ± 2,9[b]

Note: "Equilibrated" refers to samples after equilibration at 4°C for 18 hours. Different superscript letters (a, b) within the same column indicate significant differences between treatments (P<0.05).

Table3. Mean ± Standard deviation of sperm viability in Simmental bull with the addition of various doses of Indian almond or Ketapang leaf extract in Cauda Epididymis Plasma diluents

Observation Time	Viability Percentage (%) ± Standard Deviation			
	P1	P2	P3	P4
	0 mg/mL	0,013 mg/mL	0,025 mg/mL	0,038 mg/mL
Equilibrated	81,3 ± 0,6[a]	82,3 ± 0,6[a]	84,3 ± 0,6[b]	87,3± 1,2[c]
Post-thawed	67,7 ± 0,6[a]	70,3 ± 1,5[b]	74,7 ± 0,6[c]	79 ± 0[d]

Note: Equilibrated refers to samples after equilibration at 4°C for 18 hours. Different superscript letters (a, b, c, d) within the same column indicate significant differences between treatments (P<0.05).

Table 4. Mean ± Standard deviation of sperm membrane integrity in Simmental bull with the addition of various doses of Indian almond or Ketapang leaf extract in Cauda Epididymis Plasma diluent.

Observation Time	Membrane Integrity Percentage (%) ± Standard Deviation			
	P1	P2	P3	P4
	0 mg/mL	0,013 mg/mL	0,025 mg/mL	0,038 mg/Ml
Equilibrated	83 ± 1,8[ab]	81,7 ± 1,2[a]	82.9 ± 1,6[a]	84.7 ± 0,6[b]
Post-thawed	63.3 ± 0,6[a]	68 ± 2,5[b]	74,7 ± 0,6[c]	79,7 ± 1,2[d]

Note: "Equilibrated" refers to samples after equilibration at 4°C for 18 hours.
Different superscript letters (a, b, c, d) within the same column indicate significant differences between treatments (P<0.05).

Based on Table 2, the average percentage of sperm motility at two different highest motility was observed in treatment P4 (56.7 ± 2.9%), which was substantially different from the other treatments. The lowest motility was observed in treatment P1 (48.3 ± 2.9%). A similar pattern was observed at the post-thawed observation time, with the highest motility observed in treatment P4 (43.3 ± 2.9%), which was significantly different from the other treatments. In this observation times was significantly different. At the equilibrated observation time, the research, sperm motility values were higher than those reported by Novita (2020), with the highest motility value being 40%. This difference may be due to differrences in the diluent used and the duration of thawing. Although there is a trend of incre-ased motility with increasing concentration, the response pattern is not entirely linear, as indicated by the numerically lower motility

value of P3 compared to P2.

Based on Table 3, the average sperm viability differed significantly between the two observation times ($P<0.05$). At the equilibrated stage, the highest viability was observed in treatment P4 (87.3 ± 1.2%), while the lowest was in P1 (81.3 ± 0.6%), with P4 differing significantly from the other groups. After the freezing–thawing process, treatment P4 still showed the highest viability (79 ± 0%), whereas P2 had the lowest (67.7 ± 0.6%). These results indicate that the addition of 0.038 mg/mL Indian almond or Ketapang leaf extract in the CEP diluent effectively preserved sperm viability during cryopreservation. Compared with the findings of Nisa *et al*. (2022), who reported the highest post-thawed Simmental bull semen viability of 69.4 ± 11.8%, the present study demonstrated higher viability values.

The plasma membrane integrity of spermatozoa was evaluated to assess overall sperm quality, as an intact membrane indicates the sperm's ability to survive and function optimally during fertilization.

Table 4 presents data on the membrane integrity of Simmental bull sperm after adding various doses of Indian almond or Ketapang leaf extract to the CEP diluent.

Based on Table 4, the average spermatozoa membrane integrity showed significant differences at the two observation times ($P<0.05$). At the equilibrated stage, the highest membrane integrity value was observed in treatment P4 (84.7 ± 0.6%), while the lowest was in P2 (81.7 ± 1.2%), with P4 differing significantly from the other groups. After the freezing–thawing process, P4 also maintained the highest value (79.7 ± 1.2%), whereas P1 recorded the lowest (63.3 ± 0.6%), confirming the effectiveness of P4 in preserving membrane integrity during cryopreservation. Compared with the findings of Yendraliza *et al.* (2023), who repor-ted the highest post-thawed membrane inte-grity value of 44.33 ± 2.39%, the results of this study were considerably higher. This suggests that the addition of 0.038 mg/mL Indian almond or Ketapang leaf extract in the CEP diluent provided superior protection to sperm membranes against cryo-induced damage.

The fresh semen used in this study showed good physical and microscopic qualities, indicating that the Simmental bull was in normal physiological and reproductive condition. Parameters such as sufficient volume, normal pH, high motility and low abnormality rates demonstrated optimal sperm quality suitable for cryopreservation. As noted by Susilowati *et al.* (2022), semen with high initial motility and membrane integrity is more resistant to cold shock during the freezing–thawing process, leading to better post-thaw viability and fertility. Thus, the semen characteristics in this study served as a solid foundation for assessing the protective role of Indian almond or Ketapang leaf extract during cryopreservation.

The average ejaculate volume of Simmental bull is within the normal range of 9.45 mL. This is higher than the results reported by Ardita *et al.* (2024), which were 7.8 mL and 6.1 mL. The average sperm motility of 73.2% is still higher than the results of Yanuarista *et al*. (2022) at Sidomulyo Ungaran Artificial Insemination Center, which reported fresh semen motility of 69–71% for Simmental bull. The sperm concentration produced was 1.670 million/mL, categorized as thick. The frequency of collection can influence differences in sperm concentration between individuals (Komariah *et al*., 2020).

The ROS generation occurs mainly on the inner mitochondrial membrane during oxidative phosphorylation, requiring activetion of the mitochondrial electron transport chain. This process involves five protein complexes that sequentially transfer electrons from Nicotinamide Adenine Dinucleotide (NADH) to oxygen (O_2). The mitochondrial membrane potential is formed by active proton hydrogen (H^+) pumping from the matrix to the intermembrane space and when protons re-enter through complex V, the resulting protonmotive force drives ATP synthesis (Chianese and Pierantoni, 2021). However, excessive ROS production can disrupt mitochondrial function and impair

cellular activity, including sperm motility. A decrease in sperm motility from initial values is presented in Table 2, suggesting that storage at low temperatures negatively affects sperm quality. This decline in motility is likely caused by the presence of ROS, which are produced during the storage period. This finding aligns with the research report of Len *et al.* (2019), who state that prolonged storage can lead to excessive ROS production, thereby negatively impacting sperm quality.

Modification of the CEP diluent with various doses of *T. catappa* leaf extract significantly affected sperm motility in Simmental bull, both before freezing and after thawing ($p < 0.05$). The addition of 0.038 mg/mL Indian almond or Ketapang leaf extract resulted in the highest values in the sperm motility test at equilibrated (56.7 ± 2.9%) and post-thawed (43.3 ± 2.9%) stages. Post-thawed sperm motility is considered suitable for artificial insemination because the motility value exceeds 40% (SNI, 2021). Therefore, it can be suggested that the addition of 0.038 mg/mL Indian almond or Ketapang leaf extract to the CEP diluent can minimize the presence of ROS during low-temperature storage.

Flavonoids and phenolic compounds in *T. catappa* play a crucial role in maintaining sperm motility by protecting mitochondria and preserving ATP synthesis. Mitochondria are highly sensitive to oxidative stress, and the disruption of the electron transport chain by ROS reduces ATP availability, ultimately impairing flagellar movement. Flavonoids such as quercetin and kaempferol stabilise the mitochondrial membrane potential by inhibiting lipid peroxidation and enhancing endogenous antioxidant enzymes, including Superoxide Dismutase (SOD) and Catalase (CAT) (Aitken, 2017). This protective mechanism ensures sustained ATP production, which is necessary for progressive motility. Similar findings were reported by Najafi *et al*. (2018), who stated that antioxidant-rich diluents improve sperm motility during cryopreservation by preserving mitochondrial structure and function.

In Table 3 shows that sperm viability differed significantly among treatments ($P<0.05$). Treatment P4 exhibited the highest viability values at both the equilibrated and post-thawed stages, indicating that Indian almond or Ketapang leaf extract effectively preserved sperm cell integrity during cryopreservation. The differential staining observed reflects increased membrane permeability in dead spermatozoa, allowing for dye absorption, which is consistent with the findings of Hanifah *et al.* (2020). Overall, sperm viability decreased compared to the initial value, and the addition of various concentrations of Indian almond or Ketapang leaf extract in the CEP diluent influenced viability both before freezing and after thawing. The concentration of 0.038 mg/mL produced the highest viability, with values of 87.3 ± 1.2% before freezing and 79 ± 0% after thawing.

Sperm viability is closely associated with membrane stability and the ability of cells to resist ROS-induced apoptosis. Phenolic antioxidants in *T. catappa* neutralise hydrogen peroxide and hydroxyl radicals through hydrogen atom donation, thereby preventing oxidative damage to DNA and proteins that trigger cell death pathways (Jomova *et al*., 2024). According to Hassan *et al*. (2020), excessive ROS activates caspase-dependent apoptosis in spermatozoa, reducing cell survival. Flavonoids counteract this by modulating redox-sensitive signalling pathways and maintaining intracellular glutathione levels, a critical determinant of sperm viability.

Plasma membrane integrity, as shown by the average values in Table 4, decreased during storage and freezing. This parameter is a key indicator of sperm health and was evaluated using the Hypoosmotic Swelling Test (HOST) (Agarwal *et al*., 2016). According to Swelum *et al*. (2018), spermatozoa with intact tail membranes curl and swell in response to osmotic pressure, while Herdis *et al.* (2016) noted that low membrane integrity can disrupt sperm metabolism. The presence of flavonoids in Indian almond or Ketapang leaf extract helps prevent such damage by scavenging free radicals released during

peroxidation (Sholihin and Ducha, 2024). Flavonoids neutralize free radicals by donating hydrogen atoms (H), and their antioxidant activity depends on the number and arrangement of hydroxyl (–OH) groups in the core structure. The hydroxyl groups on ring B play a dominant role in capturing radicals, while rings A and C contribute less to the scavenging of superoxide anions (Arifin and Ibrahim, 2018). Falchi *et al*. (2018) explained that prolonged semen storage reduces motility, viability and membrane integrity due to nutrient depletion and ROS accumulation from oxidative metabolism. In this study, the addition of 0.038 mg/mL Indian almond or Ketapang leaf extract to the CEP diluent effectively maintained the quality of Simmental bull spermatozoa after freezing.

Antioxidants interact with ROS to stop chain reactions and prevent damage to vital molecules produced during normal body metabolism (Ibroham *et al.,* 2022). Indian almond or Ketapang leaves contain antioxidants in the form of phenolic compounds, flavonoids, terpenoids, saponins and tannins (Mulia *et al*., 2016). The flavonoids and phenolic compounds in Ketapang leaves exhibit antioxidant activity by scavenging free radicals (Widyasari *et al.*, 2019). Accor-ding to Zahra *et al.* (2024), flavonoids have the direct ability to neutralize ROS by stabilizing free radicals through the presence of phenolic hydroxyl groups. In the ROS inhibition process, flavonoids activate endogenous enzyme signaling pathways, such as CAT, SOD and Glutathione Peroxidase (GPx), to prevent the formation of hydrogen peroxide (H_2O_2) and hydroxyl radicals (.OH) (Treml and Šmejkal, 2016).

An interesting observation was found in the motility values, where treatment P3 showed a lower percentage than P2. This irregular pattern may be explained by the biphasic or hormetic effect, in which bioactive compounds exert beneficial effects at low to optimal doses but exhibit decreased effectiveness or even toxic effects at higher concentrations (Bondy, 2023). At the P3 concentration, bioactive components in Indian almond or Ketapang leaf extract, such as tannins and saponins, may have exceeded the optimal threshold, reducing the protective antioxidant effect and potentially disturbing sperm plasma membrane integrity. Such disruption could alter membrane permeability, cause osmotic imbalance, and impair mitochondrial function. Additionally, excessive antioxidant levels can shift the cellular redox state toward reductive stress, which negatively affects sperm quality similarly to oxidative stress (Li *et al*., 2022). In contrast, at the highest concentration (P4), spermatozoa appear to reach an adaptive redox equilibrium, where endogenous and exogenous antioxidant systems act synergistically to maintain optimal motility and cell function (Kowalczyk *et al*., 2022). The addition of 0.038 mg/mL Indian almond or Ketapang leaf extract (P4) to the CEP diluent produced the most favorable results, maintaining motility, viability and membrane integrity of Simmental bull spermatozoa. This effectiveness is likely attributed to the high flavonoid content, which functions as an antioxidant that neutralizes free radicals and protects spermatozoa during storage. However, this interpretation is inferred from existing literature, as direct biochemical confirmation was not performed in this study.

CONCLUSION

Indian almond or Ketapang leaf ext-ract (*T. catappa*) significantly ($p<0.05$) influ-ences the maintenance of Simmental bull spermatozoa quality during frozen storage. A CEP diluent containing 0.038 mg/mL of the extract maintained sperm motility at 43.3 ± 2.9%, viability at 79 ± 0% and membrane integrity at 79.7 ± 1.2% post-thawing. This suggests that Indian almond or Ketapang leaf extract can be used as a natural antioxidant additive in CEP diluent, with an optimal concentration of 0.038 mg/mL.

SUGGESTION

Based on the findings of this study,

further research is warranted to evaluate a wider range of Ketapang (Indian almond) leaf extract concentrations and elucidate the underlying mechanisms by which its bioactive compounds enhance spermatozoa preservation efficiency. In addition, direct application tests need to be carried out on artificial insemination programs in the field to see their effect on the success of pregnancy. Comparing the effectiveness of Ketapang leaf extract with other natural or synthetic antioxidants is also important to identify more efficient alternatives.

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