

MORINGA OLEIFERA LEAVES EXTRACT ENHANCES FRESH AND CRYOPRESERVED SEMINAL ATTRIBUTE OF JAMNAPARI BUCK

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ABSTRACT

The study was conducted to assess the impact of ethanolic extract of *Moringa oleifera* at different concentrations (2.0 and 5.0%) on the seminal parameters before and after cryopreservation of extended Jamnapari buck semen. A total of twelve ejaculates were collected twice a week from two Jamnapari bucks. Each ejaculate was diluted in a TFYG (Tris-citric-acid-fructose-egg-yolk-glycerol) extender keeping 80 million/mL and divided into three equal aliquots, which were supplemented with ethanolic extract of *Moringa oleifera* (0.0, 2.0 and 5.0%; Treatment C, T1 and T2, respectively), filled in 0.25 mL straws, cooled to and equilibrated for 4 hours at 4° C and then frozen in LN2 vapour. Frozen straws were thawed at 37° C for 30 seconds in a water bath for the post-thaw evaluation. Sperm motility, percent live sperm, sperm abnormalities, plasma membrane integrity (HOST) and enzyme leakage (AST ALT, ACP and AKP) were evaluated at both pre-freeze (on dilution) and post-thaw stages. The results indicated that the addition of ethanolic extract of *Moringa oleifera*, at both 2.0 % and 5.0% concentrations significantly ($P < 0.05$) improved sperm motility, sperm viability, and HOS-positive spermatozoa. Further, the sperm abnormalities and enzyme leakage were significantly ($P < 0.05$) lower in *Moringa oleifera* - treated groups than in control group. The results were the best with 5% ethanolic extract of *Moringa oleifera* supplementation. In conclusion, the study showed that *Moringa oleifera* at a concentration of 5% exhibited superior protection of sperm structures and functions as compared to 2.0% *Moringa oleifera* and the control group.

Keywords: Cryopreservation, Jamnapari, *Moringa oleifera*, Semen, TFYG

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Extender used for goat semen containing egg yolk is detrimental to spermatozoa due to presence of enzyme phospholipase A in seminal plasma secreted from bulbo-urethral gland. Furthermore, the phospholipase A hydrolyses the lecithin of the egg yolk to fatty acids and lysolecithin which are spermicidal and also cause coagulation of the storage medium (Roy 1957; Iritani and Nishikawa 1963), however no latest research available which suggest spermicidal effect of caprine seminal plasma at 10% egg yolk. Cryopreservation induces biochemical, functional, and ultra-structural alterations in spermatozoa (Tamburrino *et al.*, 2023). These alterations encompass the enhancement of permeability in the plasma and acrosome membranes of the spermatozoa, resulting in the disruption of their morphology, motility, and chromatin structure (Gandini *et al.*, 2006). ROS generation is the primary factor responsible for the decline in goat sperm quality during cryopreservation, as it leads to damage to the mitochondrial membranes of spermatozoa (Bansal *et al.*, 2011). Oxidative stress leads to the production of reactive oxygen species (ROS) and/or an imbalance of antioxidant defense mechanisms. This results in membrane lipids peroxidation and sperm injury (Mahanta *et al.*, 2012). Moreover, the elevated level of polyunsaturated fatty acids in the plasma membranes of

ruminant spermatozoa makes them more prone to lipid peroxidation (Bucak *et al.*, 2009). Spermatozoa possess an inherent enzymatic antioxidant defense system (Andrabi, 2009). Nevertheless, the antioxidants of seminal plasma are unable to cope up with reactive oxygen species (ROS) associated injuries (Neild *et al.*, 2005). In order to mitigate ROS associated injuries, natural antioxidants can be used as an additive (Bilodeau *et al.*, 2001). Indeed, addition of antioxidants in semen improves the seminal attributes and fertility of cattle (Iqbal *et al.*, 2016; Javed *et al.*, 2019) and caprine semen (Atessahin *et al.*, 2008). *Moringa oleifera* contains a lot of antioxidants, especially in the leaves (Das *et al.*, 2012). Ascorbic acid, flavonoids (quercetin, kaempferol, potassium, vitamin E, selenium, vitamin C, magnesium and proanthocyanidin), polyphenolics, and carotenes are found in *Moringa oleifera* leaves (Jayawardana *et al.*, 2015). These compounds lower the free radicals production and reduce the risk of sperm cell death (Wang *et al.*, 2017). Carrera-Chavez (2020) observed that a methanolic extract of *Moringa oleifera* seed improved post-thaw semen motility and antioxidant capacity in rams. To date, no literature available regarding antioxidative properties of *Moringa oleifera* leaves extract in TFYG diluter on the post-thaw seminal attributes of Jamnapari bucks.

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MATERIALS AND METHODS

Preparation of *Moringa oleifera* leaves extract

The mature, healthy and fresh *Moringa oleifera* leaves were harvested from Experimental Farm of College of Horticulture, ANDUAT Kumarganj, Ayodhya U.P. Fifty grams of dried *Moringa oleifera* leaves powder was extracted using a modified technique proposed by Khalaf *et al.* (2008).

Animals

Twelve semen ejaculates were collected from two Jamnapari buck, (2-3 years) reared at Deep Frozen Semen Laboratory, College of Veterinary Science & Animal Husbandry, ANDUAT Kumarganj, Ayodhya of Uttar Pradesh under standard management conditions.

Semen Collection, Processing and Freezing

Buck ejaculates were harvested twice in a week using AV and transfer to laboratory for analysis of mass motility (10X) and sperm concentration (Accucell photometer, IMV, France).

Selected ejaculates with visual motility above 75 % were diluted using a Tris-citric-acid-fructose-egg-yolk-glycerol (TFYG) extender. Diluted semen was split into three equal aliquots having different concentrations of 0%, 2%, and 5% of ethanolic extract of *Moringa oleifera* control (C) and treatment groups (T₁ and T₂), respectively, to make final sperm concentration of 80 million per ml. Diluted semen were automatically filled into 0.25 mL French straws (ISEVO, IMV, France) and equilibrated in a cold cabinet (Minitube, Germany) at 4° C for 4 hours. Straws were arranged horizontally and suspended in LN₂ vapour inside a foam box at 4 cm height above liquid nitrogen for 20 min, then dipped in liquid nitrogen (Najafi *et al.*, 2014).

Evaluation of semen's quality

Seminal attributes (sperm motility, viability and morphology) at post-dilution and post-thaw stage (Hafez and Hafez, 2000). The assessment of sperm plasma membrane integrity was carried out using a HOS test (Amorim *et al.*, 2009). The seminal plasma was separated from freshly diluted and frozen-thawed samples through centrifugation at 3000 rpm for duration of 20 minutes. The levels of enzyme leakage in seminal plasma (AST, ALT, AKP, and ACP) were measured using commercially available kits on the BEACON B Auto200 analyzer.

Statistical analysis

The values were presented as means $\pm$ SE for all variables. Statistical analysis was done with one-way ANOVA using Graph Pad Prism 5 software.

RESULTS AND DISCUSSION

The impacts of ethanolic extract of *Moringa oleifera* on the seminal parameters of frozen buck semen are depicted in Table 1. Semen samples cryopreserved with

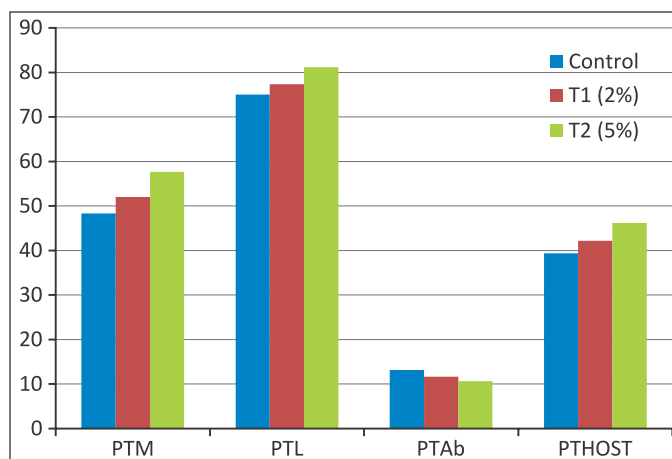


Fig. 1. Image showing post-thawed motility, livability and abnormality of control and ethanolic extract of *Moringa oleifera* (T1 and T2) treated group ($P < 0.05$)

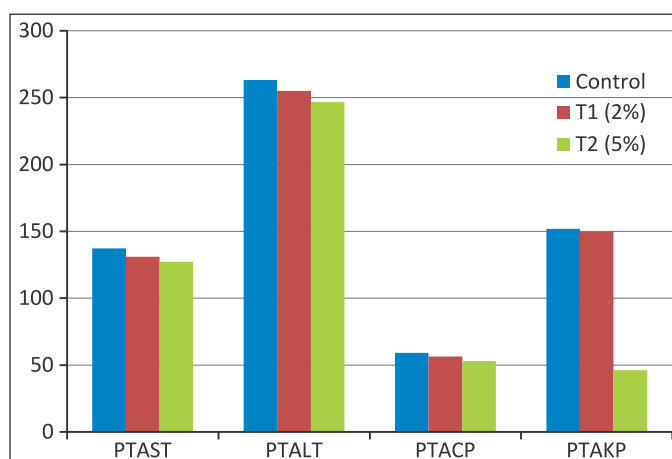


Fig. 2. Image showing post-thawed seminal plasma enzyme leakage (AST, ALT, ACP and AKP) of control and ethanolic extract of *Moringa oleifera* (T1 and T2) treated group ($P < 0.05$)

5.0% ethanolic extract of *Moringa oleifera* in TFGY extender had significantly ($P < 0.05$) higher percent post-diluted and post-thaw sperm motility, viability and morphological normal sperm followed by 2.0% ethanolic extract of *Moringa oleifera* as compared to the control extender (Fig. 1). The mean post-thaw motility recorded was in line with the findings of Hammad *et al.* (2019). Conversely lower (Mina & Husam, 2020) and higher (Wahjuningsih *et al.*, 2019) values were reported in previous studies. Our findings on sperm viability were in tune with Saurabh (2021), and lower than the findings of Mina and Husam (2020), but higher than those reported by Doidar *et al.* (2018).

Cryopreservation causes membrane dysfunction and excessive cell dehydration, leading to osmotic shock (Neild *et al.*, 2005). The damage is scribed to intracellular and extracellular ice crystals formation along with an increase in solute quantity (Mazur, 1984). However, cryopreservation can lead to alterations in sperm mitochondrial membrane fluidity, potentially leading to release of reactive oxygen species and changes in sperm membrane potential (Said *et al.*, 2010). ROS causes ATP depletion, which in turns

Table 1. Effect of ethanolic extract of *Moringa oleifera* (2.0% & 5.0%) supplementation in Tris extender on sperm quality parameters and enzyme leakage during cryopreservation of Jamnapari bucks semen

Stages	Additive concentration (%)	Progressive motility (%)	Sperm livability (%)	Sperm abnormality (%)	HOS reactive sperm (%)	AST (μmole/L)	ALT (μmole/L)	ACP KAU/100 ml	AKP KAU/100 ml
Post-diluted	Control (C)	78.67±0.8 ^a	86.33±0.6 ^a	6.83±0.60 ^b	49.00±1.0 ^a	60.33±2.7 ^c	119.3±3.20 ^c	27.17±1.8 ^c	43.17±2.6 ^c
	2.0% (T ₁)	79.67±0.8 ^b	87.17±0.70 ^b	6.33±0.67 ^{ab}	52.17±1.28 ^b	59.17±2.55 ^b	115.2±2.69 ^b	26.00±1.7 ^b	42.33±2.36 ^{bc}
	5.0% (T ₂)	81.00±0.8 ^c	88.67±0.6 ^c	5.67±0.56 ^a	55.67±1.2 ^c	56.50±2.4 ^a	110.7±2.5 ^a	24.00±1.7 ^a	39.83±2.2 ^a
Post-thaw	Control (C)	48.33±0.67 ^A	75.00±0.97 ^A	13.17±1.4 ^B	39.33±0.42 ^A	137.2±4.73 ^C	263.5±4.49 ^C	59.17±1.35 ^C	151.80±5.10 ^C
	2.0% (T ₁)	52.00±0.37 ^B	77.33±1.17 ^B	11.67±1.0 ^A	42.17±0.79 ^B	131.00±4.1 ^B	255.20±3.89 ^B	56.33±0.88 ^B	149.70±5.08 ^B
	5.0% (T ₂)	57.67±0.84 ^C	81.17±0.95 ^C	10.67±0.9 ^A	46.17±0.87 ^C	127.30±3.9 ^A	246.70±3.70 ^A	53.00±0.93 ^A	145.80±4.90 ^A

AST-ALT = Aspartate and Alanine amino-transferases, ACP-AKP = Alkaline and acid Phosphatases, Mean±SE values bearing different superscript within the stage (a, b, c / A, B, C) in a column differ significantly (P<0.05).

result in insufficient phosphorylation of the axoneme and lipid peroxidation. These effects ultimately decrease both motility and survival (El-Sherbiny *et al.*, 2022). *Moringa oleifera* leaves possess potent antioxidant properties by virtue of its ascorbic acid, carotenes, flavonoids and polyphenol content (Jayawardana *et al.*, 2015). These compounds have been shown to decrease the presence of free radicals and mitigate the possibility of cellular death (Wang *et al.*, 2017). Moreover, Carrera-Chavez (2020) observed that a 0.5 mg/mL concentration of *M. oleifera* seed extract effectively maintained the integrity of the sperm membrane.

The average percent HOS-reactive sperm cells were significantly (P<0.05) higher in extender containing 5.0% ethanolic extract of *Moringa oleifera* at both post-diluted and post-thaw stage followed by 2.0% ethanolic extract of *Moringa oleifera* than those of control extender (P<0.05, Table 1). Our findings are in tuned with Doidar *et al.* (2018), but were lower (El-Seadawy *et al.*, 2022) and higher (Kumar, 2022) values were also observed. Plasma membrane functionality and integrity are vital sperm parameters to consider when evaluating sperm quality because an intact plasma membrane is required for sperm cell survival (Makarevich *et al.*, 2010). Moreover, it plays essential roles in sperm metabolism, capacitation, acrosome reaction, eventually sperm-oocyte fusion (Forouzanfar *et al.*, 2013).

The significantly (P<0.05) lower values of AST, ALT, ACP and AKP activities in plasma of post-thawed semen were recorded in extender containing 5.0% as compared to 2.0% ethanolic extract of *Moringa oleifera* and the control extender (P<0.01, Table 1 & Fig. 2). Elevated enzyme activity in the extracellular medium indicates cold shock associated damage of spermatozoa membrane (Singh *et al.*, 1992). The assessment of enzymes also indicates the effectiveness of the extender in preserving the membrane integrity of spermatozoa during freezing and thawing (Prasad *et al.*, 2000). The addition of an ethanolic extract of *Moringa oleifera* leaves to the extender may have enhanced protection of the sperm

membrane, leading to the preservation of structural integrity and cell membrane permeability, ultimately resulting in reduced enzyme leakage. These findings align with the results reported by Srivastava and Kumar (2014). *Moringa oleifera* leaves are used for sperm preservation because they contain significant amounts of protein, calcium, potassium, magnesium, vitamin E, selenium, vitamin C, fat, and phenolic components (Ayodele *et al.*, 2014). Moreover, *Moringa oleifera* leaves contain a flavonoid compound that enhances antioxidant activity and prevent lipid peroxidation in sperm (Moyo *et al.*, 2012).

CONCLUSION

This study indicated that adding 2.0% and 5.0% ethanolic extract of *Moringa oleifera* to the extender improved the quality of post-thawed Jamnapari buck semen, highest preservation efficiency was observed in 5.0% ethanolic extract of *Moringa oleifera* compared to other treatments.

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