The Impact of *Moringa oleifera* Leaf Extract on Microbial Load in Bovine Semen at Refrigerated Temperatures

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ABSTRACT

Background: The bacterial load on semen sample be negatively impact the fertility of spermatozoa, there by resulting in total breeding failure. The Moringa olifera leaves extract (MOLE) may be a good substitute for the antibiotic component of traditional bovine semen extenders when refrigerated at refrigerated temperature. It may improve semen quality, reduce microbial load and help maintaining fertilization potential.

Methods: Present study was investigate the role of hot water Moringa extract supplementation on microbial load in liquid preserved buffalo semen. Agar well diffusion assay and standard plate count test used to evaluate the quantity of live microorganisms in the semen sample at different concentration of MOLE. The anti-bacterial activity for MOLE with 100 µl, 200 µl and 300 µl concentration as compared to control antibiotic solution with streptomycin, ranges from 44.04% to 69.48%, 62.00% to 84.03% and 32.63% to 79.60% againt *E. coli, B. cereus, E. faecalis microbes.*

Result: Our investigation is investigate the effect of *Moringa oleifera* leaves extract (MOLE) with different concentrations on semen samples at refrigerated temperatures. The higher concentration of M.O.L.E has showed significantly better anti-microbial effect for semen samples preserved up to 72 hrs. The Moringa extract addition in the tris-EYC extender significantly improved semen quality and showed antimicrobial activity during semen preservation at refrigerated temperature.

Key words: Antimicrobial activity, Microbial load, *Moringa oleifera,* Refrigerated temperature, Semen quality.

INTRODUCTION

More than 95% of the buffalo population is in Asia and play a significant role in the production milk and meat for rural livelihood. For the establishment and improvement of the indigenous non-descript breed of buffalo population, there is an enormous demand for Murrah buffalo semen. Several reproductive glands are involved in the formation of semen, but before it is expelled, it goes *via* the lengthy urethral canal (Kushwaha *et al.,* 2019; Yadav *et al.,* 2018). Semen is likely to become contaminated with bacteria during this phase while in general, bacteria move to urethral canal through the penile opening.

Staphylococcus spp., *Micrococcus spp., Corynebacterium spp., Escherichia coli, Pseudomonas spp., Proteus spp., Klebsiella spp*., *Bacillus spp*. other than *Bacillus anthracis* and *Streptococcus spp*. were among the numerous microbes found in frozen semen samples. However, *Pseudomonas, Streptococci, Staphylococci, Micrococci, Bacilli, Actinomyces, E. coli, Campylobacter, Corynebacterium pyogens*, *Trichomonas* and *Brucella* are the most commonly identified bacteria in the semen sample (Reddy *et al.,* 1971). According to Mitra *et al.* (2016), several of these microorganisms have been responsible for failure of livestock breeding, suggesting precautionary measures. Motility, shape and several others semen quality indicators are affected by microbial contamination (Najee *et al.,* 2012). Microbes present in semen compete with spermatozoa for nutrients and oxygen and affect normal growth while the formation of reactive oxygen species by macrophages, polymorphonuclear granulocytes and decrease in motility due to adherence of microbes with spermatozoa interfere the sperm function leads to reduction in fertilization potential. Microbes may have a direct effect on acrosome through toxin production (Rodeheaver, 1997; Morrell, 2006). Microorganism enters in to semen through number of routes including collection, pro-cessing or packaging of semen. The *Moringa oleifera* leaves contain higher

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concentrations of protein, carotene, vitamins, amino acids, minerals and numerous antioxidant compounds (polyphenols, flavonoids, proanthocyanidins and flavonols) (Anwar *et al.,* 2007; Khalafalla *et al.,* 2010). Strong antioxidant properties have been reported to be present in these polyphenols, flavonoids, proanthocyanidins, flavonols, vitamin C and E, carotene, zinc and selenium compounds (Dhalaria *et al.,* 2020). Supplementation of micromineral has improved qualitative as well as the quantitative aspects of semen quality in bulls (Patil *et al.,* 2023). Studies on caprine species indicated that providing Barki rams with MOLE supplements or its extract as a semen extender improved their antioxidant status, spermatozoa motility and viability and post-thaw sperm motility and viability (Shokry *et al.,* 2020; Shokry *et al.,* 2021). According to El-Seadawy *et al.* (2022) and Carrera-Chavez *et al.* (2020) sperm motility, viability and seminal antioxidant capacity in ram of ovine species were all improved when MOLE was added to semen extenders. For buffalo bulls' semen, the addition of MOLE to semen extender improved the quality and fertility of the post-thawed semen by boosting the antioxidant enzyme system's activities and decreasing cryodamage of the spermatozoa (Shokry *et al.,* 2024).

Moringa oleifera Leaf Extract may be a good substitute for the antibiotic component of traditional bovine semen extenders when refrigerated at 6°C for 72 hours. It may improve sperm motility, acrosome integrity, normal morphology and help maintaining fertilization potential. In order to improve preservation, we therefore hypothesised that MOLE incorporation in buffalo semen extender might be advantageous.

MATERIALS AND METHODS

Semen samples of four healthy Murrah buffalo bulls, maintained at ABRC, ICAR-NDRI, Karnal, were used for present study. Six ejaculates from each Murrah bull having mass activity of +3 and having more than 70% individual motility were used to study the effect of MOLE supplementation on semen diluter. Moisture free leaves of Moringa oleifera trees were dried in hot air oven, blended and kept at 4°C. Aqueous leaf extract of *Moringa oleifera* (MOLE) was made by soaking 50 grams of dry powder in 250 ml of autoclaved distilled water. The extract was incubated in hot air oven at 55°C for overnight and leaf residue were separated and kept at -20°C for further uses. To achieve the goal in the present study, two approaches were made. In the first approach, dose dependent effect of moringa hot water extract was tested for its effect on antimicrobial sensitivity against common pathogen found in semen. After the quantification of antimicrobial sensitivity, extract was supplemented in tris-EYC diluter before semen dilution, liquid preserved at 4°C up to 72 hrs. and microbial load was counted by standard plate count. Different concentrations of Moringa oleifera heat treatment aqueous extract (Control, 100 µl, 200 µl, 300 µl of extract with 2.25 ml solution) were mixed with freshly prepared tris yolk citrate extender. In second approach, semen sample was immediately diluted and stored at 4°C and the various semen quality parameters were recorded at 4 hrs, 24 hrs, 48 hrs and 72 hrs. Anti-microbial activities were assessed by agar well diffusion assay and standard plate count methods. The plates containing 15 ml nutrient agar medium containing 105 target cultures was overlaid and MOLE were added at different concentrations in to the wells and kept for proper diffusion along with incubation at 37°C for 24 hrs. At the end of incubation, zone formation surrounding the well will be measured (mm). All three extracts were quantified for its total antioxidant power by biochemical assays and select qualified extract for its chemical properties by liquid chromatography-Electrospray ionization Mass spectroscopy (LC ESI-MS) and atomic absorption spectroscopy (AAS).

One ml of extended semen samples at refrigerated at 4°C and sampled at different time intervals (4, 24, 48 and 72 hour) from each treatment was mixed with 9 ml of sterile normal saline solution (1st dilution). This diluted semen sample, 1ml was transferred to second set of 9 ml sterile saline solution to get 1/100 dilutions (10^{-2}) and so on to get a dilution of 10^{-6}.

Standard plate count test is useful for the quantitative measurement of number of viable micro-organisms in the given test semen sample. This test employs the serial dilution technique for easy quantification of the organisms in view of a wide range of bacterial population that may occur in semen. The appropriate dilutions of the semen samples are mixed with a sterile nutrient medium that can support the growth of the organisms when incubated at a suitable temperature. Each bacterial colony that develops on the plate is presumed to have grown from one bacterium or clump of bacteria in the inoculums. The total number of colonies counted on the plates multiplied by the dilution factor is taken to represent number of viable organisms present in the sample.

Tris EYC diluted buffalo semen, supplemented with or with out *Moringa oleifera* leaf aqueous extract stored at 4°C up to 72 hrs were tested at different time intervals for its effects on microbial load and analysed with two-way ANOVA (Between storage time in a treatment; between treatment at a constant storage time).

Plating the sample and preparation of plates

One ml of each required dilution (factor 10^{-1} to 10^{-5}) was transferred into a sterile petridish that was previously labeled with dilution number, sample description and date. To each petridish, 15 to 20 ml of liquified sterilized nutrient agar and 1 ml of above serially diluted samples were mixed and was poured and allowed to cool and set a soft gel. Finally, petri plates were kept in invert position and overnight incubated at 37°C for 48 hours. After the end of the incubation period the average number (taken in duplicates) of the counts of bacterial colonies were counted and multiply by the dilution factor to determine the exact number of viable bacteria in sample.

RESULTS AND DISCUSSION

In result, streptomycin showed maximum (18.33 ± 0.88 mm) diameter of bacterial zone of growth inhibition which was followed by MOLE concentration of 300 µl, 200 µl and 100 µl. However, the mean value of diameter of zone of inhibition in MOLE concentration with 300 µl) was non-significant (P>0.05) with that of streptomycin. In comparison to different concentration MOLE with 100 µl) showed significantly (P>0.05) lesser diameter zone of inhibition as compared to 200 µl and 300 µl of MOLE and the mean values were statistically significant (P>0.05) to only streptomycin but not others (Table 1).

Streptomycin showed higher sensitivity against activated *E. Coli* followed by MOLE concentration of 300 µl, 200 µl and 100 µl. Zone of inhibition with 300 µl of concentration had highest among all doses of extract but was significantly (P>0.05) lower than streptomycin and higher than100 µl concentration of MOLE. Inhibition zone with 200 µl was even higher than100 µl and lower than 300 µl of MOLE. But inhibition zone diameter with 100 µl concentration was significantly lower than 300 µl and streptomycin concentration but non-significant with 200 µl of MOLE concentration. The antimicrobial sensitivity for MOLE with different concentration showed a significant microbial sensitivity against *E. coli.* Similarly, Pal *et al*. (1995); Peixoto *et al*. (2011) and Gangwar *et al*. (2024) had also reported significant antimicrobial activity of *M. oleifera* against gram negative bacteria including *E. coli.*

B. cereus was sensitive to streptomycin and a large zone of bacterial growth inhibition was observed in agar plate. However, comparable inhibition zone was observed with MOLE and was dose independent. Among all MOCE doses, maximum zone was observed with higher concentration and mean diameter was non-significant to streptomycin, with 200 µl and 100 µl of concentration. However, mean values of inhibition zone diameter with100 µl concentration was non-significant with 200 µl concentration of MOLE. In present studies, it was observed that high specific and strong antimicrobial activity of *M. oleifera* hot water extract against *B. cereus*. As reported by Viera *et al*. (2010), a positive effect of antibacterial effect (*in vitro*) of *Moringa oleifera* against Gram positive bacteria, which was similar to present finding.

For *E. faecalis*, mean diameter of inhibition zone for 300 µl of concentration sample was non-significant to that of streptomycin control. As compared to samples with 300 µl concentration of MOLE, 200 µl and 100 µl has showed significantly smaller diameter of growth inhibition zone. The antimicrobial sensitivity test for MOLE, against E. *Faecalis*, shows positive effect against microorganism and finding were similar to report shown by Anand *et al.* (2016) and Peixoto *et al*. (2011).

Effect of *Moringa oleifera* leaf hot water extract supplementation in tris-EYC semen diluter on bacterial load in liquid semen preserved at 4°C for 72 hrs.

The result for the Effect of MOLE supplementation in tris-EYC semen diluter on bacterial load in liquid semen preserved at 4°C for 72 hrs have shown high and positive correlation exist between bacterial load and storage time (Table 2). Anti-bacterial effectiveness for MOCE was found to be directly proportional to its concentration in semen diluter. However, all three concentrations of MOLE, which were tested in our experiments had shown significantly (P>0.05) lower bacterial load for diluted semen. It was found that, for 72 hrs. of preserved semen sample, the anti-bacterial effectiveness of MOLE with 100 µl, 200 µl, 300 µl were respectively, 75.96%, 88.21%, 95.53% to that of control sample containing antibiotics. As it was indicated in above result of MOCE with different concentration, the anti-microbial sensitivity against Gram positive and Gram-

Table 1: Mean ± SEM from six experiments. Means with different letters in a row (between treatment) and column (within treatment comparisons to storage time) are significantly different at p<0.05.

Treatment	Time	Control	Moringa hot water extract		
			100 µl	200 µl	300 µl
Inhibition zone	*S. aureus*	18.33^{a}±0.88	8.33^{b}±1.20	10.33^{b}±1.20	13.33^{ab}±1.20
(mm)	*E. coli*	19.66^{a} ±0.33	8.66^{bc}±1.33	11.66^{cb}±0.88	13.66^{b}±0.66
	B. cereus	16.66^{a} ±0.88	10.33^{b}±0.33	11.33^{b} ±1.20	14.00^{c}±0.57
	E. faecalis	16.33^{a} ±1.20	5.33^{b}±0.33	9.33^{ab}±0.66	13.00^{a}±0.57

Table 2: Means with different letters in a row (between treatment) and column (within treatment in comparisons to storage time) are significantly different at P<0.05.

Treatment	Time	Control	Moringa hot water extract		
			100 µl	200 µl	300 µl
CFU×10^{3}/ml	4 hr	1.04^{a}±0.11	2.96^{b} ±0.32	1.79^{a}±0.24	1.13^{a}±0.17
	24 hr	1.75^{a} ±0.19	4.54^{b}±0.37	2.75^{a}±0.33	1.83^{a} ±0.21
	48 hr	4.04^{a}±0.18	6.75^{b} ±0.26	5.63^{bc} ±0.31	4.79^{a} ±0.30
	72 hr	12.42^{a}±0.18	15.96^{b}±0.41	14.08^{c}±0.37	13.00^{a}±0.35

Table 3: List of bioactive components present in *M. oleifera* leaf hot water extract.

Antioxidant	Antimicrobial	Effect on sperm function	
4-Phenyl-1,2,3 thiadiazole	2-Formyl-1-indanone	Methyl cinnamate	Increase sperm motility
1-Naphthol	4-phenyl-1,2,3-thiadiazole	Chelerythrine	Capacitation inhibitor
litcubine	Methyl cinnamate	Calaxin	Sperm chemotaxis, motility and fertilization
Barpisoflavone c	2-Naphthol	Naphthalene	Increase sperm CASA parameters in human
Racemoflavone	Glyphosate	Atraric acid	Positively affect sperm motility
Kanakugiol	Evoxine	Chlorohydrin	Contraceptive
Novobiocic acid	Pseudopelletierine	-	-
Phosphatidylserine	2-methylimidazole	-	-
Azulene	5-Phenyl-1,3-pentadiyne	-	-
Orsellinic acid, ethyl ester	s-Propyl 1-propanesulfinothioate	-	-
Pyrogallin	Droserone	-	-
-	Danielone	-	-
-	Quinic acid	-	-

m/z values corresponding to each biochemical component appeared in *M. oleifera* hot water extract was used as an input to mine the online MS database. Each compound identified in online database was examined in pubcam for its importance in any possible bioactivity.

negative bacteria, supplementation of *Moringa oleifera* to tris-EYC diluter significantly reduced bacterial load in liquid preserved buffalo semen. Gangwar *et al*. (2024) reported that supplementing cryopreservation solutions with *M. oleifera* aqueous extract significantly reduced the microbial load in buck semen and improved sperm motility, acrosomal integrity, plasma membrane integrity and viability. No reports are available in the use of *Moringa oleifera* extract in Murrah buffalo semen supplementation and its effects on bacteriological quality of extended semen. However, in LC-MS (liquid chromatography - mass spectroscopy) experiment on *Moringa oleifera* extract to find antimicrobial component it was observed 13 components which exhibit antimicrobial activity (Table 3). The low microbial load in extended liquid preserved semen may be due to the strong anti-microbial activities of methyl cinnamonate (Avetisyan *et al.,* 2017; Radaelli *et al.,* 2016; Wong *et al.,* 2008), glyphosate (Shehata *et al.* 2013; Sh *et al.,* 2008; Kurenbach *et al.,* 2015) and quinic acid (Rezende *et al.,* 2015; Gohari *et al.,* 2010).

CONCLUSION

Liquid preservation followed by artificial insemination (AI) of diluted semen is a very simple and economical technique, adopted by most of local semen banks, for the use superior germplasm in field. From present study, it is well evident that, supplementation of M.O.L.E. as compared to control antibiotic (streptomycin), exhibit anti-microbial sensitivity against *S. aureas, E. coli*, *B. cereus* and *E. faecalis*. However, higher concentration of MOLE has shown significantly better anti-microbial effect in samples liquid preserved semen up to 72 hrs.

Conflict of interest

All authors declare that they have no conflicts of interest.

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