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Antibiotic Potential of Moringa Leaf (*Moringa oleifera* Lam.) Crude Extract in Bull Semen Extender

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Authors' contributions

This work was carried out in collaboration between all authors. Author OAS designed and supervised the study. Author OSA prepared and packaged the manuscript for publication. Author AAL carried out the laboratory work and wrote the manuscript. Author EAA assisted with both the field and laboratory work. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The effect of *Moringa oleifera* crude extract (MOCE) was carried out on Friesian bull semen for its antibiotic potential.
Design of the Experiment: The design of the experiment was completely randomized design.
Place and Duration of Study: Artificial Insemination Laboratory of Livestock Investigation Division, National Veterinary Research Institute, K-Vom, Plateau State and teaching and research farm, university of ibadan, nigeria between april and may 2012.
Methodology: Semen obtained was diluted in glucose yolk citrate extender containing 0 ml, 4 mls, 8 mls, 12 mls and 16 mls of MOCE as treatments 1(control) to 5 respectively diluted at ratio 0.5 mls semen to 5 mls of extender and refrigerated at 6°C for 72 hours.
Sample: We included 2 Friesian bull and MOCE. Sperm concentration, percentage progressive motility, plasma membrane integrity, percentage livability, normal sperm morphology and acrosome integrity were studied at 0 hour, 24 hours, 48 hours and 72 hours.

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Results: Mean progressive motility and plasma membrane were significantly (P < .05) higher at 12 mls (97.67%) and 16 mls (99.33%) of MOCE inclusion compared to control. Percentage livability decreased significantly (P < .05) with increasing MOCE inclusion across treatments up to 48 hours. Normal sperm morphology of MOCE based Treatments were significantly (P < .05) higher than that of the control (0 ml). There was a significant (P<.05) increase in the mean values of Acrosome Integrity at 4mls and 16mls MOCE inclusion level after 24 hours.
Conclusion: 16 mls inclusion level of MOCE could be a good substitute for the antibiotic component of conventional bovine semen extender as it enhances the sperm percentage motility, acrosome integrity and normal morphology thereby preserving the fertility potential of spermatozoa when refrigerated at 6°C for 72 hours.

Keywords: Antibiotic; bull semen; crude extract; Moringa leaf; semen extender.

1. INTRODUCTION

Moringa oleifera Lam, locally known as shajna, belongs to the monogeneric family Moringaceae and is widely distributed in the Indo-Bangla subcontinent and cultivated throughout the tropical belt [1]. It is the most widely cultivated species of a monogeneric family. It is now widely cultivated and has become naturalized in many locations in the tropics. It is a perennial softwood tree with timber of low quality, but which for centuries has been advocated for traditional medicinal and industrial uses. It is already an important crop in India, Ethiopia, the Philippines and the Sudan, and is being grown in West, East and South Africa, tropical Asia, Latin America, the Caribbean, Florida and the Pacific Islands [2].

The plant possesses many valuable properties which make it of great scientific interest. These include the high protein content of the leaves twigs and stems, the high protein and oil contents of the seeds, the large number of unique polypeptides in seeds that can bind to many moieties, the presence of growth factors in the leaves, and the high sugar and starch content of the entire plant. Equally important is the fact that few parts of the tree contain any toxins that might decrease its potential as a source of food for animals or humans [3].

Previous studies have reported that various parts of *Moringa* roots, flowers, bark, and stem including seeds possess antimicrobial properties [4,5]. The leaves are also rich source of essential amino acids such as methionine, cystine, tryptophan, and lysine with a high content of proteins. The extracts of leaves, seeds and roots of *Moringa oleifera* have been extensively studied for many potential uses including wound healing, anti-tumour, anti-fertility, hypotensive and analgesic activity, antipyretic, antiepileptic, antiinflammatory, antiulcer, antispasmodic, diuretic, hypocholesterolaemic, antifungal, antibacterial, antioxidant etc., The paste of the leaves is used as an external application for wounds. Moringa leaves are a good source of natural antioxidants and very few methods have been reported for the estimation of different constituents present in Moringa plant [6]. *Moringa oleifera* leaf may also prevent oxidative stress [7].

The importance of semen extender is significant because many livestock and domestic pet breeders rely on semen obtained from animals that they themselves do not own. Transporting an animal for breeding purposes is often time-consuming and expensive, and in the case of livestock, it is often much more efficient to use artificial insemination in order to increase one's herd. Semen extender is a product that can be mixed with semen to prevent it from deteriorating while it is being held or shipped for eventual insemination. Various types of semen extender are often used by animal breeders who may need to ship semen to other breeders across the country or even around the world [8].

There is paucity of information on the assessment of the antibacterial property of MOCE in bovine semen extender. Therefore, the objective of this study is to assess the antibiotic potential in MOCE as a replacement for the antibiotic component of a conventional extender and its effects on bovine semen quality.

2. MATERIALS AND METHODS

2.1 Experimental Location

The study was carried out at the Artificial Insemination Laboratory of Livestock Investigation Division, National Veterinary Research Institute, K-Vom, Plateau State, Nigeria. The experimental site is located at 1.28

m above sea level and lies on longitude 8.45" East and latitude 9.43" North.

2.2 Experimental Animal

Semen collected from a mature bull was used for this experiment. The bull was housed in a well-ventilated pen at the dairy unit of the Livestock Investigation Division of the National Veterinary research Institute. The bull was fed with grasses and concentrate supplement concentrates containing Crude Protein 19.16%, Crude Fibre 6.63%, Fat 3.67%, Energy 2,372Kcal, Calcium 0.88%, Lysine 0.60% that met its nutritional requirement. Clean fresh water was provided *ad-libitum* and the pen was cleaned on regular basis.

2.3 Preparation of MOCE

Mature fresh, *Moringa olifera* leaves were harvested from University of Ibadan, Nigeria, and identified. The leaves were weighed and then washed properly with distilled water after which the leaves were air-dried for 24 hours, and then placed in the oven to dry at 45°C. After oven drying, moisture lost was determined before blending.

Blended dried leaves were soaked in distilled water (1 ml to 1 gram of weight loss in Moringa leaf) for 24 hours at 4°C, after which the filtrate and leaf paste were separated using a cheese cloth. The filtrate was further filtered using a whattman filter (12.5 cm) paper. The crude aqueous extract from this process was kept in the freezer until the time of usage.

2.4 Preparation of Semen Extender

Semen extender was prepared as shown on Table 1 [9]. Since semen collected for this study was preserved by chilling and not cryopreserved, glycerol was not used.

Table 1. Composition of glucose yolk citrate

Composition	Proportion
Distilled water	100 ml
Glucose	58 mg
Sodium Citrate	5 gm
Egg yolk	20 ml
Penicillin G (Procaine Propen-G ®)*	1 mg/1000 mls
Streptomycin (Matostrep ®)*	1.00 mg/ml

*Modified from Chaudhari and Mshelia , (2002); * Included only in Treatment 1 for control extender*

2.5 Treatments Groups

Treatment 1 (Control): Extender + antibiotics (0 mls of MOCE)
Treatment 2: Extender + 4 mls of MOCE
Treatment 3: Extender + 8 mls of MOCE
Treatment 4: Extender + 12 mls of MOCE
Treatment 5: Extender + 16 mls of MOCE
The design of the experiment was completely randomized design.

2.6 Semen Collection

Semen was collected from the bull using an artificial vagina. Prior to collection of the semen, the preputial pouch of the bull was cleaned with water, to remove urine and other materials that could contaminate semen during collection. The Experimental bull was stimulated with a teaser-cow. Once the penis of the bull was out of the prepuce, the artificial vagina was inserted into the penis for semen collection.

2.7 Semen Processing and Evaluation

Semen processing was done as recommended by National Animal Production Research Institute, SHIKA, Nigeria, [9]. The volume of the collected semen sample was determined using a volumetric tube after which the semen sample was placed in a water bath at 30 to 34°C and examined microscopically under a low power objective lens for wave pattern and gross motility. The Semen was diluted at ratio 0.5 ml of semen to 5 mls of extender for all the treatments. After dilution, it was then refrigerated at 6°C and readings were taken at 0 hour, 24 hours, 48 hours and 72 hours.

2.7.1 Semen colour assessment

Semen colour was assessed visually. Semen collected was checked for the presence of blood stains, dirt, hair or any other contaminant. Good semen should not contain any contaminant. The semen collected for this study was free from all contaminants.

2.7.2 Semen volume

Collected semen was determined using a graduated volumetric test tube and value obtained was recorded in mls [10].

2.7.3 Sperm concentration

The IMV® electronic photometer was used to determine the concentration. The degree of

dilution was aimed at giving an insemination dose rate of 25 to 30 million sperm cells per straw [9].

2.7.4 Progressive sperm motility

To assess sperm motility, a drop of freshly collected semen was mixed with sodium citrate buffer on a clean warm glass slide, covered with a cover-slip and examined with a microscope under X40 objective lens. The percentage progressive motility of spermatozoa were estimated and the motility score was subjectively rated between 0 and 100 [11].

2.7.5 Determination of intact acrosomes

To assess the acrosomal integrity 100 µl semen sample was fixed in 500 µl of 1% formal citrate (2.9 g tri-sodium citrate dehydrate, 1 ml of 37 % solution of formaldehyde, dissolved in 100 ml of distilled water). Normal acrosome was characterized by normal apical ridge (NAR). One hundred spermatozoa per experimental extender were counted with phase contrast microscope (X 1000) under oil immersion [12].

2.7.6 Percentage liveability and morphology

This was done by placing a drop of semen on a warm glass slide, one drop of Eosin-Nigrosin stain was added and mixed gently, it was then smeared on a slide with the edge of another clean slide, air dried and viewed under the microscope at magnification of ×400. The dead sperm cells absorbed the stain while live sperm cells did not. All sperm cells with coiled or double tail, damaged mid-piece and damaged or detached head were considered abnormal [11].

2.7.7 Plasma membrane integrity

Plasma membrane integrity (PMI) of bull spermatozoa was assessed by hypo-osmotic swelling (HOS) assay. The solution of HOS contained sodium citrate 0.73 g and fructose 1.35 g, dissolved in 100 ml distilled water (osmotic pressure ~190 mOsmol kg^{-1}). The assay was performed by mixing 50 µl of the semen sample to 500 µl of HOS solution and incubated at 37°C for 40 min. After incubation, a drop of semen sample was examined under phase contrast microscope (X 400; Olympus BX40, Japan). One hundred spermatozoa were counted for their swelling characterized by non-intact plasma membrane [12].

2.8 Statistical Analysis

Data obtained was analysed using the analysis of variance procedure of SAS [13] and means were separated using the Duncan's multiple range test of the same software.

3. RESULTS

The fresh semen collected from a Friesian bull using artificial vagina measured 9 mls with concentration of 495 x 10^6. It was milky in colour, gross motility was +++ and progressive motility was 90%. The extended bull semen parameters measured in this study includes % Progressive motility, Plasma membrane integrity, % Livability, normal Morphology and Acrosome Integrity.

3.1 Effect of MOCE on Progressive Motility (%) of Extended Bull Semen

The effect of MOCE on Progressive Motility of Extended Bull Semen is shown on Table 2. At 0 hour, Mean progressive motility for 12 mls and 16 mls were not significantly ($P > .05$) different from 8 mls but significantly ($P < .05$) different from 0 ml. Progressive Motility was significantly lowest at 4 mls. At 24 hours, Mean progressive motility was highest at 16 mls (90.00), it varied significantly ($P < .05$) from 0 ml, 8 mls and 12 mls which are significantly ($P > .05$) the same. Its lowest value is at 4 mls (70.00). At 48 hours, Mean progressive motility also varied significantly ($P < .05$) across the treatments but 4 mls and 8 mls do not vary significantly ($P < .05$). At 72 hours, Mean progressive motility of 4 mls and 16 mls varied significantly ($P < .05$) from 8 mls, 12 mls which also varied significantly ($P < .05$) from 0 ml. Progressive motility is an indication of the active metabolism and integrity of membranes [14]. Irrespective of the period of evaluation, Progressive motility is positively influenced as MOCE inclusion level increases. This could be attributed to the mild effect of the antimicrobial component in MOCE as compared to the industrial antibiotics used in the conventional extender. The effect of MOCE in extender on the progressive motility of bull spermatozoa at 0, 24, 48 and 72 hours shows that Progressive Motility values decreased with time. It thus agrees with Frydrychová et al. [15] who reported that the influence of extender and storage time on motility was statistically significant. The decrease in progressive motility recorded in this study compared favourably with those obtained by Foote et al., [16] who recorded lower motility values after 24 hours of storage

and Kevin et al. [17] who stated in an Animal science fact sheet that semen motility decreased during storage. Similar report by Muhammad et al., [12] showed that there was a significant decrease in progressive motility at 0, 2 and 4 hours after thawing. Also, Ambrogi et al. [18] also obtained similar result in which there was low sperm motility after 72 hours of storage which will significantly influence the fertilizing ability of sperm.

3.2 Effect of MOCE on Plasma Membrane (%) of Extended Bull Semen

The effect of MOCE on Plasma Membrane of Extended Bull Semen is shown on Table 3. The result shows that Plasma membrane for 0 ml, 4 mls, and 16 mls at 0 hour and 24 hours were significantly ($P < .05$) different from 8 mls and 12 mls. At 48 hours, Plasma Membrane of 0 mls varied significantly ($P < .05$) from 4 mls and 16 mls which in turn varied significantly ($P < .05$) from 8 mls and 12 mls. At 72 hours, Plasma Membrane was highest at 0 ml (60.00) and varied significantly ($P < .05$) from 4 mls, 8 mls and 16 mls which also varied significantly ($P < .05$) from 12 mls. The higher mean values of Plasma Membrane at 24 hours for 4 mls and 16 mls inclusion level with no significant difference from control suggest that MOCE has the ability to compete favourably with the conventional citrate - egg yolk extender to preserve membrane integrity. This commemorates with Muhammad et al., *[*12] who reported a significantly higher percentage of sperm cells with intact functional plasma membrane in extender containing L-cysteine 1mM as compared to 0.5, 2.0, 3.0 mM and control at 4 hours. Decreased mean values of MOCE based treatments after 48 hours in this study negates the reports of Bucak et al. [19] and Özkan et al., [20] where addition of cysteine in ovine semen resulted to high percentage of sperm cells with functional plasma membrane with significant increased catalase activity. This suggests that catalase activity could have been reduced in Moringa based treatments.

3.3 Effect of MOCE on Livability (%) of Extended Bull Semen

The effect of MOCE on Livability of Extended Bull Semen Parameters from 0 to 72 hours is shown on Table 4. Result of % Livability at 0 hours decreased significantly ($P < .05$) across the treatments. At 24 hours, % Livability was highest at 0 ml (72.33) and varies significantly ($P < .05$) from 4 mls, 8 mls and 16 mls which also varied significantly ($P < .05$) from 12 mls. At 48 hours, % Livability of 0 ml and 16 mls do not vary significantly ($P > .05$) from 4 mls and 8 mls but vary significantly *(*$P < .05$) from 12 mls. At 72 hours, % Livability of 0 ml does not vary significantly ($P > .05$) from 16 mls. At 16 mls, it does not vary significantly ($P > .05$) from 8 mls which in turn do not vary significantly ($P > .05$) from 4 mls and 12 mls. The significant ($P < .05$) decrease in percentage livability with increased MOCE inclusion across the treatments up to 24 hours indicates that Moringa contains some phytochemicals which if present in sufficient quantity, could increase mortality of sperm cells during preservation.

3.4 Effect of MOCE on Normal Sperm Morphology (%) of Extended Bull Semen

The effect of MOCE on Normal Sperm Morphology of Extended Bull Semen Parameters from 0 to 72 hours is shown on Table 5. Normal sperm morphology at 0 hour was significantly ($P < .05$) different across the treatments with the highest value at 8 mls (100.00) and lowest at 0 ml (86.33). At 24 hours, normal sperm morphology varied significantly ($P < .05$) across the treatments but 12 mls and 16 mls do not vary significantly ($P > .05$). At 48 hours, Normal sperm morphology was highest at 8 mls (97.33) and varied significantly ($P < .05$) from 4 mls, 12 mls and 16 mls which also varied significantly ($P < .05$) from 0 ml. At 72 hours, Normal sperm Morphology was highest at 8 mls and varied significantly ($P < .05$) from 4 mls, 12 mls and 16 mls which also varied significantly ($P < .05$) from 0 ml. All the mean values obtained for Normal Sperm Morphology of MOCE included Treatments were higher than that of the control (0 ml). This suggests that MOCE helps to maintain intact sperm morphology and this could be attributed to the presence of Tannins because of its astringent effect [21]. The optimum level of MOCE that best preserve the Normal Sperm Morphology is 8 mls indicating that excessive Tannins above optimum level will reduce Sperm Morphology. High percentage of normal sperm morphology is an indication of reduced microbial activities on the sperm cells.

3.5 Effect of MOCE on Acrosome Integrity (%) of Extended Bull Semen

The effect of MOCE on Acrosome Integrity of Extended Bull Semen Parameters from 0 to 72

hours is shown on Table 6. At 0 hour and 72 hours, Mean acrosome integrity was not significantly (P < .05) different across the treatments. At 24 hours, Mean acrosome integrity of 4 mls and 16 mls varied significantly (P < .05) from 0 ml, 8 mls and 12 mls. At 48 hours, Mean acrosome integrity of 4 mls and 12 mls varied significantly (P < .05) from 0 ml, 8 mls and 16 mls. The significant (P<.05) increase in the mean values of Acrosome Integrity at 4mls and 16mls MOCE inclusion level after 24 hours contradicts the findings of Kommisrud et al. [22] and Frydrychová et al. [15] who reported that the storage time significantly influenced motility and acrosome integrity. This indicates that MOCE inclusion at 4 mls competes favourably in preservation of the Acrosome Integrity better than a conventional extender.

Table 2. Effect of MOCE on progressive motility (%) of extended bull semen

Time (hours)	Moringa Inclusion (mls)					SEM
	0	4	8	12	16	
0	90.67 ^{b}	80.67 ^{c}	95.00 ^{ab}	97.67 ^{a}	99.33 ^{a}	1.876
24	80.00 ^{b}	70.00 ^{c}	81.00 ^{b}	81.00 ^{b}	90.00 ^{a}	1.704
48	50.00 ^{d}	69.67 ^{b}	71.00 ^{b}	60.67 ^{c}	80.00 ^{a}	2.733
72	46.00 ^{c}	70.00 ^{a}	59.67 ^{b}	59.00 ^{b}	69.67 ^{a}	2.358

a,b,c: Means along the same row with different superscripts are significantly (P < .05) different ; MOCE: Moringa oleifera crude extract ; SEM: Standard Error of Mean

Table 3. Effect of MOCE on plasma membrane (%) of extended bull semen

Time (hours)	Moringa Inclusion (mls)					SEM
	0	4	8	12	16	
0	73.33 ^{a}	74.33 ^{a}	64.33 ^{b}	63.33 ^{b}	75.00 ^{a}	1.439
24	70.00 ^{a}	71.00 ^{a}	60.67 ^{b}	60.00 ^{b}	70.00 ^{a}	1.323
48	71.00 ^{a}	44.67 ^{b}	41.00 ^{c}	40.00 ^{c}	45.00 ^{b}	3.078
72	60.00 ^{a}	40.67 ^{b}	40.00 ^{b}	38.00 ^{c}	40.00 ^{b}	2.194

a,b,c: Means along the same row with different superscripts are significantly (P < .05) different ; MOCE: Moringa oleifera crude extract ; SEM: Standard Error of Mean

Table 4. Effect of MOCE on livability (%) of extended bull semen

Time (hours)	Moringa Inclusion (mls)					SEM
	0	4	8	12	16	
0	77.67 ^{a}	67.67 ^{b}	66.67 ^{bc}	60.67 ^{cd}	59.00 ^{d}	1.914
24	72.33 ^{a}	58.33 ^{bc}	60.00 ^{b}	50.00 ^{c}	56.67 ^{bc}	2.171
48	56.67 ^{a}	50.00 ^{ab}	50.00 ^{ab}	46.67 ^{b}	56.67 ^{a}	1.447
72	56.67 ^{a}	43.33 ^{c}	46.67 ^{bc}	40.00 ^{c}	53.33 ^{ab}	2.000

a,b,c: Means along the same row with different superscripts are significantly (P < .05) different ; MOCE: Moringa oleifera crude extract ; SEM: Standard Error of Mean

Table 5. Effect of MOCE on normal morphology (%) of extended bull semen

Time (hours)	Moringa Inclusion (mls)					SEM
	0	4	8	12	16	
0	86.33 ^{d}	96.33 ^{c}	100.00 ^{a}	98.67 ^{ab}	97.33 ^{bc}	1.325
24	84.33 ^{d}	94.33 ^{c}	99.33 ^{a}	96.67 ^{b}	95.67 ^{bc}	1.385
48	80.67 ^{c}	91.33 ^{b}	97.33 ^{a}	92.67 ^{b}	90.67 ^{b}	1.480
72	79.33 ^{c}	89.67 ^{b}	93.33 ^{a}	90.67 ^{b}	88.67 ^{b}	1.293

a,b,c: Means along the same row with different superscripts are significantly (P < .05) different ; MOCE: Moringa oleifera crude extract ; SEM: Standard Error of Mean

Table 6. Effect of MOCE on acrosomal integrity (%) of extended bull semen

Time (hours)	Moringa Inclusion (mls)					SEM
	0	4	8	12	16	
0	98.67	100.00	98.67	98.00	100.00	0.330
24	95.67 [b]	100.00 [a]	96.00 [b]	95.67 [b]	99.33 [a]	0.532
48	90.67 [b]	95.67 [a]	91.33 [b]	94.33 [a]	91.33 [b]	0.566
72	-	91.67 [a]	92.67 [a]	91.33 [a]	92.33 [a]	1.457

a,b,c: Means along the same row with different superscripts are significantly (P < .05) different ; MOCE: Moringa oleifera crude extract ; SEM: Standard Error of Mean

4. CONCLUSION

The mean values obtained for Progressive motility, Normal Sperm Morphology and Acrosome Integrity at 72 hours across the treatments were higher than the minimum recommended by Dahmani Y. [23] which suggests that Moringa extract could preserve Motility, Morphology and Acrosome integrity of semen for more than 72 hours when refrigerated at 6°C.

16 mls inclusion level of MOCE could be a good substitute for the antibiotic component of conventional bovine semen extender as it enhances the sperm percentage motility, acrosome integrity and normal morphology thereby preserving the fertility potential of spermatozoa when refrigerated at 6°C for 72 hours.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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