



Antibacterial Potential of Aqueous Neem Leaf Extract (*Azadirachta indica* A. Juss) on Spermatozoa Quality in Extended Porcine Semen

O. D. Ilori^{1*}, O. A. Shokunbi¹, F. Alaba¹, S. Ajani² and D. Omobayo²

¹Department of Animal Production and Health, Federal University of Technology, Akure Ondo State, Nigeria.

²Department of Animal Science, University of Ibadan, Oyo State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Authors ODI and OAS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ODI, FA, SA and DO managed the analyses of the study. Author ODI managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present study was investigated to assess the antibacterial potential of aqueous neem leaves extracts (ANLE) on spermatozoa quality in extended porcine semen.

Materials and Methods: Fresh semen was collected from a mature and intact boar (age, breed, body condition score, health status) using the glove-hand technique. The collected semen samples were diluted and allotted to six treatments with three replicates per treatment in a completely randomized design and evaluated at 0, 24 and 48 h of refrigeration at 17°C. Semen quality parameters such as progressive motility (%), viability (%), morphology (%), pH, acrosome integrity (%), and bacteria load ($\times 10^4$ CFU/mL) were evaluated.

Results and Discussion: At 48 h, a significant difference ($p < 0.05$) in motility was observed across the treatments with Treatment 1 giving the highest mean value (84.67 ± 2.40) and Treatment

6 (100% ANLE) gave the least value (70.00±0.00). At 48 h, there was a significant difference ($p < 0.05$) in viability across the treatments. Treatment 2 and Treatment 3 (25% ANLE) though with a significant difference between the means has given the closest mean value (79.67±0.33 and 76.67±0.67 respectively) to Treatment 1 (80.00±0.00). At 48 h, a significant difference ($p < 0.05$) in morphology was also observed across the treatments. Treatment 2 and Treatment 3 (25% ANLE), though with a significant difference between the means has given the closest mean values (81.33±0.67 and 79.33±0.33 respectively) to Treatment 1 (82.00±1.00). At 48 h, a significant difference ($p < 0.05$) in acrosome integrity, as well as pH, was also observed across the treatments. The general trend for bacteria load is a decline as the level of ANLE increases across the treatments at 48 h of refrigeration.

Conclusion: These findings suggest that 25% of ANLE can be used in boar semen extension up to 48 h of storage at 17°C.

Keywords: Artificial insemination; aqueous neem leaf extract; bacterial load; semen quality.

1. INTRODUCTION

Microorganisms, especially bacteria constitute a major setback in the productivity of porcine in Nigeria and other tropical countries. Bacterial contamination of extended boar semen has been associated with deleterious effects on semen quality parameters [1] such as reduced motility, increased abnormal spermatozoa structure, agglutination of the sperm cell, shortened viability [2] and premature acrosome reaction [2]. This bacterial contamination shortens the shelf life of semen doses [3] and reduces fertility, conception rates, and litter size at birth [1].

The insemination of contaminated semen may be associated with vulva discharge and return to oestrus [3], embryonic or foetal death, endometritis, systemic infection and/or disease in recipient females or reduced litter size [1]. The addition of antibiotics to the semen extenders has reduced the bacterial contamination [4]. There is a considerable universal interest in reducing the utilization of synthetic antibiotics to curtail the development of antibiotic-resistant strain of bacteria. Extensive use of synthetic antibiotics has resulted in drug resistance for many bacterial species of pigs and other domestic animals such as cattle, sheep and goats.

The increase in the prevalence of resistant strains of bacteria to synthetic antibiotics necessitates the use of natural antimicrobial alternatives such as aqueous extract of neem (*Azadirachta indica* A. Juss) leaf. A biologically active component in the neem leaf that acts as an antibacterial substance in relation to semen quality profiles and fertility are azadirachtin, valassin, gedunin, salanin, meliacin and nimbin [5].

Although there is an abundance of scientific data to show the antibacterial potential of neem leaf, there is a notable dearth of information in terms of previous studies describing the antibacterial potential of aqueous extracts of neem leaf on spermatozoa quality in extended boar semen. As a result, the antibacterial potential of ANLE on spermatozoa quality in extended boar semen, appropriate inclusion level, as well as durations that maintain the quality and viability of extended boar semen, was investigated.

2. MATERIALS AND METHODS

2.1 Location of Study

Semen collection was done at the Piggery Unit of the Teaching and Research Farm, University of Ibadan, Ibadan, South Western part of Nigeria (7°20'N, 3°50'E; 200 m above mean sea level). Preparation of neem extracts and semen analysis were carried out at the Animal Physiology Laboratories of the same institution and the experiment last for 12 weeks [1].

2.2 Preparation of Aqueous Extracts from Fresh Neem Leaves

The extracts from fresh neem leaves were prepared immediately after sample collection with the following procedure; 1 kg of fresh leaves was collected, washed with distilled water and then chopped into small pieces. These were soaked into 1000 mL of distilled water in overnight and were then filtered with a cheese cloth. The filtrate was then centrifuged to remove remaining fibre in the extract, thus enhancing the visibility of spermatozoa during the microscopic evaluation and then stored at 5°C [6].

2.3 Preparation of the Boar, Semen Collection and Extension

Prior to collection of semen, the boar was thoroughly washed and the preputial pouch was cleaned with water by a milking action, to remove urine and other materials that could contaminate semen during collection. Semen was collected using the gloved hand method into a US bag inserted in a collection cup such that the pre and post sperm fractions were separated from the sperm-rich fraction. Semen and extender was mixed in a ratios 1:4, 1:4, 1:0.25, 1:0.75, 1:0.5, 1:1 as described by [1] The mixture was refrigerated at 17°C. [1,2].

2.4 Semen Evaluation

Semen evaluation was carried out using the following parameters; pH, progressive motility, liveability, morphology, acrosome integrity and microbial load at 0, 24 and 48 h of preservation (17°C).

2.4.1 pH

A pH meter (Mettler Toledo Switzerland) was used to measure the hydrogen ion concentrations produced by spermatozoa metabolic activities during the storage period.

2.4.2 Progressive motility

This was assessed by putting a drop of semen on a clean glass slide, covered with a cover slip and examined with a microscope under at 400X [B100,AmScope, USA], The progressive motility of the spermatozoa was subjectively estimated and rated between 0 and 100 [7]. 0 means low percentage of motile spermatozoa and 100 means a high percentage of motile spermatozoa which indicate that the spermatozoa have not been damaged by the process of dilution and storage [4].

2.4.3 Viability

This was determined by mixing a drop of semen with a drop of a staining solution (eosin-nigrosin) on a clean glass slide gently and a smear developed using the edge of another clean slide, air-dried and examined with a microscope at 400X [1].

2.4.4 Morphology

This was determined following the same method for liveability. Spermatozoa with coiled or double

tail, damaged mid-piece and damaged head were considered abnormal [8].

2.4.5 Acrosome integrity

Sperm was fixed with 1% glutaraldehyde in Beltsville thawing solution (BTS; 3.71 g glucose, 0.60 g trisodium citrate, 1.25 g ethylenediamine tetraacetic acid, 1.25 g sodium bicarbonate, 0.75 g potassium chloride and 100.0 ml distilled water) so as to examine acrosome integrity according to [7].

2.4.6 Bacterial load

The pour plate technique was used to determine the microbial load in each sample. From the first dilution, 1mL of the sample was pipetted into other sterile diluents containing 9 mL to obtain 10^{-2} dilution. The samples were serially diluted up to 10^{-4} . Appropriate dilution (0.1 mL) was then inoculated into sterile Petri dishes and molten plate count agar (PCA) was added and left to solidify. The plate count agar (PCA) was prepared by dissolving 22.5 g into 1000 mL of distilled water and heated in a boiling water bath. The solution was autoclave for 15 min at 121°C. The samples were in three replicates and incubated at 37°C for 24 h. The mean counts for triplicate cultures were recorded as the bacterial counts in the sample. The results were expressed as CFU/mL according to America Public Health Association.

2.5 Experimental Treatments and Design

A completely randomized design was utilized for the study, such that diluted semen was allotted to six treatments with three replicates per treatment and evaluated at 0, 24 and 48 h:

Treatment 1(Positive control): Semen + Beltsville Thawing Solution (BTS) Extender

Treatment 2(Negative control): Semen +BTS without antibiotics (BTS-A)

Treatment 3: Semen + BTS-A + 25% ANLE

Treatment 4: Semen + BTS-A + 50% ANLE

Treatment 5: Semen + BTS-A + 75% ANLE

Treatment 6: Semen + BTS-A + 100% ANLE

2.6 Statistical Analysis

Data collected were subjected to one-way analysis of variance of the Statistical Analysis System [SAS, 2003] programme. The treatment means where significant ($p < 0.05$) were separated using the Duncan's Multiple Range Test of the same software.

3. RESULTS AND DISCUSSION

3.1 Effect of ANLE on Progressive Motility of Extended Boar Semen

The result of the effect of ANLE on progressive motility of extended boar semen at 0, 24 and 48 h of refrigeration at 17°C is shown in Table 1. At 0 h, there was no significant difference in motility across the treatments. At 24 h, a significant difference ($p < 0.05$) in motility was observed across the treatments with Treatment 1 (positive control) giving the highest mean value (97.00 ± 0.33) and Treatment 6 (100% ANLE) giving the least value (70.00 ± 0.00). At 48 h, a significant difference ($p < 0.05$) in motility was observed across the treatments with Treatment 1 (positive control) giving the highest mean value (84.67 ± 2.40) and Treatment 6 (100% ANLE) giving the least value (70.00 ± 0.00). However, all the treatments gave mean values within the acceptable normal range.

Considering spermatozoa progressive motility, which is a vital feature for passage through the cervix, utero-tubal junction and even more essential, through the cumulus, the inclusion of ANLE in boar semen had no detrimental effect on sperm motility at 0, 24 and 48 h of preservation and this is probably due to presence of active constituents such as azadirachtin, valassin, gedunin, salanin, meliacin and Nimbin in ANLE which could be inhibited the growth of microorganism that could detrimentally affect the survival of sperm cells. This is in accordance with findings of [6] and [5] who reported that neem has antibacterial properties that could inhibit the growth of the microorganism. Spermatozoa motility between 50 and 70% is considered as good motility [4]. Motility above 60% is enough for fertilization to take place provided that all other semen parameters are good [9]. However, the mean values of spermatozoa motility obtained with the inclusion of ANLE in boar semen were within the acceptable normal range of 50 and 70 according to [4] throughout the

period of preservation. These high percentages of motile spermatozoa indicate that the spermatozoa have not been damaged by the process of dilution and storage and this is justified by the report of [10]. This implies that the extension of boar semen with ANLE is capable of enhancing the movement of spermatozoa through the reproductive tract of gilt or sow for effective fertilization.

3.2 Effect of ANLE on Viability of Extended Boar Semen

The result of the effect of ANLE on the viability of extended boar semen at 0, 24 and 48 h of refrigeration at 17°C is shown in Table 2. At 0 h, a significant difference ($p < 0.05$) in viability was observed across the treatments with Treatment 1 (positive control) giving the highest mean value (98.00 ± 0.00). Treatment 2 (negative control) and Treatment 3 (25% ANLE), though with a significant difference between the means gave the closest mean value (97.67 ± 0.33 and 97.00 ± 0.00 respectively) to Treatment 1 (positive control) (98.00 ± 0.00).

At 24 h, there was a significant difference ($p < 0.05$) in viability across the treatments with Treatment 1 (positive control) giving the highest mean value. Treatment 2 (negative control), Treatment 3 (25% ANLE) and Treatment 4 (50% ANLE), though with a significant difference between the means gave the closest mean value (88.67 ± 0.33 , 88.33 ± 0.67 and 84.00 ± 0.58 respectively) to Treatment 1 (positive control) (89.33 ± 0.33).

At 48 h, there was a significant difference ($p < 0.05$) in viability across the treatments with Treatment 1 (positive control) giving the highest mean value. Treatment 2 (negative control) and Treatment 3 (25% ANLE), though with a significant difference between the means gave the closest mean value (79.67 ± 0.33 and 76.67 ± 0.67 respectively) to Treatment 1 (80.00 ± 0.00). However, all the treatments gave mean values within the acceptable normal range.

Table1. Effect of ANLE on progressive motility of extended boar semen

	Time (Hours)		ANLE (%)				SEM
	(BTS)	(BTS-A)	25	50	75	100	
0	98.00	98.00	98.00	98.00	98.00	98.00	0.34
24	97.00 ^f	82.00 ^e	79.00 ^d	75.00 ^c	70.00 ^b	70.00 ^b	2.53
48	84.67 ^c	76.00 ^b	76.67 ^b	70.00 ^b	70.00 ^a	70.00 ^a	4.08
SEM	2.30	3.36	4.45	6.98	7.33	7.90	

Mean values on the same row with different superscript (a, b, c, d, e and f) are significantly different ($p < 0.05$), SD = Standard Deviation

Spermatozoa viability is also of paramount importance for effective fertilization. The inclusion of ANLE in boar semen had no detrimental effect on spermatozoa viability at 0, 24 and 48 h of preservation and this implies that the ANLE possessed active constituents that are capable of enhancing spermatozoa viability. The mean values of spermatozoa viability obtained with the inclusion of ANLE in boar semen were within the acceptable normal range throughout the period of preservation. These high percentages of viability are in agreement with the findings of [10].

3.3 Effect of ANLE on Morphology of Extended Boar Semen

The result of the effect of ANLE on the morphology of extended boar semen at 0, 24 and 48 h of refrigeration at 17°C is shown in Table 3. At 0 h, there was no significant difference in morphology across the treatment. At 24 h, there was a significant difference ($p < 0.05$) in sperm morphology across the treatments with Treatment 1 (positive control) and Treatment 2 (negative control) giving the same mean value (90.00 ± 0.00 and 90.00 ± 0.00 respectively). Treatment 3 (25% ANLE), Treatment 4 (50% ANLE), although with a significant difference between the means gave the closest mean value (88.33 ± 0.67 and 86.00 ± 0.58 respectively) to Treatment 1 (90.00 ± 0.00) and Treatment 2 (90.00 ± 0.00).

At 48 h, a significant difference ($p < 0.05$) in morphology was observed across the treatments with Treatment 1 (positive control) having the highest mean value. Treatment 2 (negative control) and Treatment 3 (25% ANLE), though with a significant difference between the means gave the closest mean values (81.33 ± 0.67 and 79.33 ± 0.33 respectively) to Treatment 1 (82.00 ± 1.00). However, all the treatments gave mean values within the acceptable normal range.

Morphological abnormalities of spermatozoa can have detrimental effects on fertilization and embryonic development. The inclusion of ANLE in boar semen was also found not to be detrimental to spermatozoa morphology throughout the periods of preservation. The lower morphological abnormalities recorded at these hours of storage could also be as a result of the presence of active constituents in ANLE which are responsible for inhibiting growth of microorganism and this is corroborated by the findings of [6] and [5] who reported that neem

has an antibacterial properties that could inhibit the growth of microorganism. This finding is also in compliance with the study of [4] who reported that high-quality semen contains a minimum number (5 to 15%) of morphologically abnormal spermatozoa whereas low-quality semen frequently contains the larger number 20% (or more).

3.4 Effect of ANLE on Acrosome Integrity of Extended Boar Semen

The result of the effect of ANLE on acrosome integrity of extended boar semen at 0, 24 and 48 h of refrigeration at 17°C is shown in Table 4. At 0 h, there was no significant difference in acrosome integrity across the treatments. At 24 h, a significant difference ($p < 0.05$) in acrosome integrity was observed across the treatments with Treatment 1 (positive control) and Treatment 2 (negative control) giving the same mean values. Treatment 3 (25% ANLE) and Treatment 4 (50% ANLE), though with a significant difference between the means gave the closest mean values (92.67 ± 0.67 and 90.00 ± 1.00 respectively) to Treatment 1 (94.67 ± 0.33) and Treatment 2 (94.33 ± 0.33). At 48 h, a significant difference ($p < 0.05$) in acrosome integrity was observed across the treatments with Treatment 1 having the highest mean value (89.33 ± 0.67) and Treatment 6 (100% ANLE) giving the least value (70.00 ± 0.00). However, all the treatments gave mean values within the acceptable normal range of 70 and 100 according to [9].

The acrosome is a secretory vesicle that is a sac-like structure below the plasma membrane and covering the anterior nucleus of the sperm head. The integrity of the acrosome is very closely associated with sperm viability because damage to the plasma membrane can trigger a disintegration of the acrosome [9]. All the inclusion levels of ANLE in boar semen had a potential of maintaining acrosome integrity by protecting acrosome from undergoing capacitation throughout the period of preservation. The mean values of all the treatments fall within the acceptable normal range and this is justified by the findings of [9] who reported that semen samples with less than 70% sperm with intact acrosome should be discarded before processing. All treatments maintained acrosome integrity by the inhibition of acrosome reactions. Acrosome reaction is related to spermatozoa fertility and is essential in the process of fertilization. This implies that the active constituents in ANLE are capable of

Table 2. Effect of ANLE on the viability of extended boar semen

	Time (Hours)			ANLE (%)			SEM
	BTS	BTS-A	25	50	75	100	
0	98.00 ^e	97.67 ^{de}	97.00 ^{cd}	96.67 ^c	94.67 ^b	95.00 ^a	0.39
24	89.33 ^e	88.67 ^{de}	88.33 ^{cd}	84.00 ^c	81.33 ^b	76.00 ^a	1.15
48	80.00 ^c	79.67 ^c	76.67 ^b	70.00 ^b	68.67 ^a	68.00 ^a	2.98
SEM	2.65	2.55	2.95	3.86	5.69	6.55	

Mean values on the same row with different superscript (a, b, c, d, and e) are significantly different ($p < 0.05$), SD = Standard Deviation

Table 3. Effect of ANLE on the morphology of extended boar semen

	Time (hours)			ANLE (%)			SEM
	BTS	BTS-A	25	50	75	100	
0	98.00 ^b	98.00 ^b	98.00 ^b	98.00 ^b	97.00 ^a	96.67 ^a	0.15
24	90.00 ^e	90.00 ^e	88.33 ^d	86.00 ^c	84.33 ^b	80.00 ^a	0.87
48	82.00 ^c	81.33 ^c	79.33 ^c	76.33 ^b	68.67 ^a	68.00 ^a	1.41
SEM	2.28	2.24	2.70	3.17	4.11	4.16	

Mean values on the same row with different superscript (a, b, c, d, and e) are significantly different ($p < 0.05$), SD = Standard Deviation

Table 4. Effect of ANLE on acrosome integrity of extended boar semen

	Time (Hours)			ANLE (%)			SEM
	BTS	BTS-A	25	50	75	100	
0	98.00	98.00	98.00	98.00	98.00	98.00	0.00
24	94.67 ^d	94.33 ^d	92.67 ^d	90.00 ^c	85.67 ^b	81.33 ^a	1.12
48	89.33 ^e	83.67 ^d	80.33 ^{cd}	79.67 ^c	75.33 ^b	70.00 ^a	1.50
SEM	1.28	2.16	2.16	2.67	3.37	4.07	

Mean values on the same row with different superscript (a, b, c, d, and e) are significantly different ($p < 0.05$), SD = Standard Deviation

protecting acrosome from undergoing capacitation during refrigeration at 17°C.

3.5 Effect of ANLE on pH of Extended Boar Semen

The result of the effect of ANLE on pH of extended boar semen at 0, 24 and 48 h of refrigeration at 17°C is shown on Table 5. At 0 and 24 h, there was no significant difference in pH across the treatments. At 48 h, there was a significant difference ($p < 0.05$) in pH across the treatments. However, all the treatments gave mean values within the acceptable normal range. All the inclusion levels of ANLE in boar semen had a potential of maintaining the pH throughout the period of preservation. It is necessary for pH to be maintained because when the pH of the semen is declined; the internal pH of the spermatozoa is also reduced leading to a decrease in sperm metabolism and motility [11] and [12]. However, the inclusion levels of ANLE in boar semen yielded mean values of pH that

are within the acceptable normal range and this is justified by the findings of [13] who reported that a pH that is higher than 8 is an indicator of poor quality semen.

3.6 Effect of ANLE on Bacteria Load of Extended Boar Semen

The result of the effect of ANLE on bacteria load ($\times 10^4$ Cfu/mL) of extended boar semen quality at 0, 24 and 48 h of refrigeration at 17°C is shown on Table 6. At 0 h, there was no significant difference in bacterial load across the treatments. There was no development of bacteria colony across the treatments. At 24 h, there was a significant difference ($p < 0.05$) in bacteria load across the treatments. There was no development of bacteria colony in Treatment 1 (positive control), Treatment 2 (negative control) and Treatment 3 (25% ANLE) but the mean values of bacteria load slightly declined in Treatment 4 (50% ANLE), Treatment 5 (75% ANLE) and Treatment 6 (100% ANLE) as the

Table 5. Effect of ANLE on pH of extended boar semen

	Time (Hours)			ANLE (%)			SEM
	BTS	BTS-A	25	50	75	100	
0	7.00	7.00	7.00	6.97	6.87	7.03	0.21
24	7.03	7.07	7.00	6.97	6.97	7.03	0.02
48	7.03	7.07	6.97	7.03	7.03	7.03	0.14
SEM	0.20	0.24	0.26	0.20	0.34	0.18	

Table 6. Effect of ANLE on bacteria load ($\times 10^4$ Cfu/mL) of extended boar semen

	Time (Hours)			ANLE (%)			SEM
	BTS	BTS-A	25	50	75	100	
0	ND	ND	ND	ND	ND	ND	
24	ND	ND	ND	6.50 ^d ±0.18	5.69 ^b ±0.23	4.09 ^b ±0.09	0.34
48	5.26 ^a ±0.14	6.76 ^c ±0.08	6.69 ^c ±0.08	6.62 ^c ±0.08	6.57 ^c ±0.09	5.65 ^b ±0.05	0.13
SEM	0.36	0.54	0.65	1.06	1.07	1.07	

Mean values on the same row with different superscript (a, b, c and d) are significantly different ($p < 0.05$), SD = Standard Deviation, ND = No Development

level of ANLE increases across the treatments. At 48 h, significant differences ($p < 0.05$) were also observed in bacterial load across the treatments with Treatment 1 (positive control), having the lowest mean value (5.26±0.14). Treatment 6 (100% ANLE), though with a significant difference between the means gave the closest mean value (5.65±0.05) to Treatment 1 (positive control). The general trend is a decline in bacteria load as the level of ANLE increases across the treatments.

Contamination of semen with bacteria is very common in collected boar ejaculates [3]. Ejaculates collected from healthy boars are usually contaminated with bacteria containing up to 10^9 microorganisms per mL [1]. The presence of bacteria in extended semen creates competition for nutrients and also results in the production of metabolic by-products that may harm the spermatozoa [2].

All the inclusion levels of ANLE in boar semen inhibited the development of bacteria colony across the treatments at 0 hours of preservation. At 24 h, 25% inclusion level of ANLE in boar semen inhibited the development of bacteria colony but the mean values of bacteria load slightly declined as the inclusion level of ANLE increases across the treatments. At 48 h of storage, all the inclusion levels of ANLE in boar semen had the potential of reducing the bacteria load as the level of ANLE increases across the treatments. This decrease in bacteria load is probably due to the presence of active constituents in ANLE which could inhibit the growth of microorganism that could detrimentally affect the survival of spermatozoa quality. This is

justified by the findings of [6] and [5] who reported that neem has antibacterial properties that could inhibit the growth of the microorganism.

4. CONCLUSION

The study has shown that the inclusion of ANLE in boar semen up to 48 h of refrigeration gave mean values which fall within the acceptable range of normal values indicative of good semen quality for all semen quality parameters. This study, therefore, recommended 25% inclusion level of ANLE in boar semen extension up to 48 h as indicated by observed mean values of all parameters, which fall within the acceptable range of normal values indicative of good semen quality.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The study was carried out with permission from the Nigeria Institute of Animal Science Welfare and Ethics Committee (Act No. 26 of 2007).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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