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Antibacterial activities of aqueous neem bark extract (*Azadirachta indica* A. Juss) on spermatozoa quality in extended porcine semen

Abstract

An experiment was conducted to assess the antibacterial activities of aqueous neem bark extracts (ANBE) on spermatozoa quality in extended porcine semen. 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 (%), acrosome integrity (%), pH, and bacteria load ($\times 10^4$ CFU/mL) were evaluated. The results revealed that at 48 hours, there was a significant difference ($p<0.05$) in progressive motility across the treatments with T1 (84.33±2.33) giving the highest mean value and T6 (41.00±1.53) giving the least value. At 48 hours, significant difference ($p<0.05$) in viability was observed across the treatments with T1 (79.67±0.33) giving the highest mean value and T6 (35.00±2.00), having the least value. At 48 hours, there was significant difference ($p<0.05$) in morphology across the treatments with treatments with T1 (85.00±0.00) having the highest mean value. T2 (83.00±0.00), T3 (82.00±1.00), T4 (81.33±0.67), T5 (80.00±0.00), T6 (78.33±0.33), though with significant difference between the mean gave the closest mean value to T1 (85.00±0.00). At 48 hours, significant difference ($p<0.05$) was observed in acrosome integrity across the treatments. At 0, 24 and 48 hours of refrigeration, there was no significant difference ($p>0.05$) in pH across the treatments. The common trend for bacteria load is a decline as the level of ANBE increases across the treatments at 48h of refrigeration. The study suggest that 25% of ANBE can be used in boar semen extension up to 48h of storage at 17°C without any detrimental effect on semen quality of boars.

Keywords: aqueous neem bark extract, bacterial load, semen quality, boar

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Introduction

Bacterial contamination of semen is a threat to semen quality, fertility and overall success of an AI program. The occurrence of bacteria in extended semen enhances competition for nutrients[1] and also results in the production of metabolic byproducts that may harm the spermatozoa.[2] The presence of contaminant bacteria in extended boar semen is associated with a decrease in sperm motility and viability,[1] premature acrosome reaction or sperm agglutination. Moreover, bacteria may cause the production of antibodies directed against the sperm glycol calix complex. Bacterial contamination have negative effects on the sperm cells in extended semen.[1,2] Failure to control bacterial contamination in semen could result in economic loss for porcine studs.[3,4] Morrell[2] reported that bacterial contamination can be controlled by addition of antibiotics to semen extenders. Antimicrobial agents are added to semen extenders to control the growth of microbes contaminating semen during collection. However, utilization of synthetic antibiotics can enhance the development of antibiotic resistance, and this resistance can, in turn, be transferred to other bacteria in other host species. Thus, unfortunately, even though combinations of antibiotics may reduce sperm toxicity, they may actually contribute more to antibiotic resistance than single agents.[2] The incessant menace of resistant strains of bacteria to synthetic antibiotics requires the use of natural antimicrobial alternatives such as aqueous extract of neem (*Azadirachta indica* A. Juss) leaf. Bioactive 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.[5,6] Several works have been carried out on antibacterial activities of neem but there is a paucity of literature on the antibacterial activities of aqueous neem bark extracts (ANBE) on spermatozoa quality in extended boar semen. In view of this, the present investigation was carried out to determine the antibacterial activities of ANBE on spermatozoa quality in extended boar semen; appropriate inclusion level; as well as durations that maintain the quality and viability of extended boar semen.

Materials and methods

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; 200m 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.

Preparation of aqueous extracts from fresh neem leaves

The extracts from fresh neem leaves were prepared immediately after sample collection with the following procedure; 1kg of fresh leaves was collected, washed with distilled water and then chopped into small pieces. These were soaked into 1000mL of distilled water in overnight and were then filtered with a cheese cloth. The filtrate was then centrifuged to remove remaining fiber in the extract, thus

enhancing the visibility of spermatozoa during the microscopic evaluation and then stored at 5°C.[6]

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, 1:0.25, 1:0.75, 1:0.5, 1:1 as described by Althouse.[1] The mixture was refrigerated at 17°C.[1,3]

Semen evaluation

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

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] 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.[1]

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]

Morphology

This was determined following the same method for viability. Spermatozoa with coiled or double tail, damaged mid-piece and damaged head were considered abnormal.[8]

Acrosome integrity

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

pH

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

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 9mL to obtain 10−2 dilution. The samples were serially diluted up to 10−4. Appropriate dilution (0.1mL) 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 22.5g into 1000mL of distilled water and heated in a boiling water bath. The solution was autoclave for 15min at 121°C. The samples were in three replicates and incubated at 37°C for 24h. 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.

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 48h:

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

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

III. Treatment 3: Semen + BTS-A + 25% ANBE.

IV. Treatment 4: Semen + BTS-A + 50% ANBE.

V. Treatment 5: Semen + BTS-A + 75% ANBE.

VI. Treatment 6: Semen + BTS-A + 100% ANBE.

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.

Results and discussion

Effect of ANBE on progressive motility of extended boar semen

Table 1 presents the result of the effect of ANBE on progressive motility of extended boar semen at 0, 24 and 48hours of refrigeration at 17°C. At 0 hour, there was no significant difference ($p>0.05$) in progressive motility across the treatments. At 24hours, significant difference ($p<0.05$) in progressive motility was observed across the treatments with T1 (97.67±0.33) giving the highest mean value and T6 (60.33±0.33) giving the least value. At 48hours, significant difference ($p < 0.05$) in progressive motility was observed across the treatments with T1 (84.33±2.33) giving the highest mean value and T6 (41.00±1.53) giving the least value. However, all the treatments gave mean values within acceptable normal range throughout the period of preservation with the exception of T5 (44.67±2.40) and T6 (41.00±1.53) which gave the mean values below acceptable normal range at 48hours of storage. The inclusion of ANBE in boar semen was found to enhanced sperm motility at 0, 24 and 48hours of preservation and this is could be attributed to availability of active constituents such as azadirachtin,valassin, gedunin, salanin, meliacin and Nimbin in ANBE which could prevent the growth of microorganism that could adversely affect the survival of sperm cells. This is in agreement with findings of Hishmanshu et al.,[5] & Hashmat et al.,[9] who reported that neem has antibacterial properties that could inhibit the growth of microorganism. Motility above 60% is enough for fertilization to take place provided that all other semen parameters are good.[8,10,11] All the treatments gave mean values within acceptable normal range throughout the period of preservation with

the exception of 75 and 100% inclusion level of ANBE which gave the mean values below acceptable normal range at 48hours of storage. This variation in percent sperm motility could be attributed to gradual reduction of active constituents in ANBE which failed to inhibits the action of bacteria on sperm cells. The presence of microorganisms especially in the ejaculation can impairs spermatozoa motility and also induces acrosome reaction.[11] Microbial contamination can also lead to production of toxins which can largely affect the motility of spermatozoa. However, these high percentages of motile spermatozoa recorded with the inclusion of ANBE in boar semen indicate that these spermatozoa have not been damaged by the process of dilution and storage and this is in compliance with the report of Khan et al., [13]

Effect of ANBE on viability of extended boar semen

Table 2 shows the effect of ANBE on viability of extended boar semen at 0, 24 and 48hours of refrigeration at 17°C. At 0hour, there was significant difference ($p<0.05$) in viability across the treatments with T1 (97.67±0.33) and T2 (97.33±0.33) having the same highest mean value. T3 (96.67±0.33), T4 (95.00±0.00), T5 (93.00±0.00), though with significant difference between the means gave the closest mean values to T1 (97.67±0.33) and T2 (97.33±0.33). However, T6 (90.67±0.67) gave the least mean value. At 24hours, significant difference ($p<0.05$) was also observed in viability across the treatments with treatment T1 (90.00±2.52), having the highest mean value. T2 (89.33±0.33), although with significant difference between the means gave the closest mean value with T1 (90.00±2.52). At 48hours, there was significant difference ($p<0.05$) in viability across the treatments with T1 (79.67±0.33) giving the highest mean value and T6 (35.00±2.00), having the least value. However, all the treatments gave mean values within acceptable range throughout the period of preservation with the exception of T5 (43.67±1.86) and T6 (35.00±2.00) which gave the mean values below acceptable normal range at 48hours of storage. The addition of ANBE to boar semen was found to enhanced spermatozoa viability at 0, 24 and 48hours of preservation and this could be due to presence of active constituents in ANBE which are capable of enhancing spermatozoa viability. All the treatments gave mean values within acceptable normal range throughout the period of preservation with the exception of 75 and 100% inclusion level of ANBE which gave the mean values below acceptable normal range at 48hours of storage. This variation in percent spermatozoa livability could be attributed to gradual depletion of active constituents in ANBE which pose a threat to spermatozoa livability at this hour of preservation. However, the high percentages of spermatozoa livability recorded with the inclusion of ANBE in boar semen are in compliance with the findings of Khan et al.,[13] This is further justified by the findings of Maes et al.,[12] who reported that semen samples should have more than 70% viable sperm by a vital stain assay prior to processing.

Effect of ANBE on morphology of extended boar semen

Table 3 presents the result of effect of ANBE on morphology of extended boar semen at 0, 24 and 48hours of refrigeration at 17°C. At 0 hour, Significant difference ($p<0.05$) in morphology was observed across the treatments with T1 (98.00±0.67) having the highest mean value. T2 (97.67±0.33), T3 (97.33±0.33), T4 (96.67±0.33), T5 (96.00±0.00), T6 (96.33±0.33), though with significant difference between the means gave the closest mean values to T1 (98.00±0.67). At 24 hours, there was significant difference ($p<0.05$) in morphology across the treatments with T1 (93.67±2.33) having the highest mean value. T2 (90.00±0.00), T3 (90.00±0.00), T4 (90.00±0.00) T5 (90.00±0.00), T6 (89.33±0.33), gave the closest mean values to T1 (93.67±2.33). At 48 hours, there was significant difference ($p<0.05$) in morphology across the treatments with treatments with T1 (85.00±0.00) having the highest mean value. T2 (83.00±0.00), T3 (82.00±1.00), T4 (81.33±0.67), T5 (80.00±0.00), T6 (78.33±0.33), though with significant difference between the mean gave the closest mean value to T1 (85.00±0.00). However, all the treatments gave mean values within acceptable normal range.

Table 1 Effect of ANBE on Progressive Motility of Extended Boar Semen (Mean±SD)

Time (hours)	ANBE (%)					
	T1	T2	T3	T4	T5	T6
	(BTS)	(BTS-A)	(25%)	(50%)	(75%)	(100%)
0	98.00^{e}±0.00	96.67^{e}±0.33	80.33^{d}±0.33	74.00^{c}±1.00	71.00^{b}±0.58	60.33^{a}±0.33
24	97.67^{e}±0.33	87.00^{d}±2.00	73.33^{c}±1.67	67.67^{b}±1.45	60.33^{a}±0.33	60.33^{a}±0.33
48	84.33^{e}±2.33	69.67^{d}±0.88	61.00^{c}±2.08	61.00^{c}±1.00	44.67^{a}±2.40	41.00^{a}±1.53

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 2 Effect of ANBE on viability of extended Boar Semen (Mean±SD)

Time (hours)	ANBE (%)					
	T1	T2	T3	T4	T5	T6
	(BTS)	(BTS-A)	(25%)	(50%)	(75%)	(100%)
0	97.67^{e}±0.33	97.33^{de}±0.33	96.67^{d}±0.33	95.00^{c}±0.00	93.00^{b}±0.00	90.67^{a}±0.67
24	90.00^{c}±2.52	89.33^{c}±0.33	84.33^{b}±0.67	82.67^{ab}±0.33	81.00^{ab}±0.00	80.00^{a}±0.00
48	79.67^{f}±0.33	68.67^{e}± 0.67	61.00^{d}±1.00	61.00^{d}±2.08	43.67^{b}±1.86	35.00^{a}±2.00

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

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The presence of ANBE in boar semen was also found to enhanced spermatozoa morphology throughout the periods of preservation. Morphological abnormalities of spermatozoa can severely influenced fertilization and embryonic development.[6] The reduction in morphological abnormalities recorded at these hours of preservation could be attributed to the presence of active constituents in ANBE which are responsible for enhancing spermatozoa morphology by inhibiting growth of microorganism and this is justified by the findings of Hishmanshu et al.,[5] & Ilori et al.,[6] who reported that neem has an antibacterial properties that could inhibit the growth of microorganism. This finding is agreement with the findings of Maes et al.,[12] who reported that ejaculates should have greater than 70% normal sperm with no more than 20% sperm with primary abnormalities.

Effect of ANBE on acrosome integrity of extended boar semen

Table 4 presents the result of effect of ANBE on acrosome integrity of extended boar semen at 0, 24 and 48hours of refrigeration at 17°C. At 0 hour, there was no significant difference ($p>0.05$) in acrosome integrity across the treatments. At 24hours, significant difference ($p<0.05$) was observed in acrosome integrity across the treatments with T1 (94.67±0.33), T2 (94.67±2.33), T3 (94.00±0.58), having the highest mean values. T4 (91.00±0.33), T5 (91.00±0.58), T6 (90.00±0.00), though with significant difference between the means gave the closest mean values to T1 (94.67±0.33), T2 (94.67±2.33) and T3 (94.00±0.58). At 48hours, significant difference ($p<0.05$) was observed in acrosome integrity across the treatments with T1 (89.33±0.67) having the highest mean value. T2 (88.33±0.33), T3 (88.00±0.00), T4 (87.00±1.00), though with significant difference between the means gave the closest mean value to T1 (89.33±0.67). However, all the treatments gave mean values within acceptable normal range. The inclusion levels of ANBE in boar semen was found to maintain 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 in accordance with the findings of Maes et al.,[12] who reported that semen samples with less than 70% sperm with intact acrosomes should be discarded before processing. All the treatments enhance acrosome integrity by inhibiting acrosome reaction. Acrosome reaction is related to sperm fertility and is essential in the process of fertilization.[6] The active constituent in ANBE is capable of protecting acrosome from undergoing capacitation during refrigeration. This finding is agreement with the findings of Ilori et al.,[6] who reported that neem has the potential of maintaining acrosome integrity of boar semen by protecting acrosome from undergoing capacitation during preservation. However, the findings disagree with the report of Khan et al.,[13] & Kommisrud et al.[14] that acrosome is more susceptible to damage during storage than organelles, being the structural basis for motility.

Effect of ANBE on pH of extended boar semen

The result of the effect of ANBE on pH of extended boar semen at 0, 24 and 48hours of refrigeration at 17°C is shown in Table 5. At 0, 24 and 48hours of refrigeration, there was no significant difference ($p>0.05$) in pH across the treatments. However, all the treatments gave mean values within acceptable normal range. The inclusion of ANBE in boar semen was found to maintain the pH throughout the period of preservation. Gadea[15] reported that decline in pH of the semen could reduce the internal pH of the spermatozoa leading to a decrease in sperm metabolism and motility. However, the inclusion of ANBE in boar semen gave mean values of pH that is within the acceptable normal range that could maintain the pH throughout the period of preservation and this is in compliance with the findings of Frunza et al.,[16] who reported that a pH that is higher than 8 is an indicator of poor quality semen. This finding corroborates the finding of Ilori et al.,[6] who reported that neem has the potential of maintaining pH of boar semen during preservation.

Table 3 Effect of ANBE on morphology of extended boar semen (Mean ± SD)

Time			ANBE (%)			
(hours)	T1	T2	T3	T4	T5	T6
	(BTS)	(BTS-A)	(25%)	(50%)	(75%)	(100%)
0	98.00^{c}±0.67	97.67^{c}±0.33	97.33^{bc}±0.33	96.67^{ab}±0.33	96.00^{a}±0.00	96.33^{a}±0.33
24	93.67^{b}±2.33	90.00^{a}±0.00	90.00^{a}±0.00	90.00^{a}±0.00	90.00^{a}±0.00	89.33^{a}±0.33
48	85.00^{e}±0.00	83.00^{d}±0.00	82.00^{cd}±1.00	81.33^{bc}±0.67	80.00^{b}±0.00	78.33^{a}±0.33

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 ANBE on Acrosome integrity of extended boar semen (Mean±SD)

Time (hours)	ANBE (%)					
	T1	T2	T3	T4	T5	T6
	(BTS)	(BTS-A)	(25%)	(50%)	(75%)	(100%)
0	98.00±0.00	98.00±0.00	98.00± 0.00	98.00± 0.00	98.00±0.00	98.00±0.00
24	94.67^{c}±0.33	94.67^{c}±2.33	94.00^{c}±0.58	91.00^{ab}±0.33	91.00^{ab}±0.58	90.00^{a}±0.00
48	89.33^{d}±0.67	88.33^{cd}± 0.33	88.00^{cd}±0.00	87.00^{c}±1.00	84.33^{b}±0.67	80.33^{a}±0.33

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

Effect of ANLE on Bacteria Load of Extended Boar Semen

The result of the effect of ANBE on bacteria load (×104 Cfu/mL) of extended boar semen at 0, 24 and 48 hours of refrigeration at 17°C is shown in Table 6. At 0, 24 and 48hours of storage, significant difference ($p<0.05$) were observed in bacteria load across the treatments. However, at 0 hour, there was no development of bacteria colony in T1, T2, and T3 while the mean values of bacteria load declined in T4 (3.24±0.12), T5 (3.53±0.27) and T6 (2.30±0.06) as the level of ANBE increases. At 24 and 48 hours, the mean values of bacteria load slightly decreased as the level of ANBE increases across the treatments. At 0 hour, up to 25% inclusion level of ANBE in boar semen inhibited the development of bacteria colony but the mean values of bacteria load slightly declined as the inclusion level of ANBE increases across the treatments. However, at 24 and 48hours of preservation, the inclusion of ANBE in boar semen was also found to reduce the bacteria load as the level of ANBE increases across the treatments. This decrease in bacteria load could be attributed to availability of active constituents in ANBE which are capable of inhibiting the growth of microorganism that could adversely affect the survival of sperm quality. This is in accordance to the findings of Hishmanshu et al.,[5] & Ilori et al.,[6] who reported that neem has antibacterial properties that could inhibit the growth of microorganism.

Table 5 Effect of ANBE fortification on pH of extended boar semen quality (Mean±SD)

Time (hours)	ANBE (%)					
	T1	T2	T3	T4	T5	T6
	(BTS)	(BTS-A)	(25%)	(50%)	(75%)	(100%)
0	7.03±0.03	7.07± 0.03	6.97±0.03	7.03±0.33	7.03±0.03	7.03±0.03
24	7.03±0.03	7.07± 0.03	7.00±0.58	6.97±0.33	6.97±0.03	7.03±0.03
48	7.03±0.03	7.03± 0.03	7.07±0.03	7.03±0.03	7.03±0.03	7.07±0.03

Table 6 Effect of ANBE on Bacteria Load (×104 Cfu/mL) of Extended Boar Semen

Time (hours)	ANBE (%)					
	T1	T2	T3	T4	T5	T6
	(BTS)	(BTS-A)	(25%)	(50%)	(75%)	(100%)
0	ND	ND	ND	3.24^{b}±0.12	3.53^{b}±0.27	2.30^{a}±0.06
24	5.00^{c}±0.00	5.45^{e}±0.07	5.26^{d}±0.14	5.13^{c}±0.62	4.73^{b}±0.10	4.28^{a}±0.09
48	5.00^{a}±0.00	6.69^{d}±0.08	6.62^{d}±0.08	6.57^{d}±0.09	5.65^{c}± 0.05	5.26^{b}±0.14

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

Conclusion

Based on the results obtained from this study, inclusion of ANBE in boar semen up to 48h of refrigeration had no detrimental effect on semen quality parameters as the semen quality parameters assessed gave mean values which fall within the acceptable range of normal values indicative of good semen quality for all semen quality parameters. Thus, 25% inclusion level of ANBE can be used in boar semen extension up to 48h as indicated by observed mean values of all parameters, which fall within the acceptable range of normal values indicative of good semen quality.

Acknowledgment

None.

Conflicts of interest

Author declares that there is no conflict of interest.

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