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ANTIOXIDATIVE EFFECTS OF SCENT LEAF (*Ocimum gratissimum)* EXTRACT ON SPERMATOZOA QUALITY AND FERTILIZING POTENTIALS OF EXTENDED BREEDER COCK SEMEN

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ABSTRACT

This study investigated the antioxidative potential of Ethanolic Scent leaf extract (ESLE) on spermatozoa quality in extended breeder cock semen. Five (5) treatments with two replicates: the control sample (T1) was diluted with Beltsview Poultry Semen Extender (BPSE) and Penicillin & Streptomycin, T2 was diluted with BPSE only, T3, T4 and T5 had ESLE at 2.50µ/mL, 2.75µ/mL and 3.00µ/mL respectively. pH, Liveability, Normal Spermatozoa (NS), Acrosome Integrity (AI), and Lipid peroxidation (LP) at 0, 3, 6, 9 and 12 hours after dilution were evaluated. The pH of T1 was significantly different from ESLE included treatments from 0 -12 hours with the highest value at 3 hours for T2 (7.01) and lowest at 12 hours for T1 (6.71) while ESLE included treatments have its highest value at 0 hour for ESLE included (7.18) and lowest at 12 hours on T4 (6.75). Liveability decreased significantly ($P<0.05$) from 0 to 3 hours with T1 having the highest value at 0 hour (93.50%) and increased across the ESLE included treatments with T4 having the highest value (84.50%). Normal spermatozoa increased significantly ($P<0.05$) from 0 hour to 6 hours but decreased significantly ($P<0.05$) from 9 hours to 12 hours for ESLE included treatments. Lipid peroxidation for 0 hour (T3) was high but decreased significantly from 3 hours to 12 hours. Acrosome integrity decreased significantly ($P<0.05$) from 0 hour to 12 hours across all treatments. Inclusion of ESLE at 2.50µ/mL gave better antioxidative potential against membrane damage for breeder cock semen up to 3hours of collection.

Key words: Antioxidant, Scent leave extract, Reactive oxygen species, Cytoprotectant

INTRODUCTION

Preservation of semen and its quality assessment based on sperm features including total and progressive motility, morphology and DNA quality, has been studied extensively in males of different species including bull, boar, ram and human (Love, 2011). Anti-oxidants are both natural and synthetic compounds with the ability to scavenge free radicals and inhibit oxidation processes (Hayat *et al.*, 2010). *Ocimum gratissimum* has been used extensively in the transitional system of medicine in many countries. In the North east of Brazil, it is used for medicinal, condiment and culinary purpose. The flowers and the leaves of this plant are rich in essential oils so it is used in preparation of teas and infusion (Rabelo *et al.*, 2003).

MATERIALS AND METHODS

Experimental location

This study was carried out at the Poultry Unit of the Teaching and Research Farm ($7^{o}20'$N and $3^{o}50'$E, 220m above the sea level) while, laboratory analysis was carried out at Animal Physiology and Bioclimatology Laboratories of the Department of Animal Science, University of Ibadan, Ibadan, Oyo State, Nigeria.

Experimental Animals
Breeder cocks were housed under standard environmental conditions (12 hours light and 12 hours dark cycle) under good hygienic environment, and they were fed and given clean drinking water *ad libitum*.

***Ocimum gratissimum* Extraction**
The fresh leaves were harvested and destalked. The destalked leaves were air dried overnight and then weighed and oven dried at 60^{o}C. The oven dried leaves were weighed again after drying. Ethanol (95%) was used as a solvent for the extraction. It was soaked for 72 hours and exposure to sunlight was totally avoided to reduce evaporation of the ethanol. Rotary evaporator was further used to obtain concentrated scent leaf extracts. The scent leaf extract was recovered and reconstituted using dimethyl sulfoxide (DMSO) and stored at 4°C.

Semen Collection
Semen was collected from all the cocks in each replicate using abdominal massage technique (Lake, 1957) and evaluated for semen quality characteristics. The abdominal massage techniques involved massaging the cloaca region to achieve phallic tumescence, followed by a cloaca stroke and a squeeze of the region surrounding the sides of the cloaca to express the semen. The semen was then milked down by firm finger pressure on either side of the vent into the labelled collecting tube.

Preparation of Semen Extender
Semen samples showing normal characteristics of motility above 65% was pooled to eliminate bull effect and to have adequate semen. Prior to semen collection from the breeder cocks, a modified Beltsview Poultry Semen Extender (BPSE) was prepared. Ejaculates were pooled, mixed thoroughly with the extender at a ratio of 1 to 4.

Experimental layout
The experimental design is a completely randomized design. Extended semen samples were distributed into five (5) treatments with each treatment having two (2) replicates, which was further reproduced five (5) times for semen quality evaluation at 0, 3, 6, 9 and 12 hours. The layout is as indicated in the treatments below:

T1: Semen + BPSE + Penicillin + Streptomycin

T2: Semen + BPSE

T3: Semen + BPSE + ESLE 2.500µ/mL

T4: Semen + BPSE + ESLE 2.750µ/mL

T5: Semen + BPSE + ESLE 3.000µ/mL

Experimental samples were stored at the refrigerator temperature (4 – 6 ^{0} C) for different storage times (3, 6, 9 and 12 hrs.). A portion of semen from each group was evaluated at 3 hrs interval for 12hrs (0hr, 3hrs, 6hrs, 9hrs, and 12 hrs.) *in vitro* storage for mass activity, individual motility, and percentages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities.

Semen Evaluation
Semen volume
The volume was determined by reading out the volume in a calibrated measuring cylinder.

Semen colour
The colour of the semen was determined by visual assessment. Coloured semen that is not pure white in colour was discarded, as it might have been contaminated by faeces or blood.

Semen pH
The semen pH of the pooled fresh semen samples of the cocks was measured using a pH meter. To evaluate the hydrogen ion concentration produced by the sperm cells.

Liveability
A drop of semen was placed on a microscope slide with a micropipette, and a drop of eosin-nigrosin stain added, smeared, immediately air-dried, and viewed under a microscope at x400 magnification. The slide field was examined with the help of the Microscope. The proportions of live (eosin-impermeable) and dead (eosin-permeable) spermatozoa in a sample was assessed on the basis of 30 cells counted.

Normal Spermatozoa
Slides of semen samples stained with eosin-nigrosin stain were viewed under x1000 magnification and normal sperm cells were counted and expressed in percentage.

Acrosome Integrity
One drop of the semen sample was mixed with one drop of the staining solution; eosin-nigrosin on a clean slide and a smear was gently created using the edge of another clean slide. The smear of the stained sample was dried and placed under a light microscope at a magnification of x1000 where the number of spermatozoa with intact acrosomes was counted using a tally counter.

Statistical analysis
Data obtained were analysed according to general linear model procedure with interaction in a complete randomised design using SAS (2013). The differences in mean values where significant were separated using the Duncan Multiple Range Test of the same software.

RESULTS AND DISCUSSION

Effects of ESLE on Quality of Extended Breeder Cocks Semen at 3 hours
For 3 hours, the data on the effect of ESLE on quality of extended breeder cock's semen is shown in Table 3. There was no significant difference ($P>0.05$) in the values of pH in treatments (T2 - T5) at 3 hours. However, there was significant difference between the treatments (T2 - T5) and treatment (T1). For liveability, there was no significant difference ($P<0.05$) between the values of treatments [T1, T2 and T5 (ESLE 3.000µ/mL)] but there was significant difference between these treatments and [T3 (ESLE 2.500µ/mL) & T4 (ESLE 2.750µ/mL)] while T4 had the lowest value. Across the table for normal spermatozoa, there was no significant difference ($P<0.05$) between the treatments with ESLE (T3, T4 and T5) for but the difference between their values and that of T2 was significant.

Effects of ELSE on Quality of Extended Breeder Cocks Semen at 6 hours
In Table 4 reveal that semen pH were influenced significantly ($P<0.05$) by the treatments. pH at T1 and T2 were statistically ($P>0.05$) similar, while significantly ($P<0.05$) higher values were obtained in T3, T4 and T5. Percentage liveability values were similar for T2, T3 and T4, the highest value was recorded at T5 and lowest at T1. The percentage normal spermatozoa on T1, T2 T3 and T5 shared statistically ($P>0.05$) similar values.

Effects of ELSE on Quality of Extended Breeder Cocks Semen at 9 hours
In Table 5 there were no significant differences ($P>0.05$) between the numerical observations for pH in all the treatments while statistically there were differences between T1, T2 and T3 T4 and T5. A similar trend was observed for Percentage liveability, while the least value for Percentage normal spermatozoa was recorded in T3 (73.33) and the highest in T1 (83.33).

Effects of ELSE on Quality of Extended Breeder Cocks Semen at 12 hours
As shown in Table 6, T1 and T2 were statistically similar ($P>0.05$), but different numerically and statistically different from T3, T4 and T5 in pH values. The percentage liveability revealed similarities in T1- T2 and T3-T5 differs statistically. Values obtained for percentage normal spermatozoa in TI, T2 and T4 were statistically similar but different from T3 and T5 with T3 having the least value and highest was T5.

Effects of ESLE on Spermatozoa Fertilizing Potential in Extended Breeder Cocks Semen at 3 hours
The effect of ESLE on Spermatozoa Fertilizing Potential in extended breeder cock's semen at 3 hours is shown in Table 8. The values of T5 (ESLE 3.000µ/mL) was significantly different ($P<0.05$) in Lipid Peroxidation (LP) from treatments (T1, T2, T3 & T4). For Acrosome Integrity (AcI), there was no significant difference between the values of T1, T4 (ESLE 2.750µ/mL) & T5 (ESLE 3.000µ/mL) but there was significant

difference between the value of T2 and the values of T1, T3 – T5. The highest value for liveability was observed from T2, while the lowest was observed from T3 (ESLE 2.500µ/mL).

Effects of ESLE on Spermatozoa Fertilizing Potential in Extended Breeder Cocks Semen at 6 hours

At 6 hours post extension (Table 9) Lipid peroxidation was considerably ($P<0.05$) higher in sample extended with BPSE (T2) than in control BPSE + Penicillin + Streptomycin (T1), whereas statistically similarity was observed in breeder cock semen extended with ESLE 2.500µ/mL- ESLE 3.000µ/mL (T3-T5). There were statistical ($P>0.05$) similarities in values obtained for Acrosome Integrity (AcI) in treatments T1, T3 and T4 which are statistically different from T2 and T5.

Effects of ESLE on Spermatozoa Fertilizing Potential in Extended Breeder Cocks Semen at 9 hours

In Table 10 there were significantly ($P<0.05$) similar values in T4 and T5, but higher than TI, T2, and T3 with T3 having the least value for LP. For AcI, statistically, similar values were recorded in T1 - T4, but significantly different from the remaining treatments.

Effects of ESLE on Spermatozoa Fertilizing Potential in Extended Breeder Cocks Semen at 12 hours

At 12 hours post extension, the LP of breeder cock semen extended with T4 and T5 were numerically and statistically similar but different from other treatments. The least LP value was recorded in T3 (Table 2). Treatment 1 had the highest value for AcI though statistically similar to T2. T3 and T5 were similar statistically. The least value was recorded in T4 (73.83).

According to Donoghue and Wishart (2000) who reported that several trials indicated that chicken sperm can tolerate a pH range of 6.0 to 8.0, many reports showed different ranges for the pH of cock semen and this variation in semen pH is said to be due to many factors. The pH of the semen across all inclusion levels was found to be within 6.75 and 7.18, which falls within the normal range. The inclusion of ESLE has a pH value close to neutral at the end of the experimental period. Liveability is an important factor in determining the fertilizing potential and quality of spermatozoa. Liveability was found to be supported by the antioxidant ability of the ESLE during the hours. All inclusion levels gave a liveability of above 80 % during the first 3 hours. At the end of the second 3 hours, all inclusion levels maintained a liveability of above 75 % which can also be explained by findings of Cerollini *et al*. (2000) that the inclusion of antioxidant sources into the diluent should prevent significant reduction in viability of cells. Semen evaluation procedures determined were volume, colour, concentration, motility, viability and morphology and their assessments correlate with the fertilizing capacity of spermatozoa when fresh semen is evaluated (Wishart, 1995a). From 0 hour to 6 hours, there was 15% increase in normal spermatozoa level of the treatments with ESLE inclusion. Lipid peroxidation was determined by estimating the malondialdehyde (MDA) levels as described by Hunter *et al.* (1963) modified by Gutteridge and Wilkins (1982). According to the result, there was decreased in the oxidative stress of ELSE included treatments. These findings suggest the rich phyto-chemical content of *O. gratissimum* and its good antioxidant activity (Akinmoladun *et al*., 2007 and Prabhu *et al*., 2009). Bansal and Bilaspuri, (2008) reported that antioxidants in general disposes, scavenges, and suppresses the formation of ROS, enhances acrosome reaction by decreasing the oxidative stress, the ESLE included treatments enhanced the acrosome integrity for the first hours.

Table 1: Composition of Experimental Extenders (g/100mL)

	T1	T2	T3	T4	T5
Fructose	0.867	0.867	0.867	0.867	0.867
Sodium glutamate	0.034	0.034	0.034	0.034	0.034
Magnesium Chloride	0.065	0.065	0.065	0.065	0.065
Potassium monophosphate	1.270	1.270	1.270	1.270	1.270
Potassium diphosphate	0.430	0.430	0.430	0.430	0.43
Sodium acetate (anhydrous)	0.064	0.064	0.064	0.064	0.064
Potassium citrate	0.195	0.195	0.195	0.195	0.195
Penicillin	0.550	-	-	-	-
Streptomycin	0.550	-	-	-	-
ESLE (µ/mL)	-	-	2.500	2.750	3.000

ESLE = Ethanolic Scent Leaf Extracts

Table 2: Effects of ESLE on Quality of Extended Breeder Cocks Semen at 0hour

Parameters	**ESLE (µ/mL)**					**Group**
	T1	**T2**	**T3**	**T4**	**T5**	**SEM**
pH	6.93^{b}	6.95^{b}	7.18^{a}	7.18^{a}	7.18^{a}	0.06
Liveability (%)	93.50^{a}	92.17^{a}	83.17^{b}	84.50^{b}	82.5^{b}	1.95
Normal Spermatozoa (%)	85.17^{ab}	84.50^{b}	85.33^{ab}	82.33^{c}	87.6^{a}	1.57

^{a,b,c}: means with different superscript on the row are significantly different ($p< 0.05$)

Table 3: Effects of ESLE on Quality of Extended Breeder Cocks Semen at 3 hours

Parameters	ESLE (µ/mL)					Group
	T1	T2	T3	T4	T5	SEM
pH	6.78^{b}	7.01^{ab}	7.16^{a}	7.16^{a}	7.16^{a}	0.06
Liveability (%)	83.33^{a}	83.00^{a}	81.83^{b}	80.17^{b}	82.50^{a}	1.95
Normal Spermatozoa (%)	85.00^{b}	83.83^{c}	86.17^{a}	86.67^{a}	85.33^{ab}	1.57

^{a,b,c}: means with different superscript on the row are significantly different ($p< 0.05$)

Table 4: Effect of SLE on Quality of Extended Breeder Cocks Semen at 6 hours

Parameters	ESLE (µ/mL)					Group
	T1	T2	T3	T4	T5	SEM
pH	6.79[b]	6.77[b]	7.12[a]	7.11[a]	7.17[a]	0.06
Liveability (%)	75.64[c]	77.33[b]	78.83[b]	77.17[b]	80.50[a]	1.95
Normal Spermatozoa (%)	83.00[b]	83.85[b]	86.67[a]	84.85[b]	84.50[b]	1.57

[a,b,c]: means with different superscript on the row are significantly different ($p < 0.05$)

Table 5: Effects of ESLE on Quality of Extended Breeder Cocks Semen at 9 hours

Parameters	ESLE (µ/mL)					Group
	T1	T2	T3	T4	T5	SEM
pH	6.79[b]	6.82[b]	6.99[a]	6.93[a]	6.95[a]	0.06
Liveability (%)	76.33[b]	76.67[b]	79.17[a]	79.83[a]	78.33[a]	1.95
Normal Spermatozoa (%)	83.33[a]	82.66[a]	73.33[c]	75.83[b]	76.00[b]	1.57

[a,b,c]: means with different superscript on the row are significantly different ($p < 0.05$)

Table 6: Effects of ESLE on Quality of Extended Breeder Cocks Semen at 12 hours

Parameters	ESLE (µ/mL)					Group
	T1	T2	T3	T4	T5	SEM
pH	6.71[c]	6.72[c]	6.79[ab]	6.75[bc]	6.82[a]	0.06
Liveability (%)	70.00[c]	71.67[c]	77.67[b]	79.83[b]	81.33[a]	1.95
Normal Spermatozoa (%)	73.83[a]	73.33[a]	68.67[c]	75.50[a]	71.83[b]	1.57

[a,b,c]: means with different superscript on the row are significantly different ($p < 0.05$)

Table 7: Effects of ESLE on Spermatozoa Fertilizing Potential in Extended Breeder Cocks Semen at 0 hour

Parameters	ESLE (µ/mL)					Group
	T1	T2	T3	T4	T5	SEM
Lipid Peroxidation	0.28[b]	0.18[c]	0.36[a]	0.26[b]	0.25[b]	0.08
Acrosome Integrity	92.83[a]	92.17[a]	92.17[a]	82.17[b]	85.67[b]	1.32

[a,b,c]: means with different superscript on the row are significantly different ($p < 0.05$)

Table 8: Effects of ESLE on Spermatozoa Fertilizing Potential in Extended Breeder Cocks Semen at 3 hours

Parameters	ESLE (µ/mL)					Group
	T1	T2	T3	T4	T5	SEM
Lipid Peroxidation	0.26^{b}	0.19^{c}	0.16^{c}	0.17^{c}	0.29^{a}	0.08
Acrosome Integrity	84.50^{b}	89.17^{a}	81.33^{c}	83.00^{b}	83.33^{b}	1.32

^{a,b,c}: means with different superscript on the row are significantly different ($p< 0.05$)

Table 9: Effects of ESLE on Spermatozoa Fertilizing Potential in Extended Breeder Cocks Semen at 6 hours

Parameters	ESLE (µ/mL)					Group
	T1	T2	T3	T4	T5	SEM
Lipid Peroxidation	0.15^{c}	0.26^{a}	0.21^{b}	0.19^{b}	0.20^{b}	0.08
Acrosome Integrity	85.50^{a}	84.50^{ab}	85.67^{a}	86.5^{a}	84.33^{b}	1.32

^{a,b,c}: means with different superscript on the row are significantly different ($p< 0.05$)

Table 10: Effects of ESLE on Spermatozoa Fertilizing Potential in Extended Breeder Cocks Semen at 9 hours

Parameters	ESLE (µ/mL)					Group
	T1	T2	T3	T4	T5	SEM
Lipid Peroxidation	0.22^{b}	0.20^{bc}	0.18^{c}	0.27^{a}	0.27^{a}	0.08
Acrosome Integrity	80.67^{a}	79.00^{a}	75.17^{b}	73.83^{c}	76.33^{b}	1.32

^{a,b,c}: means with different superscript on the row are significantly different ($p< 0.05$)

Table 11: Effects of ESLE on Spermatozoa Fertilizing Potential in Extended Breeder Cocks Semen at 12 hours

Parameters	ESLE (µ/mL)					Group
	T1	T2	T3	T4	T5	SEM
Lipid Peroxidation	0.22^{b}	0.20^{bc}	0.18^{c}	0.27^{a}	0.27^{a}	0.08
Acrosome Integrity	80.67^{a}	79.00^{a}	75.17^{b}	73.83^{c}	76.33^{b}	1.32

^{a,b,c}: means with different superscript on the row are significantly different ($p< 0.05$)

CONCLUSION

The inclusion of ESLE at 2.500µ/mL maintained liveability of spermatozoa for over 75% across the hours; pH range was maintained throughout the period of the experiment. Ethanolic extracts of scent leaf confer an inhibiting effect on reactive oxygen species (ROS) during extension of the cock semen over the hours, and also helped in scavenging free radicals that causes Lipid Peroxidation. These findings suggest the rich phyto-chemical content of *O. gratissimum* and its good antioxidant activity (Akinmoladun *et al*., 2007 and Prabhu *et al*., 2009). It also reduced the damage to the liveability of the spermatozoa because according to Alkan *et al*., (2001) the in-vitro assessment morphological sperm defects of cockerel semen include; neck bending (mid piece bending), mid piece damage, acrosome damage (bending, swelling, knotting or rounding), total head swelling and tail defects which ESLE did.

RECOMMENDATION

From the result of this study, T3 (ESLE 2.500µ/mL) is recommended because the structure of the spermatozoa is more stable throughout the experiment, suggesting it has structure maintaining ability and reduced rate of cellular damage induced by oxygen and oxygen-derived oxidants commonly known as Reactive Oxygen Species (ROS) which induced Lipid Peroxidation.

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