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Antioxidant Properties of *Vernonia amygdalina* Leaf Extract on Semen Characteristics and Seminal Plasma Biochemistry of Cockerels Reared in a Semi-Humid Environment

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Abstract: ***Rearing of birds is a major means of meeting the growing demand of animal protein in Nigeria and the world as a whole. Heat stress poses a major threat to the success of poultry farming as it causes reduced feed intake and weight gain among others. Commercial antioxidants are expensive and not readily available especially to small holder farmers, therefore there is an urgent need to source for a cheap, unconventional source of antioxidant to mitigate the adverse effect of heat stress. A total number of 45 cockerels were randomly distributed into 5 treatment groups, replicated 3 times with 3 cockerels per replicate under elevated summer temperature to investigate the effect of Vernonia amygdalina leaves extract (VALE) supplementation via drinking water on their reproductive characteristics which lasted for eight weeks. Parameters evaluated under semen qualitative analysis were: semen color, volume, concentration, motility, pH, live dead ratio, mass activity, morphology while under seminal biochemistry; lipid peroxidation (MDA) and total antioxidant capacity (GPx and SOD) were evaluated. The results of proximate analysis revealed the presence of high crude protein (23.68%), moisture (6.68%), ash (8.90%), crude lipids (0.37%), crude fibre (7.32%), CHO (53.03%) and calorific value (1295.19KJ/mole).The result of phyto chemical screening revealed saponin (+++), tannin (+++), phenol (+++), flavonoid (++), alkaloid (++), steroid (++),glycoside (++) and anthraquinone (++). Results obtained from semen quality showed that the volume (0.62ml) and concentration (615 x 10^9) were higher in birds given 90mls of VALE, also the values of SOD was higher at 90mls (1069.19u/l) while that of GPx was lowest at T_3 (12.50u/l) and T_5 (16.94u/l). In conclusion, VALE at 90mls can be used as a natural source of antioxidant. Further studies can be conducted using higher concentrations of VALE.***

Keywords: *Vernonia amygdalina*, heat stress, cockerel, leave extract, semen, motility, concentration

1. Introduction

The environmental condition of some parts of Nigeria and many other countries remains hot and humid in certain part of the year. This represent a major constraint for livestock production especially poultry, therefore adversely influencing the survival of birds, as they are more prone to changes in the environment as compared to other domesticated animals, resulting into heat stress (Askar and Ismail, 2012). Heat stress encompasses high environmental temperature and humidity, thereby hindering proper thermoregulatory processes such as mineral imbalance, increased panting, high mortality and affects semen quality and fertility in male birds (Yahav, 2000a). Influence of seasonal changes on semen quality of domestic fowl has been documented (Sahin *et al*., 2002a, b; SantiagoMoreno *et al*., 2011). Heat stress increases lipid oxidant as a result of increased free radical generation which enhances the formation of reactive oxygen species and induces oxidative stress in cells (Altan *et al*., 2003). According to McDaniel *et al*. (1996), report showed that an ambient temperature of > 31C represents a heat stress condition in poultry causing depressed rooster sperm motility, viability and fertilization potential (Sahin *et al*., 2002a, b). Also environmental temperature at ejaculation has an important effect on exogenous physiological factors influencing avian sperm motility (Ashizawa and Sano, 1990; Wishart and Wilson, 1999). Use of synthetic antioxidants such as vitamin C are not easily affordable and available to local farmers, coupled with the increasing advocacy for organic agriculture has necessitated sourcing for other viable alternative. Natural antioxidants such as vitamin C, tocopherols, flavonoids and other phenolic compounds are known to be present in certain plants. *Vernonia* amygdalina is one of such plant that has been identified to contain natural antioxidants (Farombi and Owoeye, 2011). Study conducted by Igile *et al*. (1994) and Adesanoye and Farombi (2010), showed that the antioxidant mechanism has been attributed to the presence of flavonoids. The flavones were luteolin, luteolin 7-o-β-glucuronoside and luteolin-7-0- β –glucoside, with luteolin showing greater antioxidant activity than the other two (Cook, 1996; Prabhakar, *et al*., 2006; Farombi, and Owoeye, 2011). Although, several studies have reported that the use of *Vernonia amygdalina* leaves as feed supplements in livestock (Farombi and Owoeye, 2011; Nwogwugwu *et al*., 2015), the natural antioxidant effect of *Vernonia amygdalina* leaves on the semen characteristics of cockerels reared under high ambient temperature as compared with use of a synthetic antioxidant (vitamin c) is scanty. Therefore, the objective of this study was conducted to examine the effect of various levels of *Vernonia* amygdalina leaves extract (VALE) as a possible replacement for synthetic source of antioxidant (vitamin C) on the semen characteristics and seminal biochemistry parameters of cockerels under heat stress condition.

2. Materials and Method

Experimental Site

The experiment was conducted at the Kwara State University Teaching and Research Farm, Malete, Kwara

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State, with average daytime temperature of 26-39^{0}C and relative humidity of 60-80%.

Animal Feeding and Management

Forty five Issa Brown cockerels of 24 weeks old were purchased from a reputable farm in Ilorin for the experiment. The weights of the birds ranged from 3.0-4.0kg. The birds were acclimatized for 14 days in 4 tyres cages for physiological adjustment to the environment. Grower mash was fed to the birds in relation to their body weight gain per day with *ad libitum* clean water supply from the beginning till the end of the experiment which lasted for 8 weeks.

Collection and Processing of Plant Material

The leaves of *Vernonia amygdalina* were collected fresh from Apata-Yakuba area of Kwara State. The leaves collected were rinsed with distilled water and then air dried at room temperature for seven days. The dried leaves were then blended into a powdery form with an electronic blender and kept in an air tight container. The blended portion weighed 500g.

Chemical Analysis

The proximate analysis of *Vernonia amygdalina* sample was determined chemically according to the official methods of analysis described by the Association of Official Analytical Chemist (AOAC, 1990). The *Vernonia amygdalina* leave extract was also subjected to phytochemical screening.

Extraction Procedure

Distilled water was boiled at 99^{0}C. 500mls of the boiled distilled water was then added to 50g *Vernonia amygdalina* powder so that 1g of powder received 10mls of boiled distilled water. The mixture was first stirred with a stirring rod and was later placed on a mechanical stirrer for 4-6 hours, after which the mixture was sieved using a cheese cloth. The extract obtained was then filtered using whatman filter paper into a conical flask. The extract was then stored in a refrigerator ready for use and the process was repeated every week throughout the time of the experiment. This method was adopted with some modifications (Nwogwugwu *et al*., 2015).

Administration of the Extract and Experimental Design

The birds were divided into five treatments having three replicates per treatment and three birds per replicate. Treatment one which served as the control received no extract of *Vernonia amygdalina*, while treatment 2 received 0.6g of vitamin C into 1500mls of water and treatment 3, 4 and 5 received the extract at 30mls, 60mls and 90mls in 1000mls respectively via their drinking water. VALE was administered every day. The experiment was a completely randomized design (CRD).

Semen Collection and evaluation

The semen was collected by manual massage technique. The semen was collected into small bottles and placed in a flask containing warm water maintained at 35-37^{0}C to maintain the environmental temperature of the sperm cell close to the body temperature to enhance the motility before the commencement of laboratory analysis. Visual assessment of the semen colour was carried out. The volume of the ejaculated sample was determined with the use of collection tube. Motility is the subjective estimation of the motile sperm cells in a given sperm volume. It was estimated on a microscope stage at 37^{0}C. Semen was diluted with a normal saline. A haemocytometer slide was used based on the same principle as in red blood cell count. After the appropriate dilution of semen, a drop was placed on the side. Five squares diagonally from the upper left to the lower right of the haemocytometer are counted. Dilution ratio was 1:7. Concentration equals figure obtained multiplied by 1,000 multiplied by dilution factor. The degree of acidity or alkalinity of the semen was determined using a pH, combi-9. Eosin yellow stain was prepared and wetted on microscope slide. The stained slide was air-dried and a drop of diluted semen was placed on it. The normal and abnormal sperm are viewed under the light microscope.

Lipid Peroxidation

Malondialdehyde (MDA) an index of lipid peroxidation was determined using the method of Buege and Aust (1978). 1.0ml of sample was added to 2ml of MDA reagent and boiled at 100^{0}C for 15 minutes. The reaction mixture was then allowed to cool down. The flocculent materials were removed by centrifuging at 3000 rpm for 10 minutes. The supernatants were removed and its absorbance will be read at 532nm against a blank. MDA was calculated using the molar extinction coefficient for MDATBA-complex of 1.56 x 10^5 M^{-1} cm.

Total Antioxidant Capacity

The total antioxidant capacity of sample was determined with phosphomolybdenum using ascorbic acid as the standard. The assay was based on the reduction of Mo (vi) to Mo (vv) by the extract/fractions and the subsequent formation of a green phosphomolybdate (v) complex at acidic pH 0.1ml of the extract/fraction (100ug/ml) solution was combined with 3ml of reagent (0.6M sulphuric acid, 28M sodium phosphate and 4M ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95^{0}C for 90 minutes. After the samples had cooled to room temperature, absorbance of the aqueous solution of each was read at 695nM against blank in a spectrophotometer. The blank solution contained 3ml of reagent solution and the appropriate volume of the same used for the sample and it was incubated under the same conditions as the rest of the samples (Baydar *et al.*, 2007).

3. Results and Discussion

Table 1: Proximate analysis of *Vernonia amygdalina* leaves

Parameter	Crude Protein	Crude Fibre	Ether extract	Ash	Carbohdrate	Calorific value	Moisture
Values	23.68	7.32	0.37	8.90	53.03	1295.19	6.68

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Table 1 shows the result of proximate analysis of *Vernonia amygdalina* leaves. The result revealed that *Vernonia* contain appreciable amount of crude protein (23.68%), crude fibre (7.32%), moisture (6.68%), crude fat (0.37%), ash (8.90%), carbohydrate (53.03%) and calorific value (1295.19KJ/mole). The crude protein value (32.5%) reported by Getahun(1976) was higher than the value obtained in this study. Udochukwu *et al.* (2015) also reported much higher crude protein (35.7%) in *Vernonia amygdalina* leaves. The crude fibre (7.90%), crude fat (0.48%), ash (8.98%) and moisture (6.80%) contents reported by them were also slightly higher than the values obtained in this study. The values obtained in this study are however higher than the values reported by Igile *et al.*, (1995). However, the result might vary due to geographical location, stage of plant, soil types and weather condition of the place. Application of fertilizer like NPK can also influence the nitrogen and mineral content of *Vernonia* leaves.

Table 2: Phyto chemical screening of *Vernonia amygdalina* leaves

Phytoconstituents	Saponin	Flavonoid	Tannin	Alkaloid	Phenol	Steroid	Glycoside	Anthraquinone
Qualitative Abundance	+ + +	+ +	+ + +	+ +	+ + +	+ +	+ +	+ +

Key: Present at low levels (+), present at moderate levels (++), present at high level (+++).

Table 3 shows the result of the phyto chemical screening of *Vernonia amygdalina* leaves. It revealed that *Vernonia amygdalina* contained essential compounds, saponin (+++), flavonoid (++), tannin (+++), alkaloid (++), phenol (+++), steroid (++), glycoside (++), anthraquinone (++). The presence of these compounds implies that *Vernonia amygdalina* leaves could be utilized as an unconventional source of antioxidant to alleviate heat stress effect on the semen characteristics of cockerels and also a nutritional valuable and healthy ingredient for poultry. Antioxidant activity of the compounds has also been reported (Udochukwu *et al.*, 2015). Their antioxidant activity was higher than the conventional antioxidants such as ascorbic acid, which is also present in large amounts in Vernonia leaves (Igile *et al.*, 1995). An examination of the phytochemicals of Vernonia species affords the opportunity to examine a range of fairly unique compounds (Getahun, 1976).

Table 3: Semen qualitative analysis of cockerels given *Vernonia amygdalina* leave extract (VALE)

PARAMETERS	T_1 (CONTROL)	T_2 (0.6g Vit. C)	T_3 (30mls)	T_4 (60mls)	T_5 (90mls)	SEM
Motility (%)	4.00^a	4.00^a	3.50^b	4.00^a	4.00^a	0.11
Mass Activity (%)	78.33^a	80.83^a	65.83^b	75.83^a	78.33^a	2.58
Live-dead Ratio (%)	4.89^a	4.97^a	2.40^b	3.57^{ab}	4.27^{ab}	0.64
Volume (ml)	0.48	0.60	0.60	0.48	0.62	0.06
pH	7.33	7.37	7.31	7.32	7.35	0.04
Concentration ($\times 10^9$)	585	564	540	571	615	344
Morphology	TH	T&H	T&H	T&H	T&H	
Color	CW	CW	CW	CW	CW	
Means with the same alphabet has no significant difference.						

Keys: T_1 = Control, CW- Creamy white, TH- Tailess head, T&H-Tailed and head
T_2 = 0.6g/1.5 litre of Vitamins C T_3 = 30mls of *Vernonia amygdalina* leave extract (VALE),
T_4 = 60mls of *Vernonia amygdalina* leave extract (VALE), T5=90mls of *Vernonia amygdalina* leave extract (VALE),

Table 3 shows the semen qualitative analysis of cockerels given *Vernonia amygdalina* leave extract (VALE). The result shows that the sperm motility (3.50%), mass activity (65.83%) and live-dead ratio (2.40%) of cockerels given 30mls of VALE (T_3) were significantly ($P<0.05$) lower compared to the control group (4.00%), (78.33%) and (4.89%) respectively. However, the sperm motility, mass activity and live-dead ratio values obtained for cockerels inT_3, T_4 and T_5 were significantly ($P<0.05$) similar to the control. The values recorded for the sperm volume and pH of the cockerels were not significantly ($P<0.05$) influenced across the treatment. Although a numerical increase was observed in sperm volume (0.62ml) of birds given 90mls of VALE. The semen pH values of cockerels as compared to the control group (7.33) showed no significant difference across the treatments, although while the pH of cockerels given 30mls of VALE was numerically lower (7.31) when compared to the control group (7.33), the pH values of the semen of cockerels given 90mls was numerically higher (7.35) when compared to the control group. The pH value for cockerels given 30mls of VALE was in agreement with the work of Lake and Ravie (1979) who reported maximum motility was observed in semen sample with the pH of (7.30). The semen pH of cockerels recorded by other researchers was 7.3 ± 0.01, 7.4 ± 0.02 and 7.5 ± 0.01 (Bah *et al.*, 2001, Peters *et al.*, 2008 and Tuncer *et al.*, 2008). The accessory sex gland fluid is generally alkaline. No significant effect was recorded in the sperm concentration values of the cockerels which ranged between 540-615 $\times 10^9$. The highest value (615 x 10^9) was also obtained in birds given 90mls of VALE. Supplementation of 60 mls and 90mls of *Vernonia amygdalina* leave extract in this study gave higher semen concentration (571 x 10^9 and (615 x 10^9) compared to those in the control group (540 x 10^9) and those that received vitamin C (564 x10^9). Taneja and Gowe, (1962) reported higher level of fertility can be achieved by inseminations with increased sperm concentration in excess of 100 million. Therefore, an increase in the semen concentration has a beneficial effect on reproduction. The values for semen volume obtained for this study ranges from (0.48 to 0.62ml) and no significant difference was observed. This value is lower than the value reported by Taneja and

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Gowe, (1962) which ranged from (0.02 to 0.15ml). A similar observation was reported by Omeje and Udeh (1998). They reported the effect of the volume of a single insemination of undiluted semen on fertility and hatchability with the minimum dose, increase in percentage fertility was observed with an increased semen volume. A higher value of these semen parameters is an indication of higher fertility rate, which is an important factor in animal production. Cockerels in the control group showed morphological abnormalities of head without tail (T&H), while cockerels in T_2, T_3, T_4 and T_5 showed normal morphology of head with tail. The color of the semen ejaculates did not differ significantly between the treatments under investigation and were creamy-white indicating that the massage technique used may be acceptable for cockerel semen collection to obtain good quality semen for artificial insemination. Creamy-white ejaculates observed in this study were consistent with Peters *et al.* (2008) who also reported creamy-white ejaculates in heat-stressed cockerels. Machebe and Ezekwe (2005) revealed that variations in semen color may arise in parts due to the presence of contaminants or as a result of low sperm concentration.

Table 4: Total antioxidant capacity, Lipid peroxidation, Calcium, Sodium, Superoxide dismutase and Glutathione peroxidation of cockerels given *Vernonia amygdalina* leave extract (VALE).

PARAMETERS	T_1 (Control)	T_2 (0.6g of Vit.C)	T_3 (3Omls)	T_4 (60mls)	T_5 (90mls)	SEM
TAP (µg/ml)	76.08^{ab}	63.67^{b}	89.50^{a}	96.50^{a}	74.42^{ab}	7.19
MDA (u/l)	0.076	0.070	0.071	0.093	0.073	0.00
Ca (mg/dl)	6.83	5.70	6.07	7.11	6.37	0.73
Na (mg/dl)	52.10^{a}	9.25^{b}	60.55^{a}	54.60^{a}	60.85^{a}	5.54
SOD (u/l)	746.27^{b}	761.19^{b}	728.86^{b}	1032.34^{a}	1062.19^{a}	46.36
GPx (u/l)	25.00^{b}	52.08^{a}	12.50^{c}	20.83^{bc}	16.94^{bc}	2.72

Means bearing different superscript in the same row differ significantly ($P<0.05$).

Keys: T_1 = Control
T_2 = 0.6g of Vitamins C T_3 = 30mls of *Vernonia amygdalina* leave extract (VALE)
T_4 = 60mls of *Vernonia amygdalina* leave extract (VALE)
T_5 = 90mls of *Vernonia amygdalina* leave extract (VALE)
SEM = Standard error of means TAP = Total antioxidant potential
MDA = Lipid peroxidation SOD = Superoxide dismutase
GPx = Glutathione peroxidation

Table 4 shows the level of total antioxidant potential (TAP), lipid peroxidation (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) of cockerels given *Vernonia amygdalina* leave extract (VALE). Lipids are involved in the structural and functional functions of the sperm and their profile may be modified according to physiologic events and/or diet (Mourvaki *et al.*, 2010). VALE has been found to have potential in vitro antioxidant agent. In this study, the *in vitro* and *in vivo* antioxidant activity of VALE were quantified by using traditional spectrophotometric thiobarbituric reactive specie (TBARS) assay, superoxide dismutase (SOD) activity, malondialdehyde (MDA) level and total antioxidant potential (TAP). In vitro TBARS assay showed that VALE was a moderate antioxidant agent when compared with vitamin C. Result showed that the values obtained for cockerels given 30mls (T_3) and 60mls (T_4) of VALE were significantly higher compared to the positive control group which contain synthetic pharmaceuticals (Vitamin C). Cockerels in T_5 which received 90mls of VALE were not significantly influenced when compared with the control group. This could be attributed to the presence of flavonoid component of *Vernonia amygdalina* which possess antioxidant activity as reported by Igile *et al.* (1995). The MDA and Ca values were not significantly influenced across the treatments, although a numerical increase was observed in T_3 (0.071u/l), (6.07mg/dl), T_4 (0.093u/l), (7.11mg/dl) and T_5 (0.073u/l), (6.37mg/dl) compared to T_2 (0.070u/l), (5.70mg/dl). The SOD values of cockerels given VALE at 60mls (1032.34u/l) and 90mls (1062.19u/l) were observed to be significantly higher compared with that of the control group (761.19u/l). SOD is an indicator of antioxidant capacity. Heat stress has been known to induce oxidative damage in cells of animals as a result of a depletion of natural antioxidant. *Vernonia amygdalina* has been documented to contain natural antioxidant capacity which can improve antioxidant enzymes of animals exposed to heat stress. The highest SOD value (1062.19u/l) recorded in birds given 90mls of VALE is an indication that natural antioxidant capacity improved with an increasing concentration of VALE. Glutathione peroxidase (GPx) value of T_1 (25.00u/l), T_3 (12.50u/l), T_4 (20.83u/l) and T_5 (16.94u/l) was lower when compared to that of T_2 (52.08u/l). Reduced GPx value shows its involvement in the non-enzymatic removal of reactive oxygen species (ROS) (Park *et al.*, 2006). Significantly lowest values were observed at T_3 (12.50u/l) and T_5 (16.94u/l) respectively. This is an indication that the effect of VALE to remove ROS from cockerels was promising and eminent.

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