Effects of bitter leaf (*Vernonia amygdalina*) meal as a feed additive on organ weights, serum biochemistry, organ histopathology and carcass of grasscutters (*Thryonomys swinderianus*)

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Abstract

Feed additives are added to livestock diets in order to enhance feed utilization, prevent diseases, improves health and production efficiency. Medicinal plants are recently being explored to be used as feed additives to replace synthetic antibiotics due to their abundance in the natural environment and lack of residual side effects. Therefore, this study aimed at investigating the effect of dietary bitter leaf meal (BLM) as a feed additive on organ weight, serum biochemistry and organ histopathology of weaner grasscutters. Forty weaner grasscutters of mixed sexes with a mean weight of 396 ± 4.5g were allotted randomly to four treatment groups in a completely randomized design (CRD). The BLM was administered at the rate of 0, 20, 40, and 60g/kg into the diets T_1 (control), T_2, T_3 and T_4 respectively. The grasscutters were fed the experimental diets for 10 weeks. At the end of the experiment, data were collected from four randomly selected animals per treatment. Blood samples were collected into plain bottles for serum biochemical tests. The internal organs were excised for their weight determination. Sample of the stomach, caecum and small intestine were excised and fixed for histopathological analysis. Data collected were analyzed using one-way Analysis of Variance (ANOVA) using SAS 2008 model and mean differences separated with Duncan multiple range test. The BLM supplemented diets had significant effect ($p<0.05$) on all the organ weights except the heart. Grasscutters fed 40gBLM/kg had the highest live, carcass and eviscerated weights (1004.25g, 692.5g and 698.68g, respectively), while those fed 20gBLM/kg had significantly ($p<0.05$) higher 0.91, 0.88, 0.15, 0.20, 0.21, 2.00, 4.54, 6.83 and 14.57% for kidney, lung, trachea, bile, spleen, liver, small intestine, stomach and caecum weights, respectively than those on other diets. The serum biochemical parameters were all significantly ($p<0.05$) affected by BLM supplementation except total cholesterol. BLM at 40g/kg level significantly ($p<0.05$) improved the total protein, albumin, globulin (10.95, 4.09, 6.86mg/mL) respectively, reduced creatinine (1.05mg/mL), Aspartate amino transferase (84.95U/L) and Alanine amino transferase (57.83U/L). Stomach micrographs showed mild damage to the mucosa layers, while the small intestine and caecum micrographs showed normal mucosa layers. BLM supplement at 20- 40g/kg is recommended for weaner grasscutters based on the observation that this levels improved their organ weights and general wellbeing without causing damage to their digestive organs.

Keywords*:* grasscutter; bitter leaf meal; organ weights; histopathology; serum biochemistry.

Effets de la farine de feuille d'amygdaline (Vernonia amygdalina) utilisée comme additif alimentaire sur le poids des organes, la biochimie sérique, l'histopathologie des organes et la carcasse des rongeurs (Thryonomys swinderianus)

Résumé

Les additifs alimentaires sont ajoutés à l'alimentation du bétail afin d'améliorer l'utilisation des aliments, de prévenir les maladies, d'améliorer la santé et l'efficacité de la production. Les plantes médicinales font récemment l'objet de recherches en vue de leur utilisation comme additifs alimentaires pour remplacer les antibiotiques synthétiques, en raison de leur abondance dans l'environnement naturel et de l'absence d'effets secondaires résiduels. Cette étude visait donc à étudier l'effet de la farine de feuilles amères (BLM) utilisée comme additif alimentaire sur le poids des organes, la biochimie sérique et l'histopathologie des organes chez des rongeurs-herbivores sevrés. Quarante rongeurs-herbivores sevrés des deux sexes, d'un poids moyen de 396 ± 4,5 g, ont été répartis aléatoirement en quatre groupes de traitement selon un plan d'expérimentation complètement aléatoire (CRD). Le BLM a été administré à des doses de 0, 20, 40 et 60 g/kg dans les régimes *alimentaires T1 (témoin), T2, T3 et T4 respectivement. Les gazelles ont été nourries avec ces régimes expérimentaux pendant 10 semaines. À la fin de l'expérience, des données ont été recueillies auprès de quatre animaux sélectionnés au hasard par traitement. Des échantillons de sang ont été prélevés dans des flacons simples en vue de tests biochimiques sériques. Les organes internes ont été prélevés afin de déterminer leur poids. Des échantillons de l'estomac, du cæcum et de l'intestin grêle ont été prélevés et fixés en vue d'une analyse histopathologique. Les données recueillies ont été analysées à l'aide d'une analyse de variance (ANOVA) à un facteur utilisant le modèle SAS 2008, et les différences de moyennes ont été séparées à l'aide du test de Duncan. Les régimes alimentaires enrichis en BLM ont eu un effet significatif (p < 0,05) sur le poids de tous les organes, à l'exception du cœur. Les grasscutters nourris avec 40 g de BLM/kg présentaient les poids vifs, de carcasse et éviscérés les plus élevés (respectivement 1 004,25 g, 692,5 g et 698,68 g), tandis que ceux nourris avec 20 g de BLM/kg présentaient des valeurs significativement (p < 0,05) plus élevées de 0,91, 0,88, 0,15, 0,20, 0,21, 2,00, 4,54, 6,83 et 14,57 % pour les poids des reins, des poumons, de la trachée, de la vésicule biliaire, de la rate, du foie, de l'intestin grêle, de l'estomac et du cæcum, respectivement, par rapport à ceux soumis à d'autres régimes alimentaires. Les paramètres biochimiques sériques ont tous été significativement (p < 0,05) influencés par la supplémentation en BLM, à l'exception du cholestérol total. Une dose de 40 g/kg de BLM a significativement (p < 0,05) amélioré les taux de protéines totales, d'albumine et de globuline (10,95, 4,09, 6,86 mg/mL) respectivement, et a réduit la créatinine (1,05 mg/mL), l'aspartate aminotransférase (84,95 U/L) et l'alanine aminotransférase (57,83 U/L). Les micrographies de l'estomac ont montré de légères lésions des couches muqueuses, tandis que celles de l'intestin grêle et du cæcum ont révélé des couches muqueuses normales. Un supplément de BLM à raison de 20 à 40 g/kg est recommandé pour les grasscutters sevrés, sur la base de l'observation que ces doses ont amélioré le poids de leurs organes et leur bien-être général sans causer de lésions à leurs organes digestifs.*

Mots-clés : grasscutter ; farine de feuilles amères ; poids des organes ; histopathologie ; biochimie sérique.

Introduction

The rising demand for adequate animal protein has brought attention to the domestication and rearing of wild animals (Akinloye, 2005). Globally, wildlife has huge potential for producing meat as well as an essential source of much valued protein from animal source for human consumption (Akinola *et al.*, 2015). Among the wildlife species of consideration are the grasscutter, giant African snail and Guinea fowl. Grasscutter (*Thryonomys swinderianus*) showing the greatest prospects among the considered species (Swallah, 2018).The grasscutter meat is cherished because of its peculiarly delicious taste, which makes it the majority of bush meat being consumed in West Africa (Addo *et al.*, 2007). Therefore, the domestication of grasscutters is necessary in other to conserve their wild population, preserve their natural habitats and meet increasing consumer demand (Addo, 2002). Grasscutter feeds mainly on green plants especially those with succulent stems such as elephant grass (*Pennisetum purpureum*), Guinea grass (*Panicum maximum*), and foliage of certain leguminous plants, maize husk, tubers of yam, cassava and other root crops. However, these feed resources are not readily available during the dry season; thus, regular and adequate feeding is necessary for their productivity and health performance under domestication (Okukpe *et al*., 2016; 2020).

In captivity, grasscutter experiences compromise health conditions as a result of stress and parasitic infestations resulting in suboptimal production and sometimes death. The use of feed additives and supplement have been reported to contribute to worthy production efficiency, prevention of diseases, improved health and feed utilization in poultry (PCRC 2013).The use of feed supplement in rearing young grasscutters may produce a comparable positive effects, especially during domestication that exposes the animals to considerable stress, requiring physiological adaptation to maintain and improve homeostasis (Amuda and Okunlola, 2018). However, utilization of synthetic antibiotics and supplements often results in residual deposits in the carcass, leading to restrictions on their usage (Okukpe *et al*., 2020). This has intensified the search for organic alternatives, including bitter leaf (*Vernonia amygdalina*), a tropical plant in the family *Asteraceae. Vernonia amygdalina* popularly known as bitter leaf, thrives in the humid tropics of Africa and is among several herbs used by traditional healers in Western Nigeria for treatment of various bacterial infections. Decoctions of its roots and leaves are commonly used in ethno medicine to treat fevers, hiccups, kidney problems, liver problems, stomach discomfort among other several uses (Momoh *et al*., 2010).The reported bioactivities of bitter leaf is attributable to the presence of complex active secondary plant metabolites including flavonoids, tannins, saponins, phytates, steroids and cardiac glycosides in the leaves (Orabi and Shawky, 2014).

In addition, air dried bitter leaves contains 33.47 % crude protein, 1.65% crude fibre, 9.2% ash (dry matter basis), and a good composition of macro elements (Sanni, 2024). According to Esonu *et al.* (2006), haematological and serum biochemical parameters are useful indicators of the physiological status of animals, and variations in these values can be used to evaluate how an animal's body is responding to different physiological situations. In feeding trials, toxicity level is assessed by measuring the weights of visceral organs such as kidney, heart and liver because the presence of toxins in feed might adversely affect these vital organs (Noah e*t al*., 2020). Plant based additives have been proven to improve nutrient intake and digestibility, feed conversion efficiency and subsequent weight gain in livestock (Cardoso *et al.*, 2012). Moreover, it has also been reported that the extracts of bitter leaf have antimicrobial and protective effects on the internal digestive organs (Adesanoye and Farombi 2010). In meat production system, the farmer's aim is to produce animals that meet a specific meat quality trait. Moreover, animals fed balanced and healthy diets, regardless of breed, will likely have higher dressing percentages since carcass products and by-products are good indicators of the level and quality of feeding (Amuda and Okunlola, 2018).

Thus, this study was designed to investigate the effect of graded levels of bitter leaf meal (BLM) supplementation on carcass, organ weights, serum biochemistry and organ histopathology of grasscutters.

Materials and methods

Animals and experimental design

The feeding trial was conducted at the Biological Gardens, Federal Polytechnic Offa, Kwara State**.** The institution is located within the Savannah ecological zone on Latitude 8° 7'57.91"N and Longitude 4° 42' 32.32" E. The ambient temperature ranges from 22.78 – 34.12ºC and relative humidity is 61.02%. The area is characterized by tropical wet and dry (savannah) climate with an average annual rainfall of 100.39 mm (Google Earth, 2020).

Fresh bitter leaves properly identified at the Herbarium of the Department of Plant Biology, with Voucher Number UILH/001/972, were used for the study. Mature bitter leaves were harvested from Adewole Estate in Ilorin, Nigeria. The leaves were harvested for a duration of 12 months period from January to December. The harvested leaves were thoroughly cleaned and air dried under shade for 4 – 7 days. Subsequently, the air-dried leaves were milled into powder using a food blender (Starlite, Model No: SL-999 China) and stored in an airtight container at room temperature for further use. The samples for proximate analysis and feeding were taken from the stored leaf powder. The powdered leaves were incorporated into a formulated diet as presented in Table 1 at graded levels of 0, 20, 40, and 60g /kg making a total of four (4) concentrate experimental diets: T_1, T_2, T_3, and T_4. The diets were formulated to meet the nutrient requirements for optimum production of weaner grasscutters as recommended by Adeniji (2009) and Kusi *et al.* (2012).

Forty mixed sex 8-weeks old grasscutters were purchased from a reputable farm in Republic of Benin, and used for this study. The animals were housed in a wooden single-tier cage system with cells partitioned with wood, and wire mesh floors. The cages were placed inside a fully enclosed pen made of concrete. Each cell dimension measured 40cm×80cm×50cm.Thirty -two female grasscutters and eight males in ratio 4:1 were assigned randomly into four (4) treatment groups. The animals were allotted into four dietary treatments, with each treatment group consisting of eight females and two males. The animals were arranged in five replicates per group with two grasscutters per replicate in a completely randomized design (CRD), after two weeks acclimatization period. Experimental diets (concentrates) and water were provided *ad libitum* throughout the experimental period. Elephant grass (*Pennisetum purpureum*) was offered once daily to the animals in the evening to meet their forages requirement as monogastric herbivore. The elephant grass (*Pennisetum purpureum*) contained 29.3% dry matter, 9.25% crude protein, 9.28% ash, 1.17% ether extract, 26% crude fibre, and 34.30% nitrogen free extract. Strict hygiene was maintained in the animal house by daily cleaning the animal cages, feeding troughs and drinkers, while the pens were washed and disinfected weekly using soap, disinfectant and water. The experiment lasted ten (10) weeks and was conducted with the approval of Ethical Review Committee of University of Ilorin, with the Protocol Identification Code and UERC Approval Number UERC/AGR/157 and UERC/ASN/2019/1636, respectively.

Data collection and analytical methods

Proximate analysis of the experimental diets (Table 1) was conducted at Central Research and Diagnostic Laboratory Tanke, Ilorin. Samples were analysed in triplicate. Determination of the dry matter, crude protein, crude fibre, ash, and ether extract were carried out according to the analytical methods of AOAC (2005).

Table1: Gross composition of basal experimental diets

Ingredients (%)	T1(0g)	T2(20g)	T3(40g)	T4 (60g)
Maize	50.00	50.00	50.00	50.00
Corn bran	6.00	6.00	6.00	6.00
Wheat offal	11.45	11.45	11.45	11.45
Fish meal	1.00	1.00	1.00	1.00
Soyabean meal	12.50	12.50	12.50	12.50
Palm kernel cake	7.00	7.00	7.00	7.00
Groundnut cake	8.00	8.00	8.00	8.00
Bone meal	2.50	2.50	2.50	2.50
Oyster shell	1.00	1.00	1.00	1.00
Salt	0.30	0.30	0.30	0.30
Vitamin premix	0.25	0.25	0.25	0.25
TOTAL	100	100	100	100
Bitter Leaf Meal (g)	0.00	2.00	4.00	6.00

Measurement of organ weights

At the 10th week of the trial, four grasscutters were randomly selected from each treatment group, weighed, anaesthetized with diethyl ether (used at a very low concentration (1.9%) this is produced with 0.08ml per litre of volume of a container) to reduce handling stress prior slaughtered. Diethyl ether is used because of its poor volatility which made it relatively safer to use by open drop method of applying anesthesia and reducing its residual effects on meat quality. Each animal was dissected and the different parts were weighed using an electronic Mettler weighing scale (Model No: SNR K67861 Switzerland). The parameters measured include the eviscerated, total visceral, heart, kidney, lung, bile, spleen, trachea, liver, stomach, caecum, small and large intestine. The relative organ weights were calculated using the equation below.

$$Relative\ organ\ weight\ (\%) = \frac{weight\ of\ the\ organ\ (g)}{live\ weight\ of\ the\ animal\ (g)} \times 100$$

Determination of serum biochemical variables

At the 10th week of the experiment, four animals consisting of 2 males and 2 females were randomly selected per treatment group. The animals were fasted for 10 hours, anaesthetized with diethyl ether and slaughtered by severing the carotid artery and the jugular vein. Blood samples (3mL) were collected using a 5mL sterilized disposable syringes with 21 gauges and 1.5 inches-long needles for each animal into plain sterilized tubes without EDTA for serum biochemical analyses. Serum Biochemical parameters were analysed for Total proteins were determined by the Biuret reagent using a commercial kit (Randox® Laboratories Ltd, U.K), while Albumin was evaluated using bromocresol green method. Globulin concentration was calculated as from the difference between total proteins and albumin following the method of Coles (1986). The Serum creatinine and Urea nitrogen were determined by deproteinisation and Urease-Berhelot colorimetric methods, respectively, using a commercial kit (Randox® Laboratories Ltd, U.K) as described by Fossati *et al.* (1980). Total cholesterol was determined by nonane extraction and enzymatic colorimetric methods, respectively using commercial kit (Randox® laboratories Ltd., Ardmore, Diamond Road, Crumlin Co., Auturm, UK), while the Liver enzymes, alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) were measured using the Randox® test kits as described by Reitman and Frankel, (1957).

Histopathological examination of the GIT

After slaughtering the selected grasscutters and measurement of their organ weights, tissues of some selected organs of the gastrointestinal (GIT) organ including the stomach, caecum and small intestine were cut and fixed in 10% formalin and taken to the laboratory in a sterilized universal sample bottles for Histological evaluation. After fixation, the tissues were washed and processed using the standard histological method and embedded in paraffin. The embedded tissues were trimmed of the paraffin blocks to reveal the tissue surface for microtomy, and sectioned at 7 microns to obtain tissue ribbon. Thereafter the section were stained with haematoxylin and eosin (H and E) for histopathological examination under a microscope at a magnification of x100 as described by Bancroft and Gamble (2008).

Statistical Analysis

Data obtained for proximate analysis were analyzed using descriptive statistics. All other data obtained from the feeding trial were subjected to analysis of variance (ANOVA) in a completely randomized design (CRD) using SAS (2008). Significant differences among treatment means were declared at $p < 0.05$, and means were separated using Duncan's Multiple Range Test (DMRT) within the same software.

Results and discussion

Results

Proximate analysis of the experimental diets

The proximate composition of the experimental diets are presented on Table 2. There were no significant ($p > 0.05$) difference in all the nutrient components such as the dry matter, crude protein, crude fibre, ether extract, ash and nitrogen free extract. This mean that the addition of bitter leaf meal did not contribute significant addition to the nutrient contents. This shows that the experimental diets were within the normal nutrient requirement for growing grasscutters in all the groups.

Table 2: Proximate composition of the experimental diets

Nutrients (%)	**T_1(0g)**	**T_2(20g)**	**T_3(40g)**	**T_4(60g)**
Dry matter	94.23	94.20	93.86	94.14
Ash	5.04	5.07	4.56	5.12
Ether extract	5.51	5.42	5.48	5.45
Crude fibre	5.43	4.96	4.94	4.67
Crude protein	18.12	18.78	19.31	21.50
Nitrogen free extract	60.13	59.97	59.58	57.40

T_1; 0g BLM/kg = control; T_2; 20g BLM/kg; T_3; 40g BLM/kg and T_4; 60g BLM/kg.

***Carcass and organ weights of grasscutters fed bitter leaf meal supplemented diets*.**

The effects of bitter leaf meal (BLM) supplementation on carcass and organ weights of grasscutter are presented on Table 2. The treatments effect on the organ weights were significant ($p<0.05$) except for the heart weight. Live weight, carcass and eviscerated weights of grasscutters fed with 40gBLM/kg were significantly ($p<0.05$) higher than those of the other treatments. Dressing percentages of animals on treatment groups fed with 40g and 60g BLM/kg containing diets were significantly ($p<0.05$) higher than those on 20g BLM/kg. The grasscutters supplemented with 20g BLM/kg had significantly ($p<0.05$) higher total visceral weights compared with other treatments.

Likewise, kidney, lung, trachea, bile, stomach, caecum, spleen, liver and small intestine weights of animals on 20gBLM/kg were significantly ($p<0.05$) higher than those on other treatments. Grasscutters fed 40g BLM/kg supplemented diet had the highest large intestine weight. In addition, the small intestine and large intestine lengths were longest in grasscutters fed 20g BLM/kg and were significantly ($p<0.05$) higher than those on other treatment groups.

Table 3: Carcass and organ weights of grasscutters fed bitter leaf meal supplemented diets

Parameters	T_1	T_2	T_3	T_4	SEM	P values
Live weight (g)	783.75^{b}	850.00^{ab}	1004.25^{a}	758.75^{b}	41.46	0.01
Carcass wt(g)	519.63^{b}	508.33^{b}	692.50^{a}	524.43^{b}	30.03	0.00
Eviscerated wt(g)	532.25^{b}	519.60^{b}	698.68^{a}	532.18^{b}	31.43	0.01
Dressing (%)	66.48^{a}	59.68^{b}	68.97^{a}	68.96^{a}	1.05	0.00
Visceral organs (% of live weight)						
Total visceral	28.50^{b}	35.94^{a}	25.77^{c}	23.68^{c}	0.59	0.00
Kidney	0.81^{b}	0.91^{a}	0.67^{c}	0.61^{c}	0.02	0.00
Lung	0.70^{ab}	0.88^{a}	0.80^{ab}	0.60^{b}	0.05	0.02
Trachea	0.14^{ab}	0.15^{a}	0.12^{b}	0.08^{c}	0.01	0.00
Heart	0.48	0.46	0.44	0.49	0.02	0.15
Bile	0.17^{b}	0.20^{a}	0.14^{c}	0.14^{c}	0.01	0.00
Spleen	0.14^{b}	0.21^{a}	0.13^{b}	0.14^{b}	0.01	0.00
Liver	1.83^{ab}	2.00^{a}	1.94^{ab}	1.73^{b}	0.06	0.04
Small Intestine	3.42^{c}	4.54^{a}	3.78^{b}	2.90^{d}	0.09	0.00
Large Intestine	5.00^{b}	5.24^{ab}	5.98^{a}	4.13^{c}	0.23	0.00
Stomach	5.30^{b}	6.83^{a}	4.24^{c}	3.82^{c}	0.13	0.00
Caecum	10.51^{b}	14.57^{a}	9.42^{bc}	8.23^{c}	0.32	0.00
Absolute length in cm						
Small Intestine	148.50^{bc}	174.00^{a}	153.00^{b}	142.75^{c}	1.89	0.00
Large Intestine	96.63^{c}	126.75^{a}	106.38^{b}	92.88^{c}	2.84	0.00

a, b, c, d, means with different superscript are significant($p<0.05$) along the row. SEM = Standard Error of Mean, Bitter Leaf Meal =BLM. T_1; 0g BLM/kg = control; T_2; 20g BLM/kg; T_3; 40g BLM/kg and T_4; 60g BLM/kg.

Serum biochemical variables of grasscutters fed dietary bitter leaf meal diets.

The results of serum biochemical indices of grasscutters fed bitter leaf meal supplemented diets are presented in Table 4. The treatments significantly ($p<0.05$) influenced the biochemical indices except total Cholesterol which was not significantly affected. The total protein values were highest in the group fed with 40g BLM/kg and were significantly ($p<0.05$) higher than other treatment groups. The grasscutters fed with 40g BLM/kg supplemented diet had the highest value for albumin concentration and it was statistically similar ($p>0.05$) to the control and 60gBLM/kg groups, but significantly ($p<0.05$) higher than those fed 20gBLM/kg supplemented diet. The globulin levels of grasscutters fed BLM supplemented diets were significantly ($p<0.05$) higher than those of the control. Grasscutters fed 20g BLM/kg containing diet had the lowest value for urea concentration, while those on 40g BLM/kg diet had the lowest creatinine value. The AST and ALT values of the control were significantly higher ($p< 0.05$) than those in the BLM-supplemented groups. The Alkaline phosphatase (ALP) value was highest in 60gBLM/kg group and it was significantly ($p<0.05$) higher than other treatments.

Table 4: Effects of bitter leaf meal supplement on serum biochemical variables of grasscutters

Parameters	T_1	T_2	T_3	T_4	SEM	P values
Total Protein(mg/dL)	10.20^{ab}	10.06^{b}	10.95^{a}	10.11^{b}	0.19	0.02
Albumin(mg/dL)	4.04^{a}	3.23^{b}	4.09^{a}	3.81^{a}	0.09	0.00
Globulin(mg/dL)	6.16^{b}	6.84^{a}	6.86^{a}	6.30^{ab}	0.16	0.01
Urea (mg/dL)	4.31^{c}	4.10^{d}	5.70^{a}	4.50^{b}	0.04	0.00
Creatinine (mg/mL)	1.68^{a}	2.03^{a}	1.09^{b}	1.05^{b}	0.10	0.00
Total Cholesterol (mg/dL)	241.37	160.76	148.14	144.00	29.9	0.13
AST(U/L)	98.5^{a}	91.13^{b}	86.83^{c}	84.95^{c}	0.81	0.00
ALT(U/L)	78.18^{a}	71.88^{b}	62.23^{c}	57.83^{d}	0.94	0.00
ALP(U/L)	169.77^{c}	150.75^{d}	238.55^{b}	260.63^{a}	2.90	0.00

a, b, c, d,- means in the same row with different superscripts are significantly different ($P < 0.05$); AST= Aspartate aminotransferase, ALT = Alanine aminotransferase, ALP= alkaline phosphatase. SEM = Standard Error of Mean, Bitter Leaf Meal =BLM. T_1; 0g BLM/kg = control; T_2; 20g BLM/kg; T_3; 40g BLM/kg and T_4; 60g BLM/kg.

Stomach, small intestine and caecum histopathology

Effects of bitter leaf meal (BLM) supplemented diets on histopathology of the stomach, small intestine and caecum of grasscutters are presented in the photomicrographs in plates 1-12. The slides present with delineation of the gastric wall shows the layers of the stomach; mucosa (M), muscolaris mucosa (MM) and submucosa (SM). Most of the stomach micrographs (Plates 1- 4) showed mild changes to the mucosa and submucosa layers. Photomicrographs of jejunums showed typical histological pattern similar to the entire small intestinal mucosa. The small intestine section micrographs showed the different characteristic layers of the intestinal wall such as the mucosa (M), submucosa (SM), and muscularis mucosa (MM). Most of the micrographs (Plates 5-8) showed normal mucosal layers, with mild erosion of the submucosal layer. The caecum photomicrographs (Plates 9-12) of the grasscutters showed that most of them had normal mucosa layers architecture with slight loss of the mucosa epithelial integrity and erosion of the submucosa layer in the sections.

Photomicrographs of the stomach histopathology of grasscutters fed bitter leaf meal supplemented diets

Plate 1

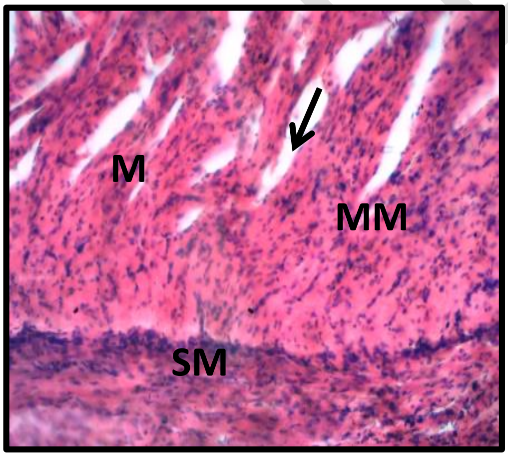


Plate 2

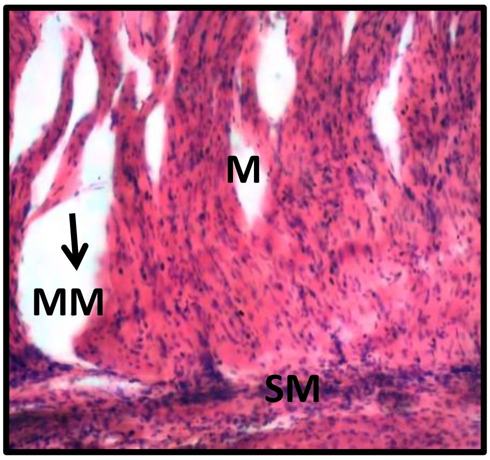
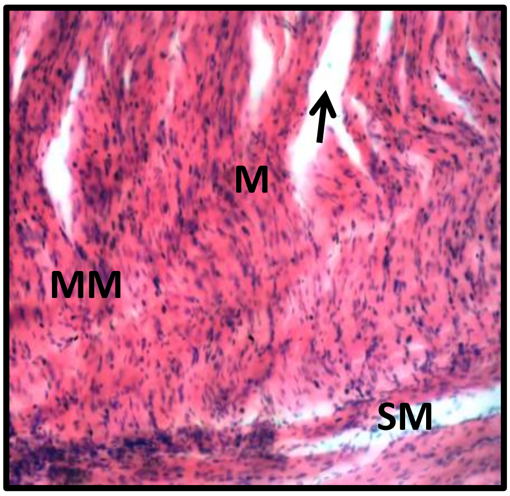
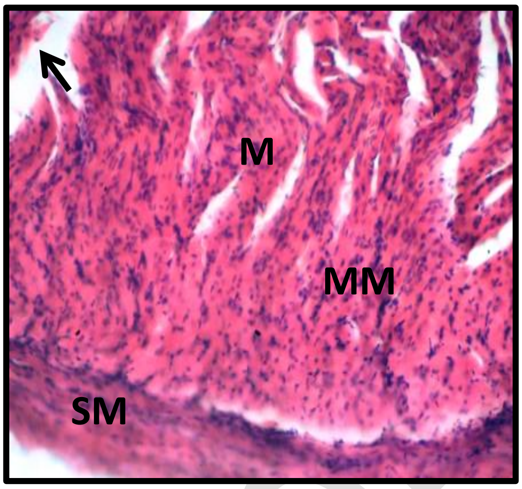


Plate 3

Plate 4



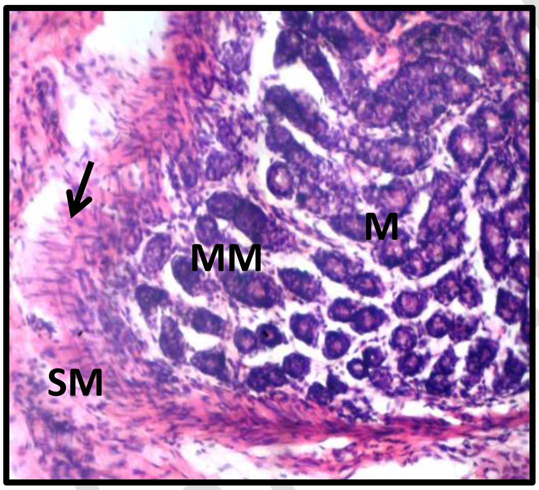


Transverse sections of the pyloric region of the stomach showing the Mucosa (M), Muscolaris Mucosa (MM) and Submucosa (SM) Layers. (Section thickness: 7µ, H&E, x100). Plate 1= T_1 (Control); Submucosa layers appear intact, there is changes (arrows) of the mucosa layer. Plate 2 =T_2 (20g BLM/kg); Extensive changes (arrows) of mucosa epithelium layer, the submucosa layers appear intact with mild changes (arrows). Plate3 = T_3 (40gBLM/kg); mild changes (arrows) of the mucosa and submucosa layers. Plate 4 =T_4 (60g BLM/kg); mild changes (arrows) of both the mucosa and submucosa layers.

Photomicrographs of the small intestine histopathology of grasscutters fed bitter leaf meal supplemented diets

Plate 5

Plate 6



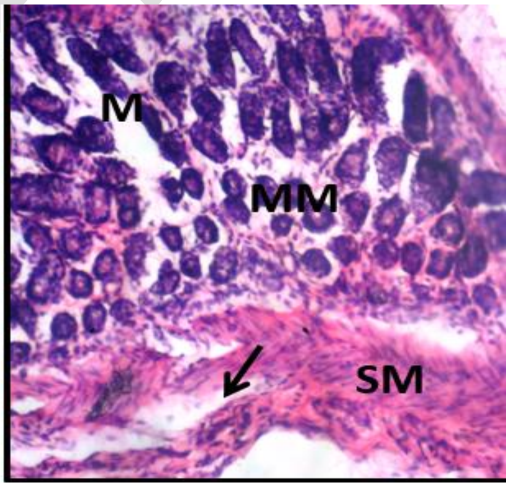
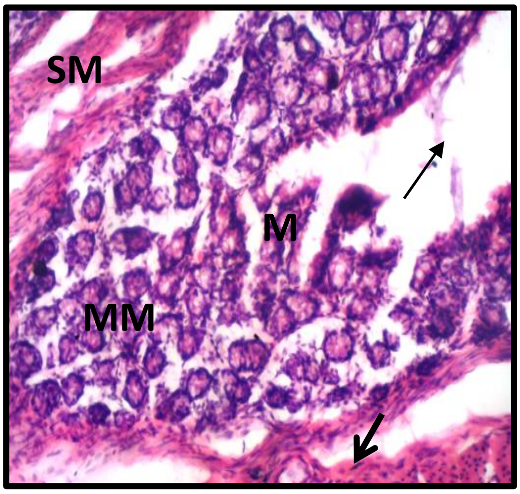
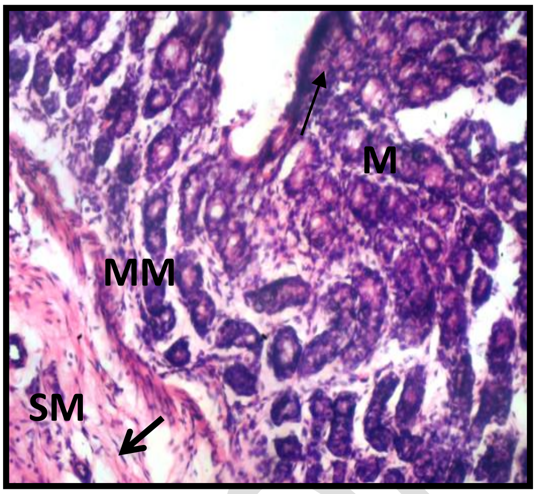


Plate 7

Plate 8





Transverse sections of the jejunums showing typical histological pattern similar to the entire small intestinal Mucosa (M), Muscolaris Mucosa (MM) and Submucosa (SM) Layers. (Section thickness: 7µ, H&E, x100). Plate 5= T_1 (Control); Mucosa layers appear normal with little loss of the mucosa epithelium, while erosion (arrows) of the submucosa layer occurs. Plate 6 = T_2 (20g BLM/kg); Normal mucosa layer with normal size and cellularity of goblet cells, and mild erosion (arrows) of the submucosa layer. Plate 7 = T_3 (40gBLM/kg); little loss of the mucosa epithelium with reduces cellularity of goblet cells and mild erosion (arrows) of the submucosa layer. Plate 8 = T_4 (60gBLM/kg); little loss of the mucosa epithelium with normal size and cellularity of goblet cells, and mild erosion (arrows) of the submucosa layer.

Photomicrographs of the caecum histopathology of grasscutters fed bitter leaf meal supplemented diets

Plate 9

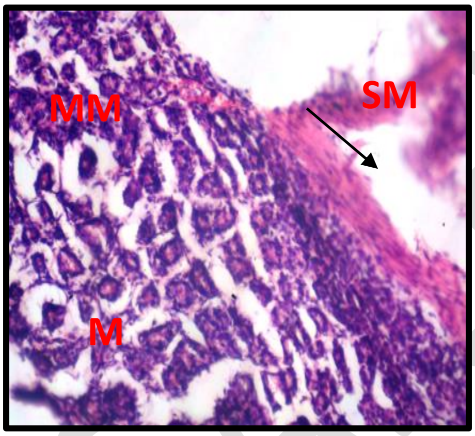


Plate 10

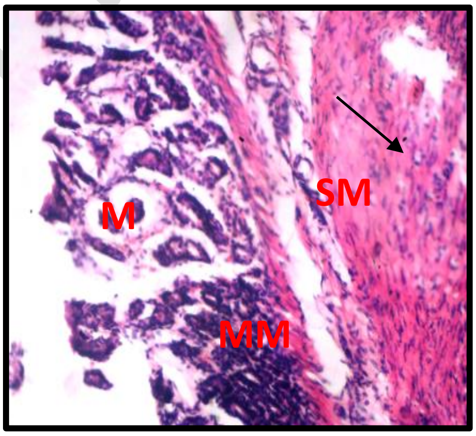


Plate 11

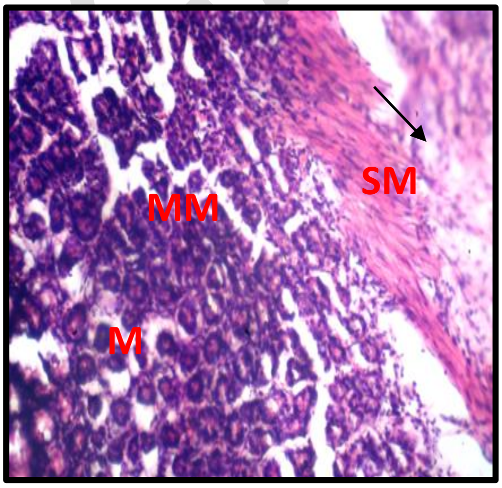
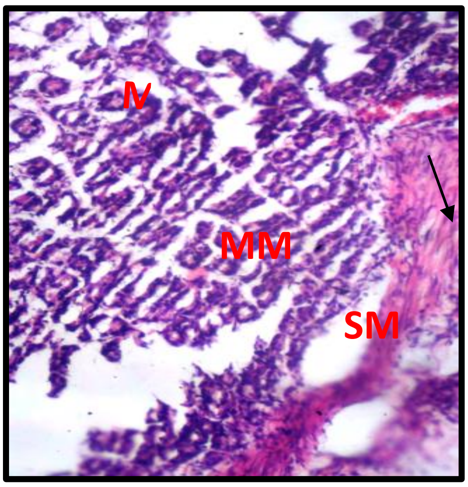


Plate 12



Transverse sections of the caecum showing the Mucosa (M), Muscularis Mucosa (MM) and Submucosa (SM) Layers. (Section thickness: 7µ, H&E, x100). Plate 9 = T_1 (Control); normal mucosa layers with the erosion (arrows) of the submucosa. Plate 10 =T_2 (20gBLM/kg); little loss of the mucosal epithelium and erosion (arrows) of the submucosa. Plate11 = T_3 (40gBLM/kg); normal mucosa layers with mild erosion (arrows) of the submucosa layers. Plate 12=T_4 (60g BLM/kg); little loss of the mucosal epithelium with mild erosion (arrows) of the submucosa layers.

Discussion

Effect of bitter leaf meal supplement on the carcass and organ weights of grasscutters

Carcass weight, eviscerated weight and final live weight of grasscutters fed 40g/kg BLM supplemented diet were the highest, while those fed 20g/kg supplemented diet have the highest total visceral weights. The significant variations observed in the live, carcass and eviscerated weights, agrees with the findings of Okukpe *et al.* (2019), who reported similar trend in grasscutter fed 1-5g bitter leaf meal per kg diets.

Dressing percentage is calculated as the proportion of weight left after deducting the weight of head, legs, internal organs, hair and blood. The observed differences in dressing percentage among treatments may be attributed to variations in the weights of blood, skin, internal organs, and undigested feed in the gastrointestinal tract. Dressing percentage ranged from 59.68 to 68.97% and increased with higher levels of BLM supplementation. The values are similar to the observed values (65.31-68%) reported by Ndelekwute *et al.* (2017), in broilers with mean live weights (1883-2152g) fed bitter leaf meal. However, they were slightly lower than (67.02-72.01%) observed by Buasilenu *et al.* (2014), in grasscutters of mean body weights of (2.75-3.09kg) fed urea as a partial replacement of protein substitute. The values were higher than the 53.1-61.7% observed by Alagbe *et al.*, (2017) in grasscutters (1.28-1.57kg final live weights) fed *Polyalthia longifolia* seed oil supplemented meal.

Measurement of dressing percentage and internal organ weight are useful assessor for determining the extent of the anti-nutritional factors reduction in feed ingredients. Also in evaluating the level of toxicity of feed offered to animals (Alagbe *et al.*, 2017). Grasscutters on 20g/kg BLM supplement had the highest values for all the internal organ weights measured except for the large intestine weights. The internal organ weights varied significantly ($p<0.05$) across the treatment except for heart weight. Generally organ weights reduced significantly ($p<0.05$) as the supplementation of BLM increased. This is suggesting that supplementation of 20g BLM/kg could be the optimum inclusion level for improved internal organs development, while higher supplementation could have negative effect on some of the organs, especially kidney. The loss weight in kidney can be a response to change in osmolarity associated with increased concentrations of bioactive secondary metabolites in bitter leaf. For kidney could adjust its size and activity in response to osmotic and excretory demands, thereby regulating urine concentration and dilution accordingly (Hall, 2006).This experiment was conducted under the tropical environmental conditions that could influence physiological response due high ambient temperature. However, the observed weight for liver, kidney, lung and spleen (1.73-2.00g, 0.61-0.91g, 0.6 - 0.88g, 0.13-0.21g,) respectively were higher than those reported by Annor *et al.*, (2013) for (1.28-1.44, 0.44-0.45, 0.52-0.64, 0.09-0.11g,) respectively, in intact and castrated male grasscutters. The organ weights were within the normal range for healthy grasscutters and BLM supplement is not toxic to the animals. This suggest that the reduction in weights might also be connected to the anti-inflammatory properties of bitter leaf due to the presence of saponins, tannins and flavonoids which possesses anti-inflammatory activity (Kim *et al.*, 2004). The non-significant ($p>0.05$) effect of dietary treatment on heart weight observed supports the observation of Poku Jnr *et al.*, (2013) in grasscutter fed different levels of protein supplement. This result indicates that BLM inclusion in grasscutter has no detrimental effect on their cardiac development.

The weights and lengths of digestive organs can be used as an indicator of feeding habits in rodents (Barnabas, 2016). In several rodent species, strict herbivores have been reported to have larger colon and caecum, while the gastrointestinal tracts of omnivorous species varies to a great extent depending on the proportions of animal foods, seed and vegetation in their diets (Wang *et al.,* 2003). This implies that the hind gut is crucial in herbivorous rodent's digestive system than in omnivorous species, and it may serve as an accurate predictor of feed preferences. These could be the reason for the higher large intestine and caecum weights observed compared to the stomach and small intestine weights since grasscutter is an herbivorous rodent. The stomach and intestine weights (3.82-6.83g, 2.9-4.54g, 3.13-5.98g) falls within the range of (1.89-4.05g and 5.53-12.91g) observed by Okukpe *et al.,* (2019) in weaner grasscutters fed 1-5g/kg diet of bitter leaf powder

The higher carcass and internal organ weights of grasscutters on BLM diets are as a result of the improved live weight gain of the grasscutters. In addition, this could be due to the protein contents of BLM supplement which resulted to an anabolic activity that enhances the building process of the body tissues and consequently weight gain. The reduction in bile weight at 40g and 60g BLM/kg suggest that higher inclusion of bitter leaf could impair fat digestion because bile is important in fat emulsification and absorption (Ndelekwute *et al.*, (2017). This may support the lipid lowering properties of bitter leaf attributed to its saponin contents. The small intestine, large intestine, stomach and caecum weights also reduced significantly. This could be related to the antimicrobial action of bitter leaf because antimicrobial agents used as feed additives have been documented to reduce the size of gastro intestinal organs due to the presence of flavonoids, tannins and phenolic compounds (Ndelekwute *et al.,* 2014). The compounds act on the organs by suppressing microbial activities which occurs highest in the caecum. Thus, it has been reported that bacteria mass could increase the caecum weight (Ndelekwute *et al.,* 2017).

Serum biochemistry of grasscutters fed bitter leaf meal supplement

Serum biochemical indices are useful in evaluating protein quality and amino acid requirements, determining the functioning level of vital organs such as heart and liver in animals. Total protein and albumin tests are also used to diagnose diseases and assess variation in health status of farm animals. Low albumin levels may signify the occurrence of renal or liver diseases (hepatic disorders), and maybe connected to the presence of an infection (Oleforuh-Okoleh *et al.,* 2015).

The total proteins and albumin values in all the treatments falls within the range of (8.03-10.51, 2.44-4.67mg/dL) respectively, observed by Okukpe *et al.,* (2019) in weaner grasscutters fed 1-5g/kg diet of bitter leaf powder except the total protein values (10.95mg/dL) obtained for those on 40g/kg BLM diets which was slightly higher. However, globulin values in the present study were higher than (5.59-5.84mg/dL) obtained by the same author. Since serum total protein is directly related to the amount of protein intake, the high total protein values observed in this study indicates that the diets offered the animals met their protein requirement for optimal growth. This may also be attributed to the high quality protein contents of bitter leaf meal, which likely contributed additional proteins to the dietary protein. The osmotic balance between the tissues and the blood in circulation is maintained by total serum protein (Kaneko, 1989). According to Mitruka and Rawnsley (1997), an elevated blood protein level above normal may indicate chronic enteropathy and hepatic dysfunction while its reduction including that of its components (serum globulin and albumin) can be due to starvation or malnutrition. This implies that the values obtained in this study are within physiological range for grasscutters, since there was no evidence of abnormalities.

The urea concentration values increased significantly as the level of BLM supplementation in the diet increased with those on 40g/kg having the highest value. Serum urea levels may be influenced by acute or chronic renal disease, exercise or high protein intake (Esievo, 2017). The high urea level observed in the BLM supplemented groups, especially those on 40g and 60g/kg may be attributed to high protein in the diets. However, the values obtained were still lower than the normal range of (21.4mg/dL) and (11.77-12.87mg/dL) obtained by Byanet *et al.,* (2008) and Lucky *et al.,* (2018) in young grasscutters respectively. Urea is a nitrogenous waste product generated during protein metabolism in the liver. The kidney is crucial in the filtrating process that helps remove urea waste from the body, which mainly leaves through the urine. Accordingly, excessive protein synthesis may put additional stress on these organs (Rao *et al.*, 2007). High blood urea nitrogen is an indication of too much protein consumption. Increased catabolism, excessive urea generation in the liver from protein-rich diet, higher protein breakdown, and decreased urea elimination from the kidney due to reduced blood flow to the kidney are some of the factors that can cause a rise in BUN (Lucky *et al.,* 2018). These authors further observed that acid-base homeostasis is significantly influenced by hepatic urea production, which uses HCO_3. The relatively low urea values observed in this study compared with previous authors shows that addition of BLM supplement improve the quality of protein in the diet of grasscutters and its inclusion up to 60g/kg in the diets did not cause metabolic or detrimental stress on the internal organs, and therefore not harmful to the grasscutters.

The serum creatinine levels decreased progressively with increasing level of BLM supplemented diets and those on 60g/kg BLM had the lowest value of 1.05mg/mL. The values obtained were within the range obtained by Byanet *et al.* (2008) and Lucky *et al.* (2018) for young grasscutters. This result agrees with the observation of Okukpe *et al.* (2016), who observed significant decrease in creatinine level of grasscutters with increasing of bitter leaf extract supplementation. Creatinine is used in assessing renal function as it reflects the difference between creatinine formation and its renal filtration by the glomerular filtration. Increased creatinine is seen in renal impairment. In contrast, high serum urea in the presence of normal creatinine levels may be an indication of dehydration (Hall, 2006). This indicates that urea and creatinine values observed in the BLM groups coupled with reduce kidney weights is an indication of dehydration and not of damage to the kidneys. This means that higher supplementation levels of BLM at 40g and 60g/kg reduce water consumption of the animals and the kidney responded by conserving water through concentrated urine production leading to reduced weights (Hall, 2006).

The lipid profile serves as diagnostic tool in detecting disease conditions such as atherosclerosis, coronary heart disease, chronic obstructive jaundice and hepatitis. According to Imaga and Bamigbetan (2013), cholesterol is the primary lipid component of atherosclerotic plaque and elevated lipid levels is one of the risk factors for coronary heart disease. In the present study, the total cholesterol levels of the BLM supplemented groups were numerically lower than the control even though not statistically significant. This finding agrees with the findings of Imaga and Bamigbetan (2013), who reported slight decrease in lipid profile of rats fed *Vernonia amygdalina* extract. Cholesterol levels of BLM groups were lower than the range of 189-194mg/mL obtained by Alagbe (2018) in grasscutters while that of the control was higher than the range. The findings were consistent with the previous studies suggesting that grasscutters on BLM were not at risk for coronary heart disease associated with high serum cholesterol, which can lead to poor health. This might be attributed to the cholesterol lowering properties of the saponins present in BLM. Saponins have been reported to reduce high blood cholesterol by preventing the absorption of cholesterol in the intestines, which would have the effect of lowering cholesterol levels (Ezeabara *et al.,* 2014).

The serum AST and ALT values significantly reduced in grasscutters fed BLM supplemented diets when compared with the control group. Grasscutters on 60g/kg BLM supplement had the lowest value of 84.95 83U/L and 57.83U/L for AST and ALT, respectively. This aligns with the work of Okukpe *et al.* (2019), who observed significant decrease in AST and ALT values in weaner grasscutters fed BLM. These enzymes are found majorly in the liver and kidney tissues (Noah *et al.*, 2020).When they are elevated above normal levels, it is an indication of some diseased conditions in the liver. However, the normal sizes and visual appearance of the livers in this study showed that the values obtained had no negative effect on the grasscutter, since they are indicators of liver health, it means that the BLM supplement improved the liver functions. The range of AST and ALT values were lower than the range 239-377U/L, 84-128 U/L observed by Okukpe *et al.* (2019) in grasscutters fed bitter leaf meal supplement while the ALP values were higher. However, the ALP values were within the range observed by Okukpe *et al.* (2016) in weaner grasscutters. ALP is found majorly in the liver and bones. Its elevation in the serum indicates inflammation or injury to the particular tissue in which they are found. ALP values had been observed to be high in growing young animals when compared with the adult, thus its high value in BLM groups which is still within normal range should not be attributed to inflammation or injury to the liver or bone tissue. The low serum levels of AST and ALT and other biochemical parameters, indicate better organs integrity in the grasscutters fed BLM supplemented diets groups as compared to the control. This finding confirms the observations of Iwalokun *et al.* (2006), and Adesanoye and Farombi (2010), that aqueous and methanolic extract of bitter leaf have hepatoprotective effects on Wistar rats. Variations in the haematological and serum biochemical parameters of animals may occur due to diet, age, genotype and physiological status (Machebe *et al.,* 2009). In general, the serum biochemical variables showed that they remained within the normal physiological range for grasscutters, consistent with the observed organ weights and histological integrity. This implies that the use of BLM supplement improved the overall health status of the grasscutters which may enhance better growth and reproductive performances in grasscutters.

Histopathological examination of the stomach, small intestine and caecum of grasscutters fed graded levels of bitter leaf meal supplement.

The histological slides showed outline of the gastric wall showing the stomach layers which includes; mucosa (M), muscolaris mucosa (MM) and submucosa (SM) histomorphology (x100). The innermost layer, the stomach (mucosa) contains the surface mucous cells which is composed of simple columnar epithelium, gastric pits, gastric glands containing parietal, chief and enteroendocrine cells, and the *lamina propria*. The glands of the mucosa are coiled and visible in cross- section beneath the pits with specialized cells for the secretion of mucus and gastric juices essential for digestion. The parietal and chief cells play key roles in chemical digestion. The muscularis mucosa and *lamina propria* are responsible for localized folding and support, respectively and they also played important role in mechanical digestion. This present findings agrees with the previous findings from the studies of Byanet *et al.* (2011) and Barnabas (2016), who reported that grasscutter fed primarily more on plant materials, rely greatly on efficient gastric digestion and intestinal architecture to breakdown the fibrous plant materials.

The photomicrographs of the jejunums showed typical histological pattern similar to the entire small intestinal mucosa. The mucosa lining is composed of simple columnar epithelium towards the intestinal lumen. The epithelium contains enterocytes and goblet cells. Enterocytes are the primary absorptive surface for nutrients and water, while goblet cells produces mucus to lubricate and protects the gut lining from pathogens. They both play vital and distinct roles in digestion, nutrient absorption and immune defense. Characteristic features observed included intestinal glands and finger like *villi* extending in the intestinal lumen. The submucosa consists of loose connective tissue, while the muscularis has an inner circular and outer longitudinal layer of smooth muscle fibres which facilitate internal movement, these findings supports Byanet *et al.* (2011) and Barnabas (2016). The absorptive capacity of the small intestine is an important determinant for the growth of grasscutters, the extension of the *villi* in the intestinal lumen observed in this study indicates an increase in surface area for absorption of nutrients in the small intestine.

The histological architecture of the caecum was similar to the entire intestinal mucosa with three mucosa layers. Simple columnar epithelium lines the mucosa (M). The mucosa is filled with enteroendocrine cells, and the surface epithelium has brush borders. The doubled layered muscularis mucosa (MM) is also seen. The submucosa of the caecum shares the same neurovascular and lymphatic configuration with the rest of the GIT. The histology findings of the stomach, small intestine and caecum observed agrees with the report of Byanet *et al.* (2011), and Barnabas (2016), who described histomorphology of normal and healthy gastrointestinal tract of domesticated grasscutter.

Histopathological evaluation of the stomach showed that damages occurs in the mucosa epithelium layers in all the treatments. Extensive damage was observed in grasscutters on 20g BLM/kg, the damages become mild as the BLM supplementation increases. The damage to the stomach mucosa layers observed may be as a result of the age of the animal and increased production of gastric acids for efficient chemical digestion of feeds. The grasscutters used in this study are still growing thus the mucosa lining and secretory glands are in the developmental stage. Imbalance may occur between the mucus secretion and acid production thus leading to the erosion of the mucosa layers. However, the damage observed become mild as the inclusion of the BLM increases. This might be credited to the secondary metabolites such as flavonoids and others found in bitter leaf. It is well documented that BLM's flavonoids and tannins have gastro-protective and anti-ulcer qualities (Adefisayo *et al.,* (2018)**.**

Flavonoids also demonstrated cytoprotective and anti-secretory activities in previous studies (Guaraldo *et al.,* 2001). This finding agrees with the report of Achuba, (2018a) and Adefisayo *et al.*, (2018) who reported gastroprotective benefits of bitter leaf in rats. The gastric mucosa layer secrete mucus that protect the epithelial lining of the stomach from auto-digestion, mechanical damage and bacterial invasion (Pavelka and Roth, 2010). According to Day *et al.* (2003), ulcer affects tissues damage deeper beyond the muscularis mucosa, while mucosa erosion is a lesion that may reach but not penetrate the muscularis mucosa. Factors such as, congenital conditions, starvation, feed structure and fiber (structure and composition), malnutrition, toxic compounds, infections, and microbial colonization are identified as predisposing factors responsible for erosion and ulceration in animals (Gjevre *et al.*, 2013). Furthermore, diets containing significant amount of highly polyunsaturated fatty acids predisposes animals to oxidative damage, while vitamin E have been documented to offer protective effects against erosion and ulcer (Gjevre *et al.*, 2013). Bitter leaf is rich in antioxidant vitamins such as vitamin C and E, and antioxidant bioactive phytochemicals such as flavonoids and tannins which could provide a protective effects against mucosa erosion.

The histopathology evaluation of the small intestine showed loss of mucosa epithelium and mild erosion of the submucosa layer across the treatment groups. This observation could be attributed to their physiological age, most essentially the young age and progressive developmental stage of the secretory glands. Also, the animals have not fully adapted to domestic rearing system, which could elevate cortisol levels and this might induce the proton pump and hydoxonium ion progeneration thereby causing erosion of the mucosa lining (Hall, 2006). This combined effects of age with the environmental factors might predispose the animals to histopathological abnormalities. It is therefore possible that the BLM would elicit this effect. The erosion of the mucosa lining observed in the study could not be attributed to the bioactive secondary metabolites in bitter leaf alone since it is observed in the control group thus other factors listed above could be responsible. The inclusion of BLM supplementation up to 60g/kg in the diets of grasscutters did not cause severe intestinal damage such as ulceration and it is not toxic at the inclusion levels. This finding agrees with Achuba (2018b), who reported the protective potential of methanolic extract of bitter leaf against petroleum induced toxicity in rats.

There is erosion of the submucosa layers of the caecum across all treatment groups. However, the erosion severity becomes mild as the BLM inclusion increases in the diets. This suggest that the bitter leaf meal exert a protective effects on the caecal mucosa. Increase in mucus secretion provides a protective mechanism against the acidic end product of microbial fermentation of fibrous feeds. Also, BLM could act as antimicrobial agent by reducing the pathogenic microorganisms that could cause infections in the caecum. It could also act as antioxidant by enhancing the capacity of the endogenous antioxidant enzymes of the animals to scavenge reactive oxygen species (ROS), thus preventing damage to the mucosa epithelial layer (Mota *et al.*, 2011). This could be attributed to the rich nutrient and phytochemical contents of BLM such as proteins, vitamins C and E, flavonoids and alkaloids that possesses antioxidant and antimicrobial properties. This finding agrees with the earlier reports by Mota *et al*, (2011), Achuba, (2018b) and Adefisayo *et al*. (2018) who reported the non-toxic, gastroprotective and antioxidative effects of bitter leaf (*Vernonia amygdalina***)** on the internal organs of model animals.

Bitter leaf meal supplementation significantly improved the carcass and organ weights, with optimal response at inclusion level of 20g/kg diet. The serum biochemical indices further revealed that the inclusion of BLM supplement up to 60g/kg diet improved the overall health status of the grasscutters without proven internal organ damage, this high inclusion level could be linked with reduced kidney weights. Histopathological evidence do not showed any pathological damage to the digestive organs of the grasscutters. In general the best inclusion levels with respect to cumulative effect on all parameters investigated were observed at 20g to 40g BLM/kg feed. Therefore, bitter leaf meal can be recommended at 20g to 40g/kg of feed as a feed additive in grasscutter diets to enhance productivity and improve optimal health status.

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