Materials and Methods

4.1 Introduction

Information on various types of feed eaten by deer were gathered from various sources including park rangers, veterinarian, veterinary officers, hunters and farmers and workers at zoological gardens, private- and government-owned farms in Malaysia (see Appendix B). Majority of these information were verified by observation on deer actually eating the plants of interest and/or as evidenced by deer biting on leaves, barks, petioles/branches of trees or shrubs.

4.2 Collection and identification of plant material

Plant samples known to be eaten by deer were collected at random from various locations including the primary and secondary forests, deer grazing fields and at farmers market (Pasar Tani) for species identification and chemical analysis. Collected leaves samples (grass, legumes, weeds, shrubs and tree fodders) were wrapped in plastic bags to minimise potential water losses through evaporation and were brought back to the laboratory for analysis.

A specimen of each plant sample collected was pressed dry and mounted (Khairuddin, 1989) and subsequently submitted to the Forest Research Institute of Malaysia (FRIM) in Kepong, Selangor, for species identification.

4.3 Proximate Analysis

Proximate analysis (PA) were performed according to methods described by the Association of Official Analytical Chemists, AOAC (1982). PA or Weende scheme is a semi-chemical approach. The feedstuff were analysed for its six components; dry matter, ash, crude protein, ether extract (Cullison, 1982), crude

fiber and nitrogen-free extract. Triplicate plant samples for each plant species were analysed for each components.

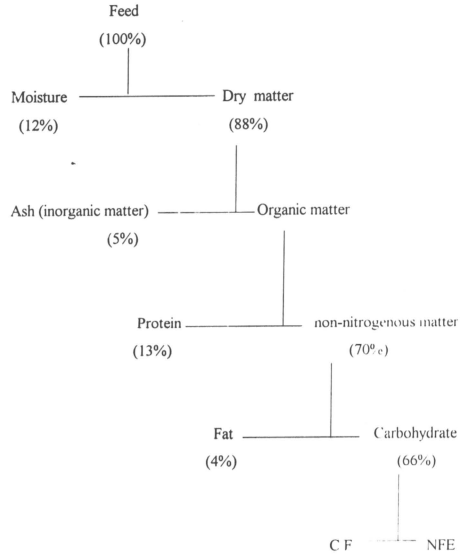

Figure 4.1 : Component of proximate analysis showing the inorganic and organic components of feed on dry basis (Figure adapted from Taylor, 1992)

4.3.1 Dry matter determination and storage of plant materials

Dry matter (DM) represent the structural component of the plant minus water. It contains fiber, fat and protein which are essential in meeting an animal's daily nutritional requirements. The moisture content was determined by drying a representative plant material of known fresh weight. The dry matter is expressed as a proportion of the initial fresh weight.

Plant sample (100-200g of fresh weight) was placed on an aluminium tray. The tray and its content was weighed before and after drying in the oven (50°C) for 72 hours.

The water or DM content was calculated and expressed in percentage :

$$\% \text{ DM} = \frac{\text{Wt of sample after drying}}{\text{Wt of sample before drying}} \times 100$$

$$\% \text{ water} = 100 - \% \text{ DM}$$

Dried plant samples were subsequently ground through a screen (1 mm) and stored in an airtight bottle. These ground samples were subsequently used in the PA described below.

4.3.2 Crude Fibre Determination

Crude fibre (CF) consists of relatively insoluble carbohydrate such as cellulose and other carbohydrates that are not easily dissolved in acid and alkaline digestion (Maynard, 1979). Its content in feed sample was determined according to Weende method (AOAC, 1990), whereby the protein, sugars and starches,

component of the feed sample were removed whereas cellulose, lignin and mineral matter were left behind in the feed residue after the extraction.

One gram of ground dried plant sample, (W_0) was placed into the Tecator glass crucible with a pore size of 2 µm. Six crucibles were then inserted into the Tecator Fibertec System 1010 Heat Extractor. One hundred milliliters of preheated reagent (0.255 N H_2SO_4) was added into each boiling column. A few drops of n-butanol, an antifoaming agent was added, to prevent bubbling. The mixture was boiled for 30 min and the reagent was filtered off with Whatman filter paper (size 40). The residues collected were washed three times with hot water (100°C).

The same extraction procedure was repeated with 100 ml of reagent 2 (0.312 N KOH). The residues obtained were filtered and washed three times with acetone in cold extractor, before drying in the oven (105°C) overnight prior to weighing (W_1). The residues were ashed in the muffle furnace at 500°C for 3 hours and the ash in the crucible (W_2) were then weighed.

The content of CF was calculated as follows :

$$\% \text{ C F} : \frac{W_1 - W_2}{W_0} \times 100$$

4.3.3 Crude Fat / Ether Extract Determination

This method determines the total lipid material present in feed samples. It extracts fat and ether soluble compounds including some non nutrient compounds such as chlorophyll, volatile oils, resin, pigments and plant waxes which are of little nutritional values to animals (Lassiter and Edwards, 1982).

Plant sample (approximately 2.0 g) was placed into an extraction thimble and weighed after oven-drying at 105°C for 24 hours. Diethyl ether (50ml) was added into each thimble and fat content in the sample was extracted in the Tecator Soxhlet System 1040 Extractor Unit. Diethyl ether was then allowed to evaporate for 1 hour and the weight of the residue (CF) that remained was determined.

EE content of the feed sample was calculated as follows:

$$\% \text{ EE} = \frac{\text{Wt of crude fat}}{\text{Wt of dried sample used}} \times 100$$

4.3.4 Crude Protein Determination

The crude protein (CP) content estimates the amount of protein, amino acids and non protein nitrogen of a feedstuff. It is based on the determination of the total amount of nitrogen calculated in accordance with the Kjeldahl method. The method is briefly described as known weight dried sample of feedstuff (0.5g) was refluxed in concentrated sulfuric acid (H_2SO_4) whereby all organic matter were digested and nitrogen in the feed constituents were converted into the form of ammonium sulfate. The digestion mixture was neutralized with sodium hydroxide (NaOH), which drove the ammonia (NH_3) over into standard acid and titrated. The amount of standard acid required to neutralize the NH_3 was used in the calculation of nitrogen content in the samples.

Powdered plant sample (0.5 g) was weighed in Tecator digestion tube in quadruplate. Fine ground catalyst mixture (4.0 g) comprising of copper sulphate ($CuSO_4$) and potassium sulphate (K_2SO_4) (7:100) was then added to the sample. Sulphuric acid (H_2SO_4; 12.5 ml) was then added and the samples were digested in a Tecator Digestion System 20, 1015 Digestor for 1 hour at

450°C. After cooling to room temperature, 75 ml of distilled water was added to the digested samples. The CP of the sample was obtained from the reading displayed on the Tecator Kjeltec Auto 1030 Analyser.

$$CP\ (\%) : \frac{\text{weight of treated sample}}{\text{weight of sample}} \times 6.25$$

4.3.5 Total Ash Determination

Ash represents the organic portion of a feedstuff and includes all the mineral nutrient. The ash constituent of a feedstuff is determined by incinerating the material to a constant weight.

Powdered plant samples (5.0 g) were weighed (weight A) and placed in dried porcelain crucibles of known weight (weight B). The samples were dried in the oven to a constant 100°C for 3-4 hours and were weighed again (weight C) prior to ignition in a muffle furnace (NEY 2 - 525) at 500°C for 3 hours. The crucibles and sample was transferred into the oven (100°C) for 3-4 hours before putting it into the dessicator to prevent absorption of atmospheric water vapour.

The crucibles were weighed (weight D) once the sample has reached room temperature and the amount of ash contents of the samples were calculated as follows:

$$\text{Ash } (\%) = \frac{\text{weight (D-B)}}{\text{weight (C-B)}} \times 100$$

4.3.6 Nitrogen-Free Extract determination

The value assigned to this portion of a feedstuff is indicative of the more soluble carbohydrate fraction. The sum of CF and NFE represents the total carbohydrate of the feed. It includes starch, sugars and other complex carbohydrates. Organic acids such as lactic acid and acetic acid are also included in this group (Morrison, 1959). NFE was determined by the difference and not by a specific analysis.

The NFE is defined as follows :

$$NFE\ (\%) = 100\% - (\%\ CP + \%\ EE + \%\ CF + \%\ Ash)$$

4.4 Principle of Fibre Analysis Determination

Fiber fractionation was performed using the sequential Goering and Van Soest (1970) technique. Forage samples were analysed for fibre composition by sequential detergent analysis without sodium sulphite (Mould & Robbin, 1981b). The detergent analysis system provide a means of dividing fibre into fractions; total cell wall as measured by neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin (which determined the lignified cell walls). Hemicellulose was determined by the difference between NDF and ADF whereas cellulose was measured as matter disappeared from ADF residue following pottasium permanganate treatment.

4.4.1 Determination of Neutral Detergent Fibre

Neutral - detergent procedure by Goering (1954) was used to estimate the dry matter digestibility (DMD). This method determines total fibre or cell wall

in plant material such as cellulose, hemicellulose, cutin and lignin. It may also contain minor cell wall components including some protein, bound nitrogen, cuticle and minerals. The soluble material, often referred to as cell wall contents is highly digestible, with the possible exception of tannin, silica and pectins.

Procedure

Air dried sample (0.5 g - 1.0 g) were weighed into a crucible. Neutral detergent solution (100 ml ; Appendix B1) and decahydrophthlene (2 ml) were added and the mixture was heated to boiling temperature for 5 - 10 min. Heat was reduced to prevent foaming and the mixture was left to reflux for 60 minutes. The mixture was then filtered and the residue was rinsed three times with distilled water and vacuum dried. The residue was then washed twice with acetone, dried by suction, and was then oven-dried (103°C) overnight. The residue was ashed in the crucible for 3 hours at 500°C and the weight of ash was recorded.

The true DMD was calculated as follows :-

True dry matter digestibility = 100 - % ND residue

4.4.2 Determination of Acid Detergent Fibre

In this method, feed samples were boiled for 1 hour in an acidic solution. Plant components soluble in acid detergent include primarily hemicellulose, cell wall proteins and the residue (ADF) includes cellulose, lignin and lignified nitrogen (indigestible nitrogen), cutin, silica and some pectins (Colburn & Evans, 1967). The procedure provides a rapid method for lignocellulose determination in feedstuff (Van Soest, 1963). It is also a prepatory step for lignin determination.

Ground air dried plant sample, W_0 (1.0 g) was weighed into Tecator glass crucible with pore sizes of 2 µm. Cold acid detergent solution (100 ml; Appendix B.2) and 2 ml decahydrophthalene were added. The mixture was heated to boiling temperature for 5 - 10 minutes. The heat was then reduced during the reflux stage (60 min). Several drops of 2 - octanol were added to prevent foaming. Tared gooch crucibles were filtered using slow suction and rinsed twice with hot water. The residue was then rinsed with acetone under vacum suction until the filtrate became colourless. The residues were broken in between rinsing so that ace tone comes in contact with all fibre particles. The residues were oven dried (105°C) overnight prior to weighing (W_1). The dried samples were ashed at 500°C for 3 hours and then the ash remained was weighed (W_2).

The ADF content of feed sample was calculated as follows :

$$\text{ADF (\%)} = \frac{W_2 - W_1}{W_0} \times 100$$

4.4.3 Determination of Lignin

A sample of ADF was used for lignin determination. Lignin estimation can be obtained from the ADF fraction using a neutral permanganate lignin (PML) (Van Soest & Wine, 1968).

Procedure

A combined permanganate solution (25.0ml; Appendix B.3) was added into crucibles of known weight containing ADF samples (0.5g ; part of W_1 samples in section 3.3.2). The crucibles were immediately immersed in an enamel pan containing 2 - 3 cm of water. Individual glass rods were used to break up particle

clumps, to ensure a maximal surface area in contact with the reacting solution. The samples were allowed to stand for 90-100 minutes at 20- 25°C in the permanganate solution with a purple colour maintained throughout the period. Exhausted permanganate solution, which turned brown, was removed using vacuum suction and replaced with fresh solution.

After the permanganate treatment, the solution was filtered off without washing the remaining residues. The crucible were then placed in a clean dry pan, half-filled with dimineralising solution and allowed to stand for 5 minutes before filtration. Residues on the sides of the crucible were carefully washed down with the demineralising solution. Yellow specks in the residues indicate the presence of lignin which necessitate a repeat of the permanganate treatment. Otherwise the residues were washed twice with an 80% alcohol solution and then followed by rinsing twice with acetone. The samples were oven dried overnight at 105°C and the weight of the crucibles and samples were determined.

The lignin content in the sample was calculated as follows:

$$\% \text{ Lignin} = \frac{\text{Wt of residue - wt of ADF}}{\text{Sample wt}} \times 100$$

4.5 Determination of mineral contents in plants

The Inductive Coupled Plasma (ICP) is an instrument used in the determination of plants mineral contents. An acid solution of a sample is aspirated into the jet of an inductively coupled plasma atomic emission spectrometer (Labtam P/L, Melbourne) and the trace element concentrations are measured at suitable wavelength for each mineral by comparison with matching matrix calibration solution.

Samples preparation for ICP

Dried plant sample (0.5 g) was weighed in Kjeldahl flask of known weight prior to the addition of 15 ml of concentrated nitric acid. The flasks were placed in a digester and were heated gradually from 70°C until the volume of the mixture was reduced to about 5 ml. The flasks were then cooled down to room temperature. The digests were then rinsed with distilled - deionised water and filtered through Whatman filter paper (9 cm). The volume was made up to 25 ml with distilled - deionised water and stored in a 50 ml polypropylene or polyethelene bottle. The samples were stored at low temperature (10°C).

ICP mineral analysis

Analysis was carried out using atomic optical emission spectroscopy (BAIRD ICP 2000, using purified Argon gas). Ultra high quality water and 1 ml of nitric acid (HNO_3) concentrate were used. All apparatus were acid washed (30% HNO_3), and rinsed with ultra high quality water.

The elements analysed were aluminium, zink, calsium, phosphorus, iron, potassium, sodium, cobalt, lead, magnesium, manganese and copper. Blank solutions were treated in the same manner as described for samples and the values obtained were subtracted from all respective sample readings.

A stock of solution for each element (1000 ppm) to be detected was diluted to 6 different concentrations; 0.1 ppm, 0.2 ppm, 0.5 ppm, 1.0 ppm, 2.0 ppm and 5.0 ppm and analysed in the same batch as the samples. Values are expressed in part per million (ppm).

4.6 The gas test method for Metabolizable Energy determination

The metabolizable energy (ME) of a feed or nutrient is the amount of energy available for maintenance and production after deducting from the total or gross energy (NE) of the feed, the amount of energy loss in faeces, combustible gases and urine.

The relationship between *in vitro* digestibility and gas production (CO_2 and CH_4) *in vivo* (when feedstuff is incubated with rumen liquor for 24 hours) can be used to estimate the digestibility of organic matter and ME content of compound samples (Menke et al, 1979). This principle was applied whereby feedstuffs were incubated with rumen liquor and the volume of the gas produced were recorded. Parallel incubations were also carried out at the same time to correct the gas production by each feed sample. The gas test method used in the present studies was adapted and modified by Yusoff et al. (1990) at the Institut Haiwan in K'ulang, Johor.

The apparatus used are as follows:

1. Electric oven/incubator (39°C)
2. A rotor with 55 - 60 holes for the graduated, glass gas syringes
3. Contruction for rolling the rotor in the incubator
4. Glass syringes (100 ml)

Dried plant samples (200 - 300 mg; ground through 1 mm sieve) was placed in graduated glass syringes which were then fitted with greased piston and subsequently filled with 30 ml of mixture of rumen liquor (from cattle) and reagents (see appendix F). Since the test must be run under an anaerobic condition at 39°C throughout the incubation, sufficient amount of CO_2 gas was

initially passed through the liquor mixture prior to sample incubation (blue or purple colour changed; first to red and then to colourless).

Each feed sample was set in triplicate on the first and second day of incubation. Gas produced from the an aerobic fermentation of the plant samples exerted pressure on the piston and the gas volume (V_8) were read at the eighth hour. The piston were readjusted back to the initial position after each reading (V_0) and the total volume was calculated to represent daily gas production. This volume was added to the reading at end of the 24h (V_{24}). Hay, concentrate and blanks standards were run in a set of 3 - 5, concurrently with the samples each day.

Differences in composition and activity of rumen liquor were monitored by three parallel measurements i.e. blank test (no substrate), standard hay meal and standard concentrate meal.

The general formula used for correcting gas production is as follows :

$$GP(ml/200mg\ DM) = \frac{V_{24} - V_0 + V_8 - (30 + GP_o) \times 200\ [F(H) + F(Con)/2]}{\text{Wt of sample in mg DM}}$$

where,

GP = Gas Production (ml/200mg DM)

F(H) = Hay factor

F(Con) = Concentrate factor

GP_o = Gas Production for blank

ME was calculated based on formula described by Menke et al. (1979)

[Roughage feeds (N = 200), (R^2% = 94)]

$$ME = 2.20 + 0.136\ GP + 0.0057\ XP + 0.0029\ XL^2$$

where;

GP = corrected gas production

XP = crude protein

XL = crude lipid

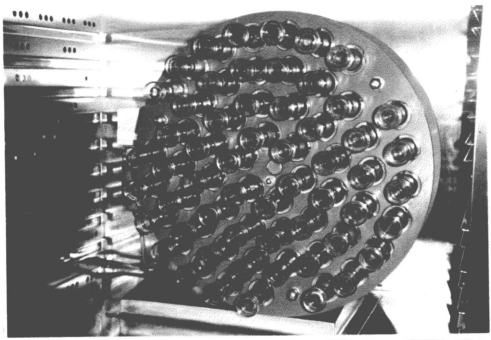

Plate 4.1 : Rotor with holes for holding glass piston syringes in oven

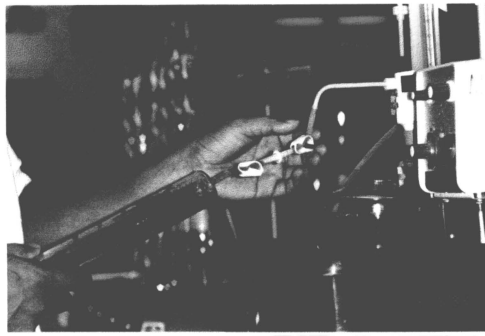

Plate 4.2 : Glass syringes were filled with the liquor mixture (with CO_2 equilibrated)

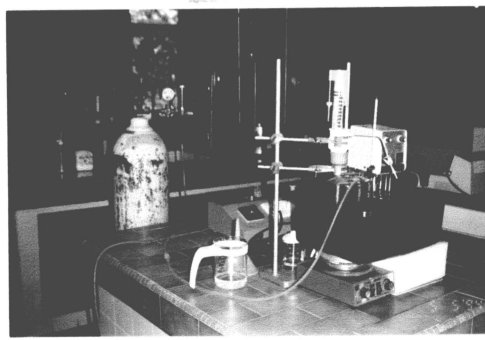

Plate 4.3 : The liquor mixture (rumen liquor and buffer) was gassed with CO_2 to maintain an anaerobic condition.

4.7 Rumen Simulation Technique

Rumen simulation technique (RUSITEC) system is an apparatus for maintaining microbial population of the deer rumen under strictly controlled condition over a long period of time. The system is widely used in the assessment of rumen microbial digestion of feed samples. The digestibility of feed samples was independent of the level of feeding of the animals from which the rumen fluid was taken whereas the fermentation characteristics were determined mainly by the proportion of roughage : concentrate consumed (Czerkawski and Breckenridge, 1977). The operational features of RUSITEC are briefly described as follows :

The RUSITEC unit (see Figure 4.2) consists of four isolated reaction vessels which are secured to the base of a water tank. The vessels were immersed in warm water (39°C) throughout the incubation. Each vessel is made up of a cylinder with an inlet at the bottom which was sealed by a flat cover. The top part of the vessel consists a screw cap cover with one inlet for artificial saliva input and 2 outlets, one each for gas output and artificial rumen outflows. At the start of RUSITEC preparation, deer rumen fluid was filled into each vessel (see Fig. 4.2).

A reagent bottle (food container) with screw cap and holes (1cm in diameter) on both bottom and wall sides was then inserted into the reaction vessel. Labelled nylon bags were filled with known weight of plant sample (≤ 1mm). Four replicates for each samples were prepared at the same time. Solid deer rumen content (80 g) were placed in four nylon bags and each was put into the four vessels along with the feed samples. The artificial saliva (see Appendix E) was allowed to flow through the system for 30 minutes prior to the initiation of the experiment.

Random movement of nylon bags as a result of contraction and relaxation of rumen was simulated by the RUSITEC system throughout the incubation. This was achieved by linear movement of the bottle up and down the vessel by means of a stainless steel rod passing through a gland which connects the screw cap of the bottle to a crank actuated by a motor. The liquid (rumen fluid and artificial saliva) passes through the bottle via holes at the bottom and on the screw cap.

The system was run continuously for 5 days but was stopped temporarily for 5 -10 min. at every 24 h incubation period for the removal of designated nylon bags. Each type of plant samples has 4 replicates, one for each vessel and at each incubation period of 24 h, 48 h, 72 h and 120 h. The nylon bags containing the plant samples were washed thoroughly with running water until the water squeezed from the bags were colourless. The nylon bags and contents were dried in an oven (52^0C) for 72 h. After drying, the bags were weighed.

The dry matter disappearance (DMD) of plant samples during the incubation period was calculated as follows :

$$DMD\ (\%) = \frac{W_0 - W_{24}}{W_0} \times 100$$

where :

DMD = dry matter digestibility

W_0 = weight of nylon bag plus sample before incubation

W_{24} = weight of nylon bag plus sample after 24 h incubation

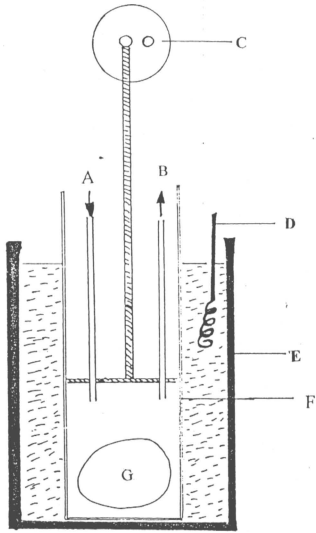

Figure 4.2 : Schematic diagram of one unit vessel in RUSITEC

A – solution B – waste C - motor D - water heater
E – water tank F - polyethylene bottle G – sample in nylon bag

4.8 Anti - nutritional factor determination

The presence of terpenoid, alkaloid and saponin were analysed using thin layer chromatography (TLC). TLC involves the separation of compound on aluminium (Al) plates coated with a thin layer of supported material. This stationary phase operate on the partition absorption. TLC plates usually developed by standing the Al sheet vertically in chromatography tank containing solvent to a 1 cm depth. The sample (extract) were applied as a spot to avoid damaging the surface. The extracts were subsequently dried and concentrated. Each extract were applied as dots on TLC aluminium plates. The plates were air - dried and subsequently dipped into a solvent system which consist of Dichoromethanes : Methanol (95:5) (Harborne, 1973).

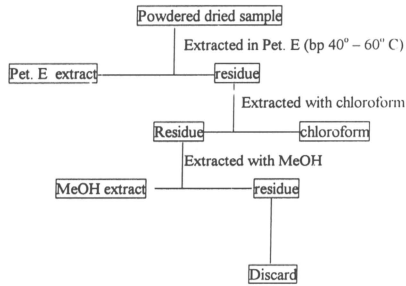

Figure 4.3 : Schematic presentation of extraction procedure (Harborne, 1973)

Each feed sample was extracted with three extraction solvents; petroleum ether, chloroform and methanol (see Figure 4.2). The solvent migrates up the surface of the TLC due to capillary and component of the samples are either swept along by the solvent or retained to a greater or lesser degree by the stationary phase. After development, the plates were air-dried and subjected to the following treatments for a direct quick method of detecting the presence of saponin, terpenoid and alkaloid in the plant samples, by spraying with an appropriate reagent.

4.8.1 Saponin

The plates were sprayed with 10 % vanilin in concentrated H_2SO_4 (see Appendix G - 1) and heated in the oven for 40 min at 120°C. The presence of black spots indicates the presence of saponin.

A faster method to detect the presence of saponin is by shaking powdered plant samples with warm mixture of ethanol and water (1:1). The presence of saponin was indicated by the presence of persistent foam.

4.8.2 Terpenoids

The plates were sprayed with 10 % vanilin in concentrated H_2SO_4 reagent (see Appendix G - 1), then heated to 120°C in the oven for 10 min. The presence of purple /blue spots indicated the presence of terpenoids.

4.8.3 Alkaloids

The plates were sprayed with Dragendorff's reagent (see Appendix G -2). The presence of orange spots indicated the presence of alkaloids.

4.9 Statistical Analysis

Statistical analysis were done on PA and fiber digestibility on three set of treatment, which is among leaves/ twigs/ shoots; leaves/ shoots and leaves/ twigs. Analysis were also done on the effect of different places for *Sapium baccatum*. All result were expressed as means ± standard deviation. Differences between the treatment means were tested for significance by one way ANOVA (Steel and Torrie, 1980). Chemical and fiber constituents and digestibility were analysed by ANOVA and presented as mean ± SE among plants species and places. These analyses were conducted both within samples and these collected from different places.

Analysis was also carried out on nutrient contents in various parts of plants; either twigs/leaves on five samples, leaves/shoots on three samples and twigs/leaves/shoots on three samples. The experimental model was included the final efficiency of different parts of plants and two/three way interactions.