Evaluation of Toxicological Effects of Leaf Meal of an Ethnomedicinal Plant-Neem on Blood Chemistry of Puberal Chinchilla Rabbit Does

Ifeanyi Princewill Ogbuewu*, Ifeanyi Charles Okoli, Michael Uwaezuoke Iloeje

Department of Animal Science and Technology, Federal University of Technology,
P.M.B.1526, Owerri, Imo State, Nigeria.
princiano2001@ yahoo.com

Abstract: The livestock industry in Nigeria, in the last decade, has been greatly affected by high cost of feed. The provision of feed alone has been reported to account for 60 - 80% of the total cost in most livestock production in Nigeria and this emphasize the interest to develop local feedstuffs. In view of this, there is increased interest by Nigerian livestock farmers to harness unconventional feed ingredients such as neem leaf meals. Neem has been reported to contain several biologically active constituents such as azadirachtin, meliantriol, salanin, nimbin as well as nimbidin. The present study aimed to investigate the effect of long term feeding of neem leaf meal based diets on blood chemistry chinchilla rabbit does. Thirty-six clinically healthy rabbits were divided into four groups. Rabbits on group 1 served as a control whereas those on group 2, 3 & 4 were used for the determination of toxic effect of neem leaf meal on blood chemistry. Blood samples were collected to obtain serum for biochemical studies and heparinized blood for hematological investigations. The neutrophil counts of rabbits on group 2, 3 & 4 were significantly ($p<0.05$) reduced. Serum cholesterol and serum alkaline phosphatase concentrations were significantly ($p<0.05$) affected by the treatment. The serum globulin and serum glucose concentrations of group 4 rabbits were significantly ($p<0.05$) lowered relative to the group 1 (control) rabbits. These results indicate that neem leaf meal based diets had visible deleterious effects on blood chemistry of chinchilla rabbit does. [Report and Opinion 2010:2(2):29-34]. (ISSN: 1553-9873).

Keywords: rabbits; neem leaf meal; blood chemistry; phytotoxicity

1. Introduction

Scarcity of feed resource has been the main limitation in the production of livestock products to meet the animal protein requirements of human and other industrial needs. The conventional cereal and vegetable protein sources being used in animal feeds are under pressure of competition through their use in human diets. The conventional vegetable protein sources such as soybean and groundnut cake are very expensive in developing countries like Nigeria due to high exchange rate as many of them still import these commodities. In view of this, there is increased interest by Nigerian livestock farmers to harness unconventional feed ingredients. One of such unconventional feed ingredient is leaf meal of ethnomedicinal plants such as neem (Esonu *et al*., 2006).

The neem plant is native of India and Burma and adapted favorably to the sub-sahelian Nigeria. The stem bark is an astringent and the root bark and young fruits are used in a similar way (Ketkar and Ketkar, 1995). Fresh twigs are often used for cleaning teeth. The seed is a stimulant and is also applied externally in the treatment of rheumatism and skin diseases. The leaves can be used in the treatment of abscesses and can also apply topically after castration (Kausik *et al*., 2008). Neem has been identified among the tropical plant that has been used as livestock feed resource (Sokunbi and Egbunike, 2000a, b; Akpan *et al*., 2008; Kausik *et al*., 2008; Ogbuewu *et al*., 2008; Ogbuewu *et al*., 2009). Chemically analysis revealed that neem leaf meal is relatively high in crude protein (20.69%) (Esonu *et al*., 2006) and low in metabolisable energy (0.34KJ / Kg DM) (Bakshi *et al*., 2006).

Biologically active compounds isolated from different parts of the plant include: azadirachtin, meliacin, gedunin, salanin, nimbin, valassin and many other derivatives of these principles. Azadirachtin forms the bitter principles of neem leaf. Azadirachtin has also shown direct detrimental and histopathological effects on most insect tissues e.g. muscles, body fat and gut epithelial cells (Mordue (Luntz) and Blackwell, 1993). The seed also contain tignic acid (5-methyl- 2-butanic acid) responsible for the distincitive odour of the oil (Akpan *et al*., 2008). These compounds belong to natural products called triterpenoids (Limonoids). The active principles are slightly hydrophilic, but freely lipophilic and highly soluble in organic solvents like, hydrocarbon, alcohols, ketones and esters (Akpan *et al*., 2008).

Neem leaves like most tropical tree leaves contain bioactive compounds (Kausik *et al*., 2008; Akpan *et al*., 2008) which may affect nutrient utilization. These bioactive compounds may also alter the hematological and serum biochemical parameters of animals. The blood contains a myriad of metabolites and other constituents which provide a valuable medium for clinical investigation and nutritional status of an individual hence (WHO, 1963) recommended the use of blood parameters for medical assessment.

Therefore, this study was designed with specific objectives of investigating the hazards effect of neem leaf meal on blood chemistry of chinchilla rabbit does.

2. Materials and Methods

2.1 Experimental location

An investigation was conducted at the Rabbit Unit of the Teaching and Research Farm, Department of Animal Science and Technology, Federal University of Technology, Owerri, Imo State. Imo State lies between latitude $4^{o}4'$ and $6^{o}3'$N and longitude $16^{o}15'$ and $8^{o}15'$E. Owerri, the capital city of Imo State is located in the south-eastern agro-ecological zone of Nigeria. Owerri is about 91m above sea level with annual rainfall, temperature and humidity ranging from 2300 - 2700 mm, 26.5 – 27.5^{o}C and 80 - 90% respectively. Owerri has a three month dry season duration (i.e. month with < 65mm rainfall) and this covers December – February (MLS, 1984; Ibeawuchi *et al*., 2005; Ibeawuchi *et al*., 2007).

2.2 Collection and preparation of neem leaf meal

Fresh matured neem leaves were harvested in and around the Federal University of Technology, Owerri. The fresh neem leaves were immediately shade dried in an open clean concrete floor space until moisture content became constant at 13%. The sun-dried leaves were later milled using a commercial feed milling machine (Artec, model 20) into neem leaf meal according to the procedures described (Esonu *et al*., 2006; Herbert *et al*., 2005).

2.3 Experimental ration formulation

The feed ingredients used in ration formulation were purchased locally from reputable commercial feed ingredient dealers in Owerri, Imo State. The NLM was sourced as earlier discussed. All diets were formulated to contain identical crude protein content (iso-nitrogenous) and metabolisable energy (isocaloric). Group1 (control diet) was formulated without neem leaf meal. Group 2, 3, and 4 were formulated such that they contained 5%, 10% and 15% levels of neem leaf meal respectively (Table 1).

Table 1. The composition of experimental diets.

Ingredients	Diets (% Neem leaf meal)			
	Group1 (0% NLM)	Group 2 (5% NLM)	Group 3 (10% NLM)	Group 4 (15% NLM)
Spent grain	55.00	50.00	45.00	40.00
Neem leaf meal	0.00	5.00	10.00	15.00
Calculated analysis				
Crude protein	18.87	18.70	18.53	18.37
Crude fibre	10.1	10.78	11.02	11.27
Ether extract	5.97	5.95	5.93	5.91
Calcium	1.41	1.39	1.38	1.36
Phosphorus	0.66	0.62	0.58	0.53
ME (MJ/kg)	10.42	10.38	10.33	10.22

Each diet contained 35% maize, 3% local fish meal and groundnut cake each, 2% bone meal, 1% oyster shell, 0.50% vitamin./mineral premix, 0.5% common salt. Vitamin / mineral premix contributed the following per kg of feed: vitamin A, 10,000iu; vitamin D_3, 2000iu; vitamin E, 5iu; vitamin K, 2mg; riboflavin, 4.2mg; vitamin B_{12}, 0.01mg; panthothenic acid, 5mg; nicotinic acid, 20mg; folic acid, 0.5mg; Choline, 3mg; magnesium, 56mg; iron, 20mg; copper, 1.0mg; zinc, 5.0mg; cobalt, 1.25mg; iodine, 0.8mg.

2.4 Experimental design

Thirty six Chinchilla rabbit does were randomly divided into four treatment groups of nine animals each. Each group was further divided into 3 replicates of three animals. Each treatment group was randomly assigned to one of the four neem leaf meal based diets at Group 2, Group 3 (10%), Group 4 (15%) and Group 1 (control) in a completely randomized designed (CRD) experiment.

2.5 Management of experimental rabbits

The experimental rabbits were housed in a 2 - tier rabbit hutches measuring 50 cm × 50 cm × 30 cm. The hutches are made up of wooden frame with wire gauze of 0.3 mm covering both sides of the hutch. The experimental rabbits were stabilized for two weeks before the commencement of the actual experiment. During this period, the rabbits were dewormed subcutaneously with Ivomectin at the dose of 0.2 ml / Kg and also treated prophylactically against coccidiosis with amprodon according to manufacturer's instructions. Water and feed were given *ad libitum.*

2.5 Blood collection

On the 112^{th} day of feeding trial, between the hours of 9am and 10am, 3 rabbit does were randomly selected from each treatment group for blood collection. About 12 mL of blood was collected from the marginal ear vein using a scalp vein needle set after swabbing with methylated spirit. The blood was quickly collected into a vial bottles treated with or without heparin. Samples were transported in an ice pack immediately to the Federal Medical Centre Owerri, Nigeria for hematological and serum biochemical test.

2.6 Hematological analysis

The packed cell volume (PCV), red blood cell count (RBC), white blood cell (WBC) and hemoglobin (Hb) were determined were determined as outlined by Schalm *et al*. (1975) and Kelly (1979). The blood indices: Mean cell hemoglobin (MCH), Mean cell volume (MCV) and Mean cell hemoglobin concentration (MCHC) were

computed using appropriate formulae as described by Jain (1986).

2.7 Serum biochemical analysis

The serum biochemical assay were carried out using standard chemical procedures: Total serum protein by Golgberg refractometer method (Kohn and Allen, 1995), albumin by Bromocresol green (BCG) method (Peters *et al*., 1982), creatinine (Boisness and Taussky, 1985), urea nitrogen (Baker and Silverton, 1985), serum glucose (Toro and Ackerman, 1979), Sodium ions and Potassium ions by flame photometry, bicarbonate ions and chloride ions (Schales and Schales, 1941), serum enzymes by spectrophotometric method (Rej and Hoder, 1983).

2.8 Data analysis

Data collected were subjected to one way analysis of variance (Steel and Torrie, 1980) and means were separated using the Duncan's New Multiple Range Test (1990).

3. Results

The effect of neem leaf meal diets on the hematological values of rabbit does is shown in Table 2. The PCV, hemoglobin concentration, WBC and RBC count of group 4 rabbits were not significantly ($p>0.05$) different from the group 1 rabbits. The white blood cell count and hemoglobin concentration were similar ($p> 0.05$) among the groups. The neutrophil counts of rabbits on group 2, 3 & 4 were significantly ($p<0.05$) reduced. The rabbits on group 2 & 4 had significantly ($p<0.05$) lower MCV value when compared with rabbit does on group 1.

Table 2. Hematological values of female rabbits on neem leaf meal based diets

Parameters	Inclusion levels of Neem leaf meal (NLM)				S.E.M
	Group 1 (0% NLM)	Group 2 (5% NLM)	Group 3 (10% NLM)	Group 4 (15% NLM)	
Hemoglobin (g/dl)	12.20	12.60	12.40	12.00	0.07
PCV (%)	36.00	37.00	37.00	35.00	0.32
RBC ($\times10^6$/mm^3)	3.60	4.20	3.80	4.00	0.07
MCV (fl)	105.6^a	88.00^b	97.40^a	87.50^b	2.16
MCH (pg)	33.90	30.00	32.60	30.00	0.49
MCHC (%)	32.10	34.10	33.50	34.30	0.25
WBC($\times10^9$/mm^3)	8.80	9.90	8.70	8.50	0.16
Lymphocytes (%)	61.00^{ab}	66.00^{ab}	73.00^a	58.00^b	1.64
Neutrophil (%)	39.00^a	30.00^b	34.00^b	36.00^{ab}	1.37

^{a,b,c} Means within a row with different superscripts differ significantly ($p<0.05$); PCV - Packed Cell Volume; RBC - Red Blood Cells; WBC - White Blood Cells; MCV - Mean Cell Volume; MCH - Mean Cell Hemoglobin; MCHC -Mean Cell Hemoglobin Concentration.

The data on the effects of neem leaf meal on serum biochemical characteristics of rabbit does are presented in Table 3. The serum total protein concentration of rabbits on the group 4 was numerically reduced. The group 4 rabbits had significantly ($p<0.05$) lower serum globulin value when compared with the control rabbits (group 1). The serum cholesterol value for the group 2, 3 and 4 were significantly ($p<0.05$) reduced. The serum glucose value of rabbits on group 4 was adversely affected ($p<0.05$) by the neem leaf meal diet. Serum sodium concentration of group 2 (58.20 mmol/l) and group 3 (66.40 mmol/l) were significantly ($p<0.05$) depressed by the treatment diets. The serum chloride values of rabbits on group 3 and 4 were significantly ($p<0.05$) elevated. The serum alkaline phosphatase values of rabbit does on group 2, 3 and 4 was significantly ($p<0.05$) higher than those on group 1. Serum total bilirubin concentration of rabbits on group 3 was significantly ($p<0.05$) higher than the control rabbits.

Table 3: Serum biochemical values of rabbit does fed neem leaf meal.

Parameters	Inclusion levels of Neem leaf meal				S.E.M
	Group 1 (0% NLM)	Group 2 (5% NLM)	Group 3 (10% NLM)	Group 4 (15% NLM)	
Total protein (g/dl)	7.50	6.20	5.60	4.10	0.35
Globulin (g/dl)	4.30^a	2.60^{ab}	2.40^{ab}	0.70^c	1.37
Albumin (g/dl)	3.20	3.60	3.20	3.40	0.05
Urea (mg/dl)	66.40^{ab}	55.50^b	68.40^{ab}	77.30^a	2.24
Creatinine (mg/dl)	1.10	1.00	1.10	1.20	0.02
Cholesterol (mg/dl)	130.00^a	95.40^b	72.10^c	64.30^d	7.37
Glucose (mg/dl)	89.80^a	91.40^a	85.00^a	50.30^b	4.85
Sodium (mmol/l)	89.00^a	58.20^b	66.40^b	80.10^a	3.44
Potassium (mmol/l)	4.30	4.40	3.80	3.50	0.11
Chloride (mmol/l)	86.50^b	96.40^{ab}	100.00^a	103.60^a	1.84

Bicarbonate (mmol/l)	25.80	26.40	24.00	22.10	0.49
Total bilirubin (mg/dl)	0.30^{b}	0.30^{b}	0.50^{a}	0.40^{ab}	0.04
Conj. bilirubin (mg/dl)	0.20	0.20	0.30	0.30	0.01
ALT (µl)	6.00	8.00	8.00	7.00	0.24
AST (µl)	8.00	11.00	13.00	9.00	0.55
ALP (µl)	52.10^{d}	81.60^{b}	93.70^{a}	65.80^{c}	4.54

[a,b,c] Mean within a row with different superscripts differs significantly ($p<0.05$), AST- Aspartate Transferase, ALT- Alanine transferase, ALP – Alkaline phosphatase.

4. Discussion

The present study was carried out to establish base line data on the toxicological effect of neem leaf meal based diets on hematological and serum biochemical characteristics of chinchilla rabbit does. Hematological parameters are good indicators of the physiological status of animal and its changes are of value in assessing the response of animals to various physiological situations (Esonu *et al*., 2006). The results of the present study showed that neem leaf meal had mild depressive effect on the hemoglobin concentration and packed cell volume of female rabbits at 15% inclusion level. This mild depressive effect indicates that these animals were slightly stressed by the test diet (neem leaf meal). The slight reduction in packed cell volume and hemoglobin concentrations observed in the present study was not in support with Esonu *et al*. 2006 who reported slight increments in the values of hemoglobin and packed cell volume of laying birds fed neem leaf meal diets at 0% to15%. The differences observed in the two studies could be attributed to breed differences (WHO, 1963).

The neutrophils are concerned with day to day immunological defense against pathogens. The significant reduction in neutrophil counts of rabbits on group 2 and 3 implies that the ingestion of neem leaf meal may have decreased the production of these blood components. The lymphocyte count of group 3 rabbits was slightly above the normal range of (53.5 - 65.8%) recommended by Kronfield and Mediway (1979) for healthy rabbits raised in the temperate climate. It is probable that the raised lymphocyte count could be an indication that the rabbits were immunologically challenged. The elevated lymphocyte counts of rabbits on group 3 could be a physiological adjustment presented by the animals against negative antigenic effect associated with the neem leaf meal bioactive compounds.

Serum biochemical investigations have been explored extensively to distinguish normal state from stress and disease conditions in animals. Dietary components have also been shown to have measurable effects on blood components (Awosanya *et al*., 2000) hence the serum biochemical metabolites are used to detect the existence of heart attack, liver damage and to evaluate protein quality and amino acid requirements in animals (Harper *et al*., 1999). The slight increment in serum urea and creatinine values of rabbit does on group 4 was an indication that neem leaf meal diet was of poor quality relative to the control diet. This suggests that there was little breakdown of muscle tissues and those animals could be surviving at the expense of its body reserve. The relative increase in serum urea at group 3 and 4 observed as the dietary level of neem leaf meal was increased in the ration is an indication that the animals were tending towards negative nitrogen balance. This could be attributed to the presence of some of neem leaf bioactive compounds (Azadirachtin, nimbin, salanin) which have been reported to block the energy metabolic pathway in animals, thus making it difficult for the animals to meet their energy requirement (Ogbuewu *et al*., 2008).

The serum cholesterol and serum glucose value were observed to decrease progressively with increasing levels of neem leaf meal in the diets. The reduction in serum cholesterol concentration (130.00 – 64.30 mg/dl) agreed with the hypocholesmic effects of neem earlier reported by Ogbuewu *et al*. (2008) in rabbit bucks and Oforjindu (2006) in broiler birds. It appeared that neem leaf meal indirectly inhibit HMG - COA reductase, a key enzyme in cholesterol biosynthesis. The hypoglycaemic activity of neem leaf meal observed in the present study was in agreement with the values reported by Ogbuewu *et al.* (2008) in buck rabbits. The significant changes in the serum alkaline phosphatase (ALP) concentrations rabbits fed neem leaf meal diets relative to those on control diet did agreed with the report that serum ALP could be influenced by changes in the physiological state of an animal.

4. Conclusion

It appears that rabbit does cannot tolerate the neem bioactive compounds for a long period. It therefore concluded that neem leaf meal had visible deleterious effect on blood chemistry of female rabbits. However, the observed alterations in blood chemistry of rabbits receiving neem leaf meal are a source of concern, and therefore should be given adequate attention while incorporating neem leaf meal in the ration of rabbit does.

Acknowledgement:

We express our unreserved gratitude to the staff of Clinical Chemistry and Hematology Laboratory, Federal Medical Centre, Owerri, Nigeria. We also wish to thank the non teaching staff of Rabbit Unit, Department of Animal Science and Technology, Federal University of Technology, Owerri for the invaluable services they provided during the execution of this study.

Correspondence to:

Ogbuewu Ifeanyi Princewill
Department of Animal Science and Technology,
Federal University of Technology,

PMB 1526,
Owerri, Imo State,
Nigeria.
Cellular phone: +2348035441864

Email:princiano2001@yahoo.com

Date of submission: 27th January, 2010.

References

[1] Akpan MJ, Enyenihi GE, Obasi OL, Solomon IP, Udedibie ABI. Effects of dietary neem leaf extract on the performance of laying hens. Proceedings of 33rd Annual Conference Nigerian Society Animal Production March 20-25, 2008; 396-398.

[2] Awosanya B, Joseph JK, Apata DF, Ayoola MA. Performance, blood chemistry and carcass quality attributes of rabbits fed raw and processed *Puereria* seed meal. Tropical Journal of Animal Science 2000; 2: 89 - 96.

[3] Bakshi MPS, Wadha M, Rana KK. Nutritive value of tree leaves for livestock from a semi-hilly arid region of India. In: Improving productivity by supplementary feeding of multi-nutrient blocks, controlling internal parasites and enhancing utilization of alternate feed resources 2006.

[4] Boisness RW, Taussky HHJ. Determination of creatinine in plasma and urine. J. Biol. Chem.1985; 581-582.

[5] Baker FJ, Silverton RE. Introduction to Medical Laboratory Technology, 6th edition. Butterworth, England 1985.

[6] Esonu BO, Opara MN, Okoli IC, Obikaonu HO, Udedibie C, Iheshiulor OOM. Physiological responses of laying birds to Neem (*Azadirachta indica*) leaf meal based diets, body weight, organ characteristics and hematology. Online J. Hlth. Allied Sci; 2:4 http//www.ojhas.org/issue 18/2006-2-4.htm 2006.

[7] Harper AE, Rodwell B, Mayes PA. Review of physiological chemistry. 17th ed. Lang Medical, Los Altos, California 9442, 1999:60-81.

[8] Herbert U, Ozoje MO, Adejumo DO. Effect of *Leucaena* and *Gliricidia* leaf meals on the seminal characteristics, testis weights and seminiferous tubule diameters of rabbits. Animal Research 2005; 54:173-178.

[9] Ibeawuchi II, Obiefuna JC, and Ofoh MC. Effect of row spacing on yield components of okra (*Abelmoschus esculenta*) and mixture groundnut (*Arachis hypogea*). Journal of Agroecology 2005; 4(4): 304-307.

[10] Ibeawuchi II, Dialoke SA, Ogbede KO, Ihejirika CO, Nwokeji EM, Chibundu IN, Adikwu NC, Oyibo PO. Influence of yam/cassava based intercropping systems with legumes in weed suppression and disease/pest incidence reduction. Journal of American Science 2007; 3 (1): 49-59.

[11] Jain NC. Veterinary Hematology. 4th edition. Baillere, Tindall 1986; 297.

[12] Kausik B, Ishita C, Ranajit KB, Uday B. Biological activities and medicinal properties of Neem (*Azadirachta indica*). Review article 2008.

[13] Kelly WR. Veterinary Clinical Diagnosis. 2nd edition, Bailliere Tindall London 1979; 266 - 267.

[14] Ketkar AY, Ketkar CM Source of unique natural products for integrated pest management, medicine, industry and other purposes In: H Schmutter (ed). The Neem tree 1995; 518-525.

[15] Kohn RA, Allen MS. Enrichment of proteolytic activity relative to nitrogen in preparations from the rumen for in vitro studies. Animal Feed Science Technology 1995; 52(1/2):1-14.

[16] Kronfield OW, Mediway NC. Blood chemistry. In: Textbook of Veterinary and clinical pathology Publ. Williams and Williams Co. Baltimore 1979; 18-96.

[17] MLS. Atlas of Imo State. Ministry of Lands and Surveys, Owerri, Nigeria 1984.

[18] Obi UI. Statistical methods of detecting differences between treatment means. Snnap press 2nd ed. Enugu, Nigeria, 1990; 24 - 35.

[19] Oforjindu O. The toxicity of graded levels of graded levels of Neem (*Azadirachta indica*) leaf meal. B. Agric. Tech. Project Report, Federal University of Technology, Owerri 2006.

[20] Ogbuewu IP, Okoli IC, Iloeje MU. Serum biochemical evaluation and organ weight characteristics of buck rabbits fed graded levels Neem (*Azadirachta indica*) leaf meal diets. Vet-on - line - The International Journal of Veterinary Medicine 2008.

[21] Ogbuewu IP, Okoli IC, Iloeje MU. Semen quality characteristics, reaction time, testis weight and seminiferous tubule diameter of buck

rabbits fed neem *Azadirachta indica*) leaf meal based diets. Iranian Journal of Reproductive Medicine 2009; 7(1): 23-28.

[22] Peters T, Biomonte CT, Doumas BT. Protein (total protein) in serum, urine and cerebrospinal fluid, albumin in serum: In selected methods of clinical chemistry, volume 9. W.R. Faulkner and S. Meites (eds.) Washington D.C. American Association of Clinical Chemist 1982.

[23] Schales O, Schales SS. A simple and accurate method for determination of chloride ion in biological fluid. Journal of Biological Chemistry 1941; 140:874.

[24] Sokunbi OA, Egbunike GN. The performance of growing rabbits fed diets containing sun cured Neem leaf meal. Proceedings of the 5th Ann. Conf. ASAN., Sept.19-22, Port Harcourt, Nigeria 2000a; 113-116.

[25] Sokunbi OA, Egbunike GN. Physiological response of growing rabbits to Neem leaf based diets. Hematology and Serum Biochemistry. Tropical Animal Production and Investigation 2000b; 3: 81-87.

[26] Rej R, Hoder M. Aspartate aminotransferase. In: Methods of enzymatic analysis. 3rd ed. H.U. Berg Meyer and M. Grassl (eds.). Weinheim Verlag-Chemie, 1983; 1(3): 416 - 433.

[27] Schalm OW, Jain NC, Carol EJ. Textbook of Veterinary Hematology. 2nd edition, Lea and Febiger. Philadelphia 1975; 192 - 250.

[28] Steel RG, Torrie JHT. Principles and Procedures of Statistics. An Approach. 2nd ed. McGraw-Hill Book Co.Inc. New York 1980.

[29] Toro G, Ackerman A. Practical chemical chemistry, 1st edition. Little Brown and Company, Boston 1979; 237-238.

[30] WHO. Technical Report Series, No. 842 (Expert Committee on Medical Assessment of Nutritional Status). WHO Geneva 1963.

12/20/2009