International Journal of Food Science and Nutrition
ISSN: 2455-4898
Impact Factor: RJIF 5.14
www.foodsciencejournal.com
Volume 3; Issue 6; November 2018; Page No. 43-49

Effect of protein deficiency on teratogenicity obtained from the ethanolic extract of the seed of *Garcinia kola*

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Abstract
The effect of a single subcutaneous dose of 25 mg kg-' the ethanolic extract obtained from the seed of *Garcinia kola* given on day 6 of gestation were compared in weanling rats fed 5% protein for 3 weeks followed by normal protein diet (referred to as 5% normal protein diet) for the remainder of the experimental period (GROUP 1), rats maintained on 10% protein diet (GROUP 2), and rats on a normal protein diet throughout (GROUP 3). Administration of the Ethanolic extract obtained from the seed of *Garcinia kola* resulted in significant depression of fetal body weights in rat fed 10%protein diet. Malformations (gross and skeletal) occurred in all the treated groups, Major gross malformations after treatment were hydrocephaly, microphthalmia and anophthalmia. The major skeletal defects involved vertebrae, sternebrae, and ribs the highest incidence of skeletal malformations occurred in treated rats maintained on 10% protein diet throughout the experiment.

Keywords: protein, *Garcinia kola*, teratogenicity, malformations, diet

1. Introduction
Protein is essential in the development of a healthy baby as it forms the structural basis for all new cells and tissues in the mother and fetus. It is important to ensure the adequate balance of protein to energy as high protein alone can cause harm to the fetus (Ota *et al*, 2012) [17] and protein deficiency can result in thin babies (Godfrey *et al*, 1997) [7]. A balanced intake of energy and protein seems to improve fetal growth (Ota *et al*, 2012) [17]. However, the evidence is emerging on the relationship between the type of protein and fetal growth. Consumption of processed meats (such as sausage, burgers and chicken nuggets) increases the risk of small for gestational age babies (Knudsen *et al*, 2008) [9] while fish and eggs seem to reduce the risk (Ricci *et al*, 2010) [18]. Choosing foods high in fat, salt and sugar seem to further increase the risk of small for gestational age baby (Thompson *et al*, 2010) [20]. Women from lower socioeconomic groups are at higher risk of inadequate protein intake due to the associated costs. They are also more likely to choose less expensive processed foods which would put them at risk of small for gestational age babies. The recommended daily allowance for dietary protein is based on the requirement that dietary protein, be provided by a mixture of both animal and plant proteins. The addition of 20 to 30% of animal protein to a 7:3 combination of cereal to legume seed meal, increases, ultimately the nutritive value of the food, and is consistent with the Protein Advisory Group guidelines for weaning foods, which states that dietary protein content of weaning foods, should be at least 20% (on a dry weight basis) (FAO/WHO, 1971) [6].
Campbell TC *et al*., 1976 stated that dietary protein deficiency is known to modify the response to the pharmacology-toxicological activities of drugs and foreign compounds, due in part to altered rates of metabolism. The dietary composition has been shown to represent an important determinant of pharmacological and toxicological activities of xenobiotic. The objective of this work is to utilize diets prepared from available and affordable plants and animal sources to know how protein deficiency affects the susceptibility of animals to teratogenic effects, To know how protein deficiency can modify a response to the pharmaco-toxicological effects of wild plants, Data from well-nourished laboratory animals cannot be extrapolated to the malnourished human without knowledge of how various nutritional deficiencies might alter the toxic response. Therefore, more information is needed about fundamental, biochemical and physiological mechanisms to understand the interaction between nutritional deficiencies and toxic response of chemicals. Teratogenicity is the presence of major congenital malformation. Major malformations are those that are either life-threatening, require major surgery, or have serious cosmetic effects. The more inclusive term of all these major defects is congenital anomalies or "birth defects" Teratology is the study of abnormal development in embryos and the causes of congenital malformations or birth defects. These anatomical or structural abnormalities are present at birth although they may not be diagnosed until later in life. They may be visible on the surface of the body or internal to the viscera. Congenital malformations account for approximately 20% of deaths in the perinatal period. Approximately 3% of newborn infants will have major malformations and another 3% will have malformations detected later in life. (Barrow-v *et al* 1971) [2] Recognition of human teratogens offers the opportunity to prevent exposure at critical periods of development and prevent certain types of congenital malformations. In general, drugs, food additives, and pesticides are tested to determine their teratogenicity to

minimize teratogenic means to prove that the frequency of congenital malformations in women exposed to the agent is prospectively greater than the background frequency in the general population. Therefore, testing is often done in animal models and often times administered at higher than the usual therapeutic doses.

Some plants/crops resources have been widely exploited and used as food crops, while others, mainly the tree crops of which *Garcinia kola* is an important member have been underexploited and still harvested from the wild. The seed of *Garcinia kola* is variously named by different/ethnic tribes across the continent (Africa). In Nigeria, it is called oje in Bokyi, Edun or safari in Efik, eerie in Ejagham-Ekin, cida Goro in Hausa, efiat in Ibibio, emiale in Icheve, igoligo in Idoma, Aku-ilu or ugolo in Ibo, akaan in Ijo-Izon, okain in Isekiri, and orogbo in Yoruba. *Garcinia kola* or bitter kola, a name sometimes also used for G. afzelii is species of flowering plant in the Clusiaceae or Guttiferae family. It is found in Benin, Cameroon, the Democratic Republic of the Congo, Ivory Coast, Gabon, Ghana, Liberia, Nigeria, Senegal and Sierra Leone. Its natural habitat is subtropical or tropical moist lowland forests. *Garcinia kola* seeds contain bioflavonoid (Kolaviron) capable of having anti- ovulatory and anti-inflammatory properties (Olatunde *et al*., 2002, Terashima *et al*., 2002) [16, 19]. Constituents of the seed of *G. kola* include 1-3, 8-11 benzophenones, Garcinia bioflavonoids (GB-1, GB-2) and kola flavonone. Akpantah *et al*. 2005 suggest that *Garcinia kola* seed may block ovulation by inhibiting cyclooxygenase activity and prostaglandin synthesis. Some flavonoids suppress the formation of cyclooxygenase – 2, thus playing an important role in the prevention of cancer and inflammation.

2. Materials and method

2.1 Equipment / Apparatus

Test tubes, Beakers (500ml), Conical flasks (250ml), Round Bottom flasks, Measuring cylinders (250ml), Masking tape, Volumetric flasks (50ml and 100ml), Separating funnels, Whatman Filter paper (No. 1), Electronic weighing balance, Water bath, Foil, Constant temperature magnetic stirrer, Micro slides, Petri dish, Refrigerator, Needle and Syringe, Dissection sets, Microscope, Nose mask, Hand gloves, Wooden blocks, Microtome, Oven

2.2 Reagents

Bouin's fluid, Chloroform, 70% Ethanol, Distilled water, Alizarin red, 10% Tween 80, Ammonia, Haematoxylene, Eoxin, Benzene or Xylene, Paraffin Waxes.

2.3 Collection and preparation of Material

Plant material Fresh fruits of *G. kola* were bought from Bere market Ibadan, Nigeria and identified by the biochemistry laboratory ODUDUWA University. The fruits were peeled to remove the shell covering the pulp which was then chopped to small pieces and air - dried. Thereafter, the dried pulp were blended using a blender and the powdered samples were stored in polythene bags and placed at room temperature until they were used.

2.4 Ethanolic extraction from the seed of *Garcinia kola*

The dried seeds were ground to fine powder and extraction was done using 70% alcohol, 600g of the powdered *Garcinia kola* was macerated in 3000ml of ethanol and left for 72 hours after which it was sieved using filter paper and a second filtration was done using No 1 Whitman filter paper. The filtrate was concentrated by evaporation in a water bath and dried to solid form. A yield of 46g was gotten from the extraction corresponding to a 7.7% yield.

2.5 Method

75, Approximately 3 weeks old weanling female Wistar albino rats obtained from an animal house in modakeke. These animals were maintained, after weaning, on diets having different protein concentrations. 5% and 10% protein diets were prepared by adding an appropriate quantity of lactogen to protein-free test diet. These animals were distributed into three groups. Group, I animals were maintained on 5% protein diet for 4 weeks followed by the normal protein diet for the remainder of the experimental period. Group II animals were maintained on 10% protein diet throughout the experimental period, and Group III animals were fed on normal 27% protein diet throughout the experimental period. The animals were weighed at weekly intervals and examined for their physical condition. When sexual maturity was achieved, the rats were mated with mature males of the same strain in stainless steel breeding cages housed in a temperature-controlled and artificially illuminated room (12 h light/l2 h dark) free from known sources of toxic contamination. The day on which a vaginal plug was found was designated day 0 of pregnancy. Pregnant females received single subcutaneous injections of the Ethanolic extract obtained from the seeds of *Garcinia kola* (25mg kg-' in 10% tween80) on gestation day 6. All injection volumes were 0.1 ml 100 g-l body weight. Some animals from each feeding group were untreated and a few animals of group I were injected with the solvent 10%Tween80. Body weights of pregnant rats were monitored daily to assess their general health. Pregnant rats were killed with an overdose of chloroform on day 20 of gestation. The uterine horns were exposed and subjected to visual examination for resorptions. Live fetuses were counted, removed from the uterus, blotted dry, weighed and examined for gross abnormalities. Every third fetus was fixed in 95% ethanol, cleared and stained with alizarin red, prior to examination for skeletal defects. The remaining fetuses were preserved in Bouin's fluid for subsequent internal soft tissue anomalies by the method of histology. Data for fetal body weights, number of implants, and number of live fetuses, Number of resorptions, gross, skeletal and visceral abnormalities were evaluated statistically by analysis of variance ANOVA. The fetus was used as the experimental unit. In all statistical tests, a probability of $p \leq 0.05$ was accepted as significant. This project was conducted in accordance with the internationally accepted principles for laboratory animal use and care National Institute of Health (NIH) Publication (1985).

2.6 Experimental Design

Table 1: Experimental design

Protein level %	Untreated	Solvent treated (10% tween 80)	Ethanolic extract (25 $mgkg^{-1}$)
Group I (5% protein diet- Normal protein diet	10	7	10
Group II (10% protein diet)	12	-	12
Group III (Normal protein diet)	12	-	12

2.7 Feed Composition

Table 2: Feed composition

Feed Composition	Control Normal protein DIET (27%)	5% Protein Diet	10% Protein Diet
Maize flour(g)	43	65	60
Groundnut cake(g)	30	30	30
Lactogen (g)	27	5	10
Total(g)	100	100	100

2.8 Dosage Calculation

According to the OECD's (organization of economic corporation and development's) guidelines, the dosage of drug (mg) should be constituted in an appropriate volume not usually exceeding 10 ml/kg (1 ml/100g) body weight of experimental animals (mice and rats) for the non-aqueous solvent in an oral route of administration. However in the case of aqueous solvents, 20 ml/kg (2 ml/100g) body weight can be considered, stock solutions and doses of a plant extract (With selected doses, 200 mg/kg and 400 mg/kg) for a rat weighing 120g be calculated as follows; (OECD, 2000).

$$^{x}/_{1000} = 25mg, \text{ (Body weight of animal = } x \text{)}$$

Where 25mg/kg is the average standard dose of *Garcinia kola*

2.9 Uterus contents and ovaries examination

The cesarean cut was through the peritoneal section to the diaphragm. Uterus was dissected longitudinally and the number of live fetuses, number of dead fetuses (absence of movement when touched with no visible degeneration), number of post-implantation resorptions degenerating recognizable dead fetuses or implantation site but no recognizable fetus), and total implantation sites were recorded. The placenta and each fetus (live or dead fetuses and resorptions) were then detached, weighed, and recorded. The weight of the live fetuses was recorded as well. The live fetus was removed and fixed in bouin fluids and every third fetus was placed in 95% ethanol. The fetuses were assessed in terms of congenital abnormalities after staining using Alizarin red, Haematoxylene and Eoxin. The skeletal abnormalities and internal soft tissues were examined. (R.Ola Lawal.; 1997)

2.10 Fetal Preparation

At the end of the caesarian procedure, the fetuses were prepared either for skeletal or visceral and internal examination. Half of the fetuses were assigned to visceral evaluation using Bouin's solution as a fixative and every third fetal were fixed in 95% ethanol. Regarding skeletal evaluation, right after weighting each fetus, they were placed in a dish containing 95% ethanol solution for dehydration, where they remained for four days and they were subsequently placed in another dish containing ethanol 100% for additional four days to complete dehydration. After dehydration was completed, the fetuses were rinsed in distilled water and allowed to dry before clearing by the use of xylene solution. After this, infiltration is done by the use of paraffin wax at 60ºc in the oven for 1hour. The fetuses are embedded in the molten paraffin wax, trimmed and blocked. The next phase is sectioning and this is done through the use of a Microtome and water bath. The blocked fetuses were sectioned using microtome on the micro slide and dipped into warm water bathe for few minutes getting ready for staining.

2.11. Alizarin Red Staining

Involves (2) two processes

i) **Hydration process:** The fetuses on the slides were placed in a jar containing xylene for the least 10minutes; the slide was removed and placed into 100% ethanol for 2minutes--> placed 90% ethanol for 2minutes-->70% ethanol for 2mins-->50% ethanol for 2minuutes-->Haematoxylene for 15minutes-->Acid alcohol (2 drops of acid) for 5minutes and finally Eoxin for 2minutes.

ii) **Dehydration process:** This is done by repeating the hydration process in descending order and some were stained with alizarin red. After twenty-four hours the stained tissues were very clear and visible in the microscope.

3. Result and Discussion

Table 3 is showing the group distribution of rats fed different rations of protein in relation to fertilization, normal protein diet had the highest rates of rats that conceived in the group with 10% protein diet having the highest level of rats that were unsuccessful in conceiving. Table 4 showing the effects of a single dose (25 mg kg-‘) of the ethanolic extract of *Garcinia kola* on of live and dead fetuses and fetal body weights on the 6th day of gestation in rats maintained on different rations of protein, group 2b (10% protein diet treated) having the highest percentage of resorptions 40%, and the lowest number of live fetuses 60%, while group 3a which is the untreated normal protein diet followed by group 1a the 5% untreated protein- normal protein diet had the highest number of live fetuses respectively 78.50%,77.53%, which can be attributed to the fact that protein helps in the formation of structural formation of fetuses, group 2b also had the least fetal weight 2.20g.

Table 5 showing the Percentage of fetuses with gross visceral and skeletal anomalies from maternal subcutaneous single dose (25 mg kg-‘) of the ethanolic extract of *Garcinia kola on the* 6th day of gestation in rats maintained on different rations of protein, group 2b had the highest percentage of malformed

fetuses 81.81% for external examination of fetuses while group3a,1b and 1a where negative for external malformations, skeletal examination showed group 2b and 2a had the highest percentage of skeletal malformations 80,45.45% while group 1a,1b,3a where negative for skeletal malformation, internal soft tissue examination showed that group 2b and 2a respectively had the significantly highest percentage of soft tissue malformation 83.33% and 50% respectively, Table 6 showing the Fetal malformations (gross, internal soft tissue and skeletal) associated with prenatal exposure to the ethanolic extract of *Garcinia kola* (25 mg kg-l) on day 6 in rats= maintained on different rations of protein, Major gross malformations after treatment were hydrocephaly, microphthalmia and anophthalmia with group 2b having the highest 38.98%,21.19%,8.22% respectively, major internal soft tissue malformation were internal hydrocephalus and deposits in the brain with group2a and 2b having the highest percentage, major skeletal malformation were incomplete ossification of the sternebrae, bipartite vertebra centra, bipartite sternebrae with group 2b having the highest incidence 30%,21.45%,22.55% respectively.

From the above results group 2a and 2b when compared to the control group 3a and 3b and also when compared to the rehabilitated group 1a, b and c, there was an incidence of significant increase in malformations both gross, skeletal and visceral, there was a significant increase in the level of success of mating between group 2 and the control, with group 2 having the lowest success of conceiving across the group, with shows that protein deficiency can altered the response to the pharmaco-toxicological activities of drugs, wild plants and foreign compounds, due in part to altered rates of metabolism.

The findings in this investigation suggest that protein deficiency may increase the susceptibility of the animal to the teratogenic effects of ethanolic extract obtained the seed of *Garcinia kola* with the major increased susceptibility related to skeletal development which relates with Mayura *et al*., 1982, 1983 reported that a single dose of 1.75 mg kg-' subcutaneous injection of Ochratoxin A, was teratogenic in rats, and also Lawal R.O. 1997 reported that a single dose of 2.5 mg kg-' subcutaneous injection of Treculia africana was teratogenic in rats fed varying levels of dietary protein.

Figure 2a represents a picture showing normal histology of the brain, Figure 2b showing hydrocephalus (brain) which is caused by excessive accumulation of fluid in the brain, characterized by increased intracranial pressure, increased cerebrospinal fluid (CSF) volume and dilation of CSF known as cerebral ventricles, Figure 2c is showing microcephaly of the brain, figure 2d shows deposits in the brain, Figure 3a represents a picture showing complete ossification of sternebrae Figure 3b represents incomplete ossification of sternebrae.

Table 3: Showing the % group distribution of different rations of protein to fertilization in the rat. (Numbers in parentheses indicate percentage.

Protein level	Total No. of rats	% of rats unsuccessful in mating	% of rats positive For mating	No of rats that conceived
5% protein diet - normal protein diet	27	0	27(100)	25(92.60)
10% protein diet	24	9(37.5)	15(62.5)	8(33.33)
Normal protein diet	24	0	24(100)	23(95.83)

Table 4: Effects of a single dose (25 mg kg-') of the ethanolic extract of *Garcinia kola* on the live and dead fetuses and fetal body weights on the 6th day of gestation in rats maintained on different rations of protein.

Treatment	No of implants	Resorptions		Live fetuses		Fetal body weight
		Total Number	%Of Implant	Total Number	%Of Implant	Average (G)
Group 1 A	89	20	22.47±0.15	69	77.53±0.37	3.27±0.03
Group 1 B	50	19	38.00±0.42	31	62.00±0.20	2.98±0.04
Group 1 C	67	23	34.33±0.18	44	65.67±0.11	3.10±0.05
Group 2A	47	18	38.30±0.29	29	61.70±0.48	2.38±0.04
Group 2 B	35	13	37.14±0.25	22	62.86±0.29	2.20±0.10
Group 3 A	93	20	21.50±0.14	73	78.50±0.08	3.53±0.03
Group3 B	100	32	32.00±0.09	68	68.00±0.12	3.45±0.08

Table 5: Percent of fetuses with gross visceral and skeletal anomalies from maternal subcutaneous single dose (25 mg kg-') of the ethanolic extract of *Garcinia kola on the* 6th day of gestation in rats maintained on different rations of protein**.**

Treatment	External Examinations		Skeletal examinations		Internal soft tissue	
	No of fetuses examined	% malformed	No of fetuses examined	% malformed	No of fetuses examined	% malformed
Group 1A	69	0	20	0	49	0
Group 1B	31	0	10	0	21	9.52±0.33
Group 1C	44	34.10±0.25	21	23.80±0.25	23	31.52±0.29
Group 2A	29	17.24±0.33	11	45.45±0.33	18	50.00±0.33
Group 2B	22	81.81±0.25	10	80.00±0.40	12	83.33±0.37
Group 3A	73	0	20	0	53	1.89
Group 3B	68	22.10±0.29	32	21.87±0.0.37	36	26.56±0.29

Table 6: Fetal malformations (gross, internal soft tissue and skeletal) associated with prenatal exposure to the ethanolic extract of *Garcinia kola* (2.50 mg kg-l) on day 6 in rats maintained on different rations of protein**.**

Treatment	Group 1A	Group 1B	Group 1C	Group 2A	Group 2B	Group 3A	Group 3B
External No Examined	69	31	44	29	22	73	68
% Hydrocephalus	-	-	13.80	5.67	38.98	-	9.22
Anophthalmia	-	-	5.50	4.00	21.19	-	3.0
Ectopia Cordis	-	-	2.00	1.44	5.93	-	1.0
Microphthalmia	-	-	7.30	3.73	8.22	-	6.56
Microcephaly	-	-	5.5	1.40	4.49	-	2.32
Internal Soft Tissue Examined	49	21	23	18	12	53	36
Internal Hydrocephalus	-	-	21.93	32.00	53.45	-	23.78
Deposits in the brain	-	9.52	9.59	18.00	29.88	1.00	2.78
Skeletal No. Examined	20	10	21	11	10	20	32
Incomplete Ossification Of Sternebrae	-	-	13.30	18.45	30.00	-	10.80
Bipartite Vertebra Centra	-	-	3.00	5.00	21.45	-	-
Bipartite Stemebrae	-	-	2.00	6.00	22.55	-	-
Rudimentary Rib	-	-	1.00	3.00	-	-	2.00
Fused Ribs	-	-	0.50	3.00	-	-	0.20
Extra Ribs	-	-	1.00	4.00	1.00	-	0.80
Broken Ribs	-	-	2.00	4.00	4.50	-	5.00
Missing Ribs	-	-	0.50	2.00	0.50	-	3.00

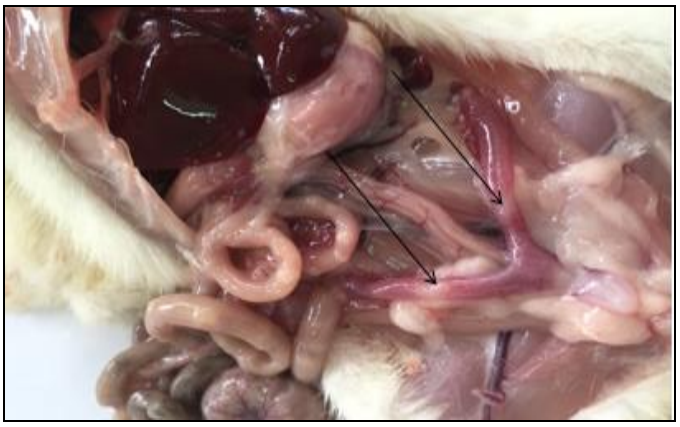

Fig 1: Arrows Indicating Resorptions Sites and Presence of Fat Around The Gonadal Area

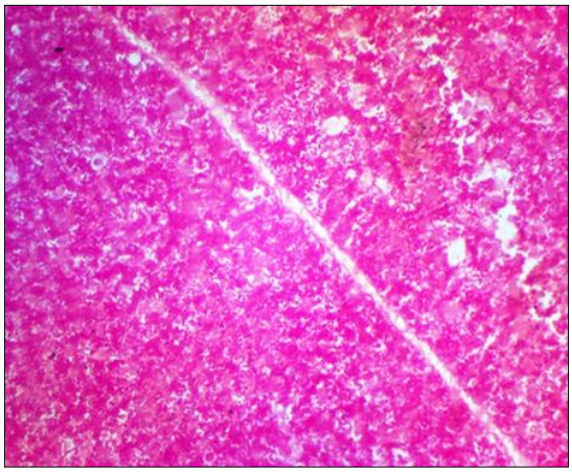

Fig 2B: Hydrocephalus

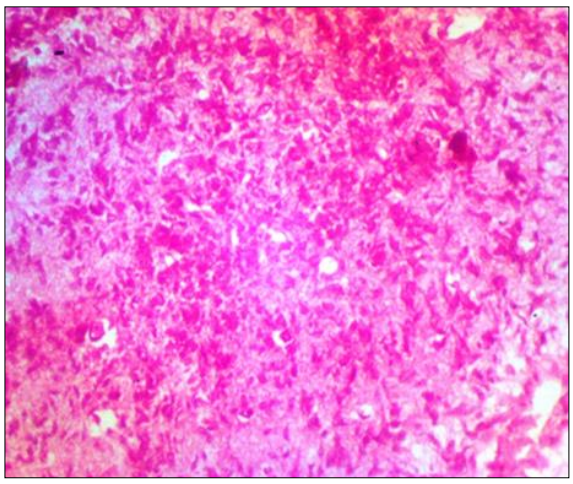

Fig 2A: Normal Rat Brain

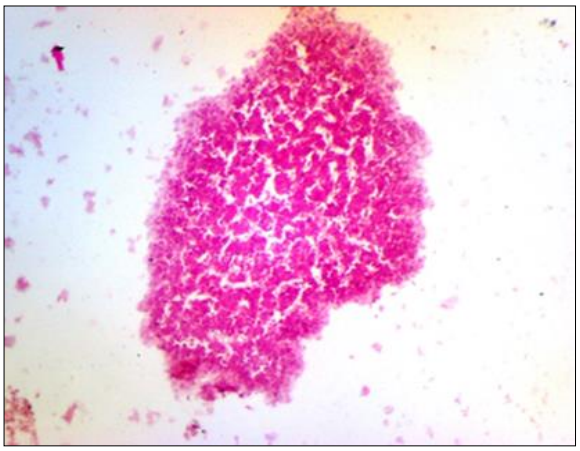

Fig 2C: Microcephaly

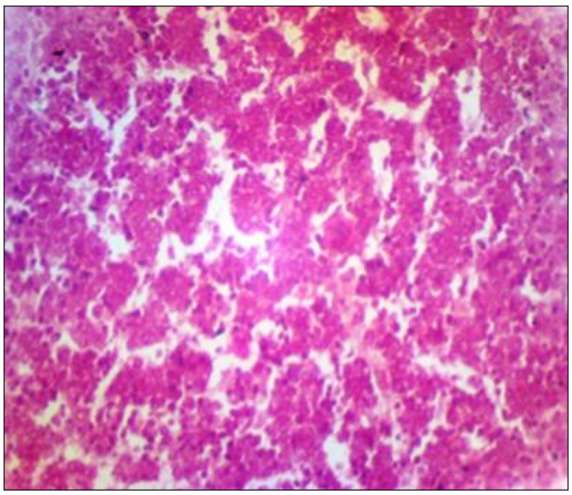

Fig 2d: Deposits in the Brain

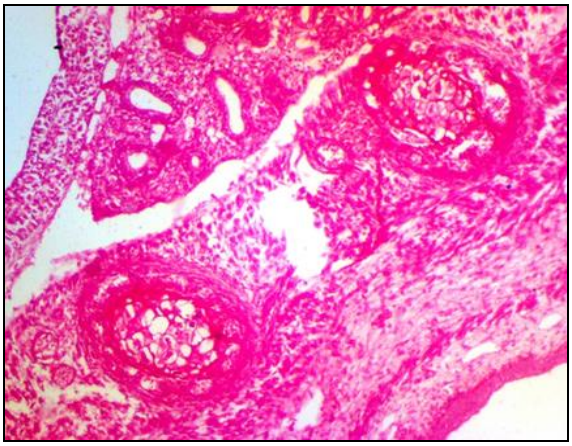

Fig 3A: Complete Ossification of the Sternebrae

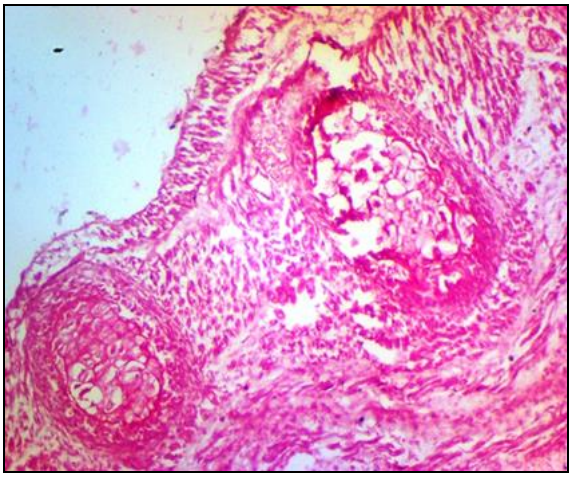

Fig 3B: Incomplete Ossification of the Sternebrae

4. Conclusion

The results shows that protein deficiency can modify the response to the pharmaco-toxicological effects of wild plants such obtained from the ethanolic extract from the seed of *Garcinia kola* and also various gross, visceral and skeletal malformations might be induced by extract administration in rats fed different levels of proteins. Pregnant women are advised to avoid taking of herbs and concoctions, they should adopt a balanced diet which is in accordance with the standard given by the world health organization (W.H.O)

5. Acknowledgments

Special thanks goes to the almighty for the strength provided to carry out this work, R.O. Lawal for writing assistance and general guidance, Stanley Okorie for language and material help and Oduduwa university for accepting us.

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

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