Effect Of Unfermented *Pakia biglobosa* Seeds On Calcium And Lipid Levels And Pain Perception In Adult Male Wistar Rats

A.L. Oyewole, V. O. Adeyinka, S. B. Kayode, and B. V Owoyele.

Department of Physiology, Faculty of Basic Medical Sciences, University of Ilorin, Ilorin, Kwara, Nigeria.

Abstract

Parkia biglobosa seed is commonly used as seasoning for traditional soups in the west coast of Africa. However, its effects on serum calcium, lipid profile, pain and anxiety related behaviour have not been clearly elucidated. The present study therefore aim to investigate its effects on these serum parameters and behaviours.

Fifteen male Wistar rats with an average weight of 180-240g were divided into three groups of five rats and used for the study. The groups were treated as follow: Group 1, the control, took 10ml/kg of distilled water, Group 2 was treated with 250mg/kg of *P. biglobosa* and Group 3 was given 500mg/kg of *P. biglobosa*. All treatments were administered orally and once daily for 21 days. Weights of the animals were measured once a week. Tail immersion pain study and behavioural study on open field maze were carried out three hours post treatment on the 21st day and finally, blood was collected from each animal via cardiac puncture for lipids and Ca^{2+} assay.

The results showed a significant ($p<0.05$) increase in mean value of weight in Group 2 which had no association with the lipid profile that showed a significant ($p<0.05$) reduction of LDL cholesterol, total cholesterol, triglycerides and significant ($p<0.05$) elevation in HDL cholesterol in all the treated groups compared with the control. Serum Ca^{2+} level was also significantly ($p<0.05$) increased in the treated group compared to the control and there was significant ($p<0.05$) decrease in reaction time to pain, in our pain related behavioural study. However, there was no significant ($p<0.05$) difference between the control and the treated group in the anxiety related behavioural study. In conclusion, this findings point to possibility that consumption of *P. biglobosa* seed, a popular food seasoning among local folks in various African countries may be of benefit to the people with regards to regulation of their lipids.

Correspondence to:

A.L. Oyewole
Department of Physiology,
University of Ilorin,
Ilorin, Kwara, Nigeria.
e-mail- oyewole.al@unilorin.edu.ng;
Mobile no: +2348075113375.

Keywords: Lipids; *Parkia biglobosa;* rats; serum; weight

Introduction

Parkia biglobosa, commonly known as African locust bean tree is a perennial tree legume of sub-family *Mimosoideae* and family *Leguminosae*[1]. The tree preference habitants spread widely in the savannah region of West Africa up to the southern edge of the Sahel zone[2]. Though not usually cultivated, couple of *Parkia* trees can be seen scattered around in the savannah region of Nigeria[3]. The locust bean tree or African locust bean tree as it is commonly called, normally bears yellow pulp that contains the seeds sealed in zip-like pods on a yearly basis. The yellow pulp is naturally sweet and the trapped seeds are processed into a valuable traditional food and soup seasoning paste known as *iru* and *daddawa* among the Yoruba and Hausa people of Nigeria, respectively[4,5]. The seeds of locust bean was reported to contain vitamin B2, protein, carbohydrate, lipids and lysine in its fermented form[6]. Past studies indicated that various parts of the locust bean tree are used for medicinal purposes[7]. The plant is documented for its wound-healing properties in South-Western Nigeria and its antimalarial properties in Guinea[8,9]. In rats, the stem bark decoction of *Parkia biglobosa* was implicated for its anxiolytic[10], analgesic and anti-inflammatory activities[11]. Ethnopharmacological studies also reported the seeds and stem bark to have cardio-inhibitory[12] and antidiabetic[13] activities, and it also prevented myocardial infarction[5]. The effect of unfermented *P. biglobosa* seeds on body weight in relation to lipid profile in male Wistar rats has not been properly elucidated in the literature.

The body lipid profile had many a time been correlated with the body weight in various studies in the literature. The effect of weight loss on body lipids for instance differs from one person to another[14]. Loss of about 10% of body weight in an overweight person leads to reduced low density lipoprotein cholesterol (LDL) and triglycerides (TGs), and increased high density lipoprotein cholesterol (HDL)[15]. This observation is backed by data from WHO where intentional weight loss of 10 kg in overweight persons was claimed to result in a 15% reduction in LDL cholesterol, 30% reduction in triglycerides, 10% reduction in total cholesterol, and 8% increase in HDL cholesterol[16]. Thus on the other way round, intentional

weight gain might lead to increase lipids in the body. Also, low concentrations of HDL, and high concentrations of circulating TC, TG and LDL are established biomarkers of risk for cardiovascular disease[17]. A good example of such risk is atherosclerosis where inflammation causes oxidative modification of LDL, a process believed to be crucial in its genesis[18]. Hence, weight and the level of circulating lipids have a stake in an individual's health status which in turn influences one's behavioural activities. In alloxan-induced diabetic rats, fermented *Parkia biglobosa* seeds was associated with weight gain and improvement in body lipid profile[11]. In normal rats however, the effect of the unfermented seeds is relatively unexplored.

Calcium (Ca^{2+}), a naturally occurring chemical element is the fifth-most-abundant element by mass in the earth's crust and in the seawater[19]. Because of its high reactivity, freely occurring Ca^{2+} metal hardly exit in nature. In cell physiology, calcium acts as a second messenger that regulates almost all known cellular processes that define life and death of every cell[20-22]. These varieties of functions executed by Ca^{2+} depends on the speed, amplitude, and spatiotemporal pattern of Ca^{2+} signals and by interactions between Ca^{2+} and other signaling pathways[21]. In growing neurons, intracellular Ca^{2+} is an important part of the response to attractive guidance cues in neurite outgrowth[23], and as a messenger in axon growth cone mobility and on bidirectional axon growth cone turning[24,25]. In skeletal muscle contraction, Ca^{2+} triggers contraction by reacting with regulatory proteins that would have prevented interaction of actin and myosin if Ca^{2+} is absent[26]. Contraction of skeletal muscles is vital in animals' locomotion and other physical displays. Movements generated from this muscular contraction can be interpreted into behavioural activities when conducted in the appropriate maze.

Generalized anxiety disorders are the most common psychiatric disorders in human[27]. The development of tool to assess anxiety in animal studies is of great importance to detect potential anxiolytic drug. Open field maze is such tool that assess the anxiety level in rodents by systematically assessing novel environment exploration, general locomotor activity, and provide an initial screen for anxiety-related behaviour[28]. Also, the presence of pain in human and animals has been linked to attitudinal and behavioural changes in the various past studies. The experience of pain depends on the balance between pro-nociceptive and anti-nociceptive inputs, this under normal physiological conditions allow us to recognise and react to pain. The nociceptive balance can however be altered in a state of injury or disease[29]. Such alteration can be quantified with cold and hot tail immersion model of pain evaluation.

As a result of convincing evidences on central effects of stem bark decoction of *Parkia biglobosa*[10,11] the present studies were targeted to find out possible central effects of unfermented *Parkia biglobosa* seeds in male Wistar rats. Thus, we focused on the effects of unfermented *Parkia biglobosa* seeds on anxiolytic related behavioural changes, pain related behavioural changes, body weight as it relates with the serum lipid profile, as well as the effects of unfermented *Parkia* seeds on calcium level.

Materials and Methods

Animal Preparation

Fifteen (15) male adult Wistar rats weighing between 180-240g were used for this experiment. The rats were obtained from the Department of Biochemistry, University of Ilorin, Ilorin, Kwara State, Nigeria. Animals were randomly divided into three groups of five rats and were labeled Group 1, 2 and 3. The animals were housed in the College of Health Sciences' Animal House, University of Ilorin where the research was carried out. The animals had free access to water and food throughout the experimental period. Principles of laboratory animal care that guides animals' experiment was strictly followed as prescribed in the University of Ilorin's Guide for the Care and Use of Laboratory Animals.

Seeds Collection, Drug Preparation and Treatment

Unfermented locust bean was bought from a local dealer around Elekoyangan area, Ilorin, Nigeria sometime in the month of December. The identification and authentication were done by Mr. Stephen Adebayo of Botany Department, University of Ilorin and voucher number UITH/001 was deposited at the herbarium of the university for future reference. Unfermented locust bean (15g) was weighed and milled with mortar and pestle without adding water. The smoothened paste of the beans was finally dissolved in 30ml of distilled water. The dosage was calculated base on this stock solution and fresh stock solution was prepared each day throughout the treatment period to avoid any slight chance of seeds fermentation. The method of Odetola *et al.*[30] was modified and used for these studies. While Group 1 (Control) was given 10ml/kg of distilled water, Group 2 and 3 were treated with 250mg/kg and 500mg/kg respectively. All treatments were done through oral route using oral canula and administration was done once daily for 21days. Three (3) hours after the last administration, the rats were sacrificed under an anaesthetic agent (chloroform). Blood was collected from each animal into a plain bottle via cardiac puncture. Serum lipid profile and calcium level were investigated in the samples collected.

Toxicity Test

The method described by Parasuraman (2011) for acute

toxicity test was modified and used for this study[31]. In this test, a group of five animals was treated with high dose (1000mg/kg) of unfermented *Parkia biglobosa* seeds orally for 14 days and was kept under close watch at day 1, day 3, day 7 and day 14.

Biochemical assays of Serum Lipid and Calcium

Blood was collected via cardiac puncture in a plain bottle for the determination of triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C) and total cholesterol (TC). The blood was centrifuged at 3000 × g for 15 min and the serum was extracted. TG, TC, HDL-C and LDL-C concentrations were measured by standard enzymatic-colorimetric method using enzymatic kits supplied by Randox laboratory Ltd (Co. Antrim, UK). The detection limit was 3.88mmol/L (150mg/dL). Also, serum calcium level was measured using standard enzymatic-colorimetric method with the kit produced by Abcam Plc, Kuwait.

Tail Immersion

The method described by Pizziketti *et al.*[32], *and* Sewell and Spencer[33] was used to study pain in this study. Three hours after the oral administration of the extracts and the normal saline, the tail of the animals were immersed in 0°C and 55°C water baths. The average time spent by each group before the animals flicked or removed their tails from the water bath was documented as reaction time to pain stimulus.

Anxiety Evaluation

Anxiety was evaluated using File's method[34]. Briefly, the open field apparatus used in the present study consisted of a square area (72x72 cm) with walls 36 cm high. The four walls were made with transparent Plexiglas. The wooden floor was painted white; it was divided by lines into 16 equal squares by parallel and intersecting blue lines. A central square was drawn in the middle of the open field. Animals were put at this middle individually with each facing away from the handler. Animals were allowed to explore the maze for 15 minutes during which anxiety related parameters were recorded.

Statistical Analysis

Results were presented as mean ± standard error of mean (SEM). Statistical difference between the control and the treated groups was carried out using one-way ANOVA on SPSS Statistical 16.0 for Windows (SPSS Inc, Chicago, IL, USA). Statistically significant differences were accepted at *p less than* 0.05.

Results

Toxicity effects of unfermented Parkia biglobosa seeds

Within 24 hours of treatment with 1000mg/kg dose of unfermented *Parkia biglobosa* seeds, there was no noticeable change in the food and water consumption in the animals used. Also, all the animals appeared to be as active as they were before the commencement of the study. At day 3, the animals were still active as day 1.

At day 7, the weight of the animals decreased significantly while food and water consumption remained the same. The faeces also became soft and

Table 1. Effect of unfermented *Parkia biglobosa* seeds on serum lipids level.

GROUP	TG (mmol/L)	CHOL (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
Group 1	1.180 ± 0.091	2.200 ± 0.089	0.288 ± 0.023	0.552 ± 0.099
Group 2	1.300 ± 0.070	1.540 ± 0.040*	0.482 ± 0.023*	0.184 ± 0.016*
Group 3	1.920 ± 0.080*	2.860 ± 0.120*	0.488 ± 0.021*	0.216 ± 0.018*

Each value is the mean ± S.E.M. of 5 Wistar rats; * = $p < 0.05$ compared with control, ANOVA. Group 1 - Distilled water (5ml/kg b.w.); Group 2 - 250mg/kg of unfermented *Parkia biglobosa* seeds; Group 3 - 500mk/kg of unfermented *Parkia biglobosa* seeds.

Table 2. Effect of unfermented *Parkia biglobosa* seeds on Calcium ions

GROUP	Calcium (mmol/L)
Group 1 (5ml/kg of distilled water)	2.99 ± 0.09
Group 2 (250mg/kg of *Parkia biglobosa* seeds**)**	3.36 ± 0.14*
Group 3 (500mg/kg of *Parkia biglobosa* seeds**)**	3.33 ± 0.20*

Each value is the mean ± S.E.M. of 5 Wistar rats; * = $p < 0.05$ compared with control, ANOVA.
Group 1 - Distilled water (5ml/kg b.w.); Group 2 - 250mg/kg of unfermented *Parkia biglobosa* seeds;
Group 3 - 500mk/kg of unfermented *Parkia biglobosa* seeds.

Table 3. Effect of unfermented *Parkia biglobosa* seeds on pain induced by tail immersion

Group	Reaction Time in Cold Immersion (s)	Reaction Time in Hot Immersion (s)
Group 1	40.20 ± 12.13	5.20 ± 0.58
Group 2	10.60 ± 0.51*	4.40 ± 0.67
Group 3	13.00 ± 2.98*	2.80 ± 0.58*

Each value is the mean ± S.E.M. of 5 Wistar rats; * = $p < 0.05$ compared with control, ANOVA. Group 1 - Distilled water (5ml/kg b.w.); Group 2 - 250mg/kg of unfermented *Parkia Biglobosa* seeds; Group 3 - 500mk/kg of unfermented *Parkia Biglobosa* seeds.

Table 4. Effect of unfermented *Parkia biglobosa* seeds on anxiety indices using open field maze

Group	Ambulatory	Rearing Frequency	Rearing Latency (s)	Grooming Latency (s)
Group 1	26.8±8.61	7.00±1.78	16.00±2.30	92.80 ±22.65
Group 2	17.60±6.50	5.20±2.15	13.00±3.60	83 . 00 ± 30 . 56
Group 3	28.20±9.24	8.60±1.74	22.60±4.40	1.65±17.80*

Each value is the mean ± S.E.M. of 5 Wistar rats; * = $p < 0.05$ compared with control, ANOVA. Group 1 - Distilled water (5ml/kg b.w.); Group 2 - 250mg/kg of unfermented *Parkia Biglobosa* seeds; Group 3 - 500mk/kg of unfermented *Parkia Biglobosa* seeds.

wet but activities in animals appeared to remain the same as in day 1.

At day 14, weight loss became more obvious across all the animals. Food and water consumption became drastically decreased and animals showed low activity. Also, the faece still remained soft and wet. In summary, at dose 1000mg/kg of *P. biglobosa* seeds, there was no dose related lethality though behavioural signs of discomfort was noticed in the animals.

Effect of unfermented Parkia biglobosa seeds on serum lipids level.

While a significant ($p<0.05$) decrease in LDL was recorded in both treated groups, a significant increase in HDL was observed also in all the treated groups compared to the control group (Table 1). This study also showed significant increase in TC in the high dose and decrease in the low dose compared to the control. Significant ($p<0.05$) increase in TG was also observed in the high dose compared to the control group.

Effect of unfermented Parkia biglobosa seeds on Calcium ions

Serum calcium ions was significantly ($p<0.05$) increased in all the treated groups (3.36 ± 0.14; 3.33 ± 0.20) compared to the control group (2.99 ± 0.09) (Table 2).

Effect of unfermented Parkia biglobosa seeds on weight

Changes in weight were monitored each week for duration of the study. A significant ($p<0.05$) increase in weight was recorded at the third week in the Group 1 and Group 2 compared with the initial weights (Fig. 1).

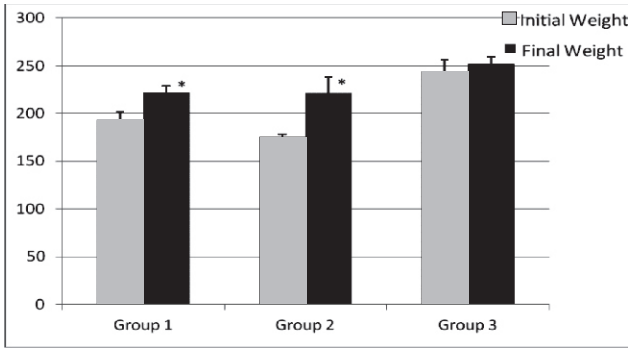

Fig. 1. Effect of unfermented *Parkia biglobosa* seeds on weight
Each value is the mean ± S.E.M. of 5 Wistar rats; * = $p < 0.05$ compared with control, ANOVA.
Group 1 - Distilled water (5ml/kg b.w.); Group 2 - 250mg/kg of unfermented *Parkia Biglobosa* seeds;
Group 3 - 500mk/kg of unfermented *Parkia Biglobosa* seeds.

Effect of unfermented Parkia biglobosa seeds on pain

The seeds extract lowered the pain threshold in the treated animals. There was a significant ($p<0.05$) decrease in reaction time for the animals in treated groups (10.60 ± 0.51; 13.00 ± 2.98) when subjected to cold tail immersion compared to the animals in the control (40.20 ± 12.13). However following hot tail immersion test, only the high dose treated group (2.80 ± 0.58) showed a significant ($p<0.05$) decrease in reaction time compared with the control (5.20 ± 0.58) (Table 3).

Effect of unfermented Parkia biglobosa seeds on anxiety indices

Behavioural observations of the treated animals on the

open field maze gave no significant ($p<0.05$) difference when compared with the control group (Table 4).

Discussion

Apart from the rigorous physical efforts required, millions of dollars is spent annually by the overweight and obese people to shed their weight. This is not unconnected to the amount of information available to the people from the scientific community that over weight can lead to obesity which in turn can lead to chronic diseases like type 2 diabetes, coronary heart disease, hypertension, and some forms of cancer[16]. Despite several previous studies targeted to solving over weight, obesity and behavioural disorders, there is paucity of effective drugs in view of its purpose in the literature thus ethno-pharmacology study on this purpose is always welcome.

In this present study, animals administered 1000 mg/kg of *P. biglobosa* seed extract in the toxicity test lost weight. This observed decrease in weight was not observed in the real study as the final weight of the Group 2 animals significantly ($p<0.05$) increased compared with the initial weight of the same group prior to treatment. The same cannot be said for Group 3. The increased weight in Group 2 may not be due to the treatment with 250 mg/kg of *P. boglobosa* seed extract. This is because the control group that was treated with 10ml/kg of distilled water recorded similar significant ($p<0.05$) increase in weight. The weight gain can thus be viewed as part of normal growth process. Also, this study revealed the ability of the extract to significantly ($p<0.05$) decrease LDL, TG, CHOL and significantly ($p<0.05$) increased HDL. The data provided by WHO on overweight individuals shows that intentional weight loss of 10 kg led to reduction of LDL cholesterol (15%), cholesterol (10%), triglycerides (30%)and elevated HDL cholesterol (8%)[17]. Our study recorded the same pattern of reductions and elevation of various lipids despite the fact that the animals (180-240g) were neither overweight nor loss weight. In fact, the animals rather gained weight though this might not be associated with the treatment as stated earlier.

In relation to weight, this study established that *P. biglobosa* seed extract has no associating significant effect on weight. Also, reduction of LDL cholesterol, cholesterol, triglycerides and elevation HDL cholesterol in rats does not lead to loss of weight as documented in humans but might rather be an index of "good growth". "Good growth" in the sense that the recorded reductions and elevation of lipids are positive health adjustments. The results also showed that unfermented *P. biglobosa* seeds at the doses used in this study has positive effects on healthy lipids balance which may be helpful in treatment of diseases with underlying imbalance in "bad" and "good" lipids.

We observed in this study that treatment with *P. biglobosa* seed extract led to significant ($p<0.05$) increase in serum Ca^{2+} level compared to the control group. This might be due to parts of the seeds constituents that was absorbed via the GIT or maybe the seeds has ability to free Ca^{2+} from its storage organ such bone. While drugs like phenobarbital and oral glucocorticoid were documented with ability to release Ca^{2+} from its storage, grape products[35-37] and combined extract of *Morus alba* and *Polygonum odoratum* leaves were shown to increase serum Ca^{2+} level.

To the best of our knowledge, no previous study has linked increased serum Ca^{2+} level with increased muscular contraction activities. We postulated a correlation between the two since Ca^{2+} is necessary for muscular contraction[38], though Ca^{2+} needed for muscular contraction reside in the sacorlemma/endoplasmic reticulum of such muscle[26]. At the end, it was not possible to establish any reasonable association either between the observed increased serum Ca^{2+} and behavioural changes on open field maze or that of pain-related behavioural response. All behavioural indices for anxiety or depression on our open field maze test were not significant ($p<0.05$) in the treated groups compared with the control. However, in both the hot and cold tail-immersion pain models, the reaction time to pain which is usually identified by pain related behavioural response (tail flicking) significantly ($p<0.05$) decreased in the treated groups compared with the control. Though, this is with the exception of the low dose group in the hot water tail immersion model. Why unfermented *P. biglobosa* seeds decreased pain threshold is not yet clear and it needs to be investigated. It is not surprising however as mustard oil that is also consumed as food was documented with the same effects[39].

Our new findings based on the doses used herein suggest that *P. biglobosa* seed has no effect on the studied behaviours. Furthermore, *P. biglobosa* seed extract was capable of elevating serum Ca^{2+} level via a mechanism we are still investigating in our laboratory. Also, *P. biglobosa* seeds is potent in the reduction of pain threshold as well as the reaction time to pain stimulus.

In conclusion, unfermented *P. biglobosa* seed, a popular food seasoning among local folks in various African countries stands as a promising solution to the future accomplishments in the adjustment and stability of lipids profile and Ca^{2+} level in the body towards positive health attainment, as this is needed for nervous system development, memory formation and adipocytes-cross talk in the brain.

References

1. Alabi DA, Akinsulire OR, Sanyaolu MA. Qualitative determination of chemical and nutritional composition of Parkia biglobosa (Jacq.) Benth. *African Journal of Biotechnology*. 2005; 4 (8)

pp. 812-815.
2. Campbell-Platt G. African locust bean and it's West African fermented products- Dadawa. Eco. *Food Nutr*. 1980; 9: 123–132.
3. Schnell BS. Plants alimantaries et vie Agricolade L'Afriqu Noire larose. *Paris.* 1957; 3: 110–115.
4. Olaniyan, A. Locust Bean Products. *Non-Wood News*-2013; No.10.
5. Adi K, Metowogo K, Eklu-Gadegbeku K, Lamboni C, Napo-Koura G, Aklikokou A K, Gbeassor M. Fermented seed of Parkia biglobosa (Jacq.) Benth (African Mustard) diet prevents myocardial infarction in rat. *Pakistan Journal of Nutrition* 12(4): 322-328, 2013.
6. Akande FB, Adejumo OA, Adamade CA, Bodunde J. "Processing of locust bean fruits: Challenges and prospects". *African Journal of Agricultural Research*, 2010; Vol.5, pp.2268-2271.
7. Builders M, Wannang N, Aguiyi J. Antiplasmodial activities of Parkia biglobosa: In vivo and in vitro studies. *Ann. Biol. Res*. 2011; 2:8-20.
8. Asase A, Alfred A, Yeboah O, Odamtten GT, Simmonds SJ. (2005). Ethnobotanical study of some Ghanaian antimalarial plant. *J. Ethnopharmacol*. 2005; 99, 273-279.
9. Jullian V, Bourdy G, George G, Maurel G, Sauvain M. Validation of use of a traditional antimalarial remedy from French Guiana, Zanthoxylum rhoifolium Lam. *J. Ethnopharmacol.* 2006; 106:348-352.
10. Tijani AY, Samuel EO, Salawu OA. Neuropharmacological effects of standardized aqueous stem bark extract of Parkia biglobossa in Wistar rats. *Avicenna J Phytomed*. 2014; 4(1): 59–71
11. Konadio, F., Kanko, C., Juge, M., Grimand, N., Jean, A., Guessan, Y.T., Petit, J.Y., 2000. Analgesic and anti-inflammatory activities of an extract from Parkia biglobosa used in traditional medicine in the Ivory Coast. Phytother. Res. 14, 635–637.
12. Bonnah B, Aklikokou KA, Akpagana K, Gbeassor M. (1998). Contribution a l'etude des proprietes pharmacologiques des extraits hydroalcooliques de graines de parkia biglobosa. *Sc. Med. Rev. CAMES*, 1998; 00:12-15.
13. Odetola AA, Akinloye O, Egunjobi C, Adekunle WA, Ayoola AO. Possible antidiabetic and antihyperlipidaemic effect of fermented Parkia biglobosa (jacq) extract in alloxan- induced diabetic rats. *Clin. Exp. Pharmacol. Physiol*., 2008; 33:808-812.
14. Mateo-Gallego R, Perez-Calahorra S, Cofan M, Baila-Rueda L, Cenarro A, Ros E, Puzo J, Civeira F. (2014). Serum Lipid Responses to Weight Loss Differ between Overweight Adults with Familial Hypercholesterolemia and Those with Familial Combined Hyperlipidemia. 49. *J. Nutr.* 2014; 144: 1219–1226,
15. Haslam DW, James WP. Obesity. *Lancet* 2005; 366:1197–209
16. Hongu N, Kitts DD, Zawistowski J, Dossett CM, Kopec A, Pope BT, Buchowski MS. Pigmented Rice Bran and Plant Sterol Combination Reduces Serum Lipids in Overweight and Obese Adults. *Journal of the American College of Nutrition*, 2014; 33(3) 231–238.
17. Ahaneku GI, Ahaneku JE, Osuji CU, Oguejiofor CO, Anisiuba BC, Opara PC. Lipid and Some Other Cardiovascular Risk Factors Assessment in a Rural Community in Eastern Nigeria. *Ann Med Health Sci Res*. 2015; 5(4):284-91.
18. Itabe H. Oxidative modification of LDL: its pathological role in atherosclerosis. *Clin Rev Allergy Immunol.* 2009; 37:4–11.
19. Dickson AG, Goyet C. "Chapter 5" Handbook of method for analysis of the various parameters of the carbondioxide system in sea water. 1994;version 2 ORNL/CDIAC-74
20. De Smedt H, Verkhratsky A, Muallem S. (2011). Ca(2 +) signaling mechanisms of cell survival and cell death: an introduction. *Cell Calcium* 50:207–210.
21. Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signaling. *Nat Rev Mol. Cell Biol*. 2000; 1:11–21.
22. Toescu EC, Dayanithi G. Neuroendocrine signalling: natural variations on a Ca(2 +) theme. *Cell Calcium*. 2012; 51:207–211.
23. Rimantas T, Andrius K, Emmanuel J, Dominique B, Saulius S. R-Type Calcium Channels Are Crucial for Semaphorin 3A–Induced DRG Axon Growth Cone Collapse. *PLOS ONE.* 2014; (7)e102357.
24. Henley J, Poo MM. Guiding neuronal growth cones using Ca2+ signals. *Trends in cell biology*. 2004;14:320–330.
25. Tojima T, Hines JH, Henley JR, Kamiguchi H. Second messengers and membrane trafficking direct and organize growth cone steering. Nature reviews. *Neuroscience.* 2011; 12:191–203.
26. Szent-Gyorgyi AG. Caicium regulation of muscle contraction. *Biophys J.* 1975; 15(7): 707-723.
27. Kelley SP; Bratt AM; Hodge CW. Targeted gene deletion of the 5-HT3A receptor subunit produces an anxiolytic phenotype in mice. Eur J Pharmacol 2003; 461(1): 19-25.
28. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: A review. European Journal of Pharmacology. 2003;463 (1–3):3–33.
29. Hansen R. R., Malcangio M., (2013). Review Astrocytes—Multitaskers in chronic pain. *European Journal of Pharmacology* 716 : 120–128
30. Odetola AA, Akinloye O, Egunjobi C, Adekunle WA and Ayoola AO. Possible antidiabetic and antihyperlipidaemic effect of fermented parkia

biglobosa (jacq) extract in alloxan-induced diabetic rats. *Clinical and Experimental Pharmacology and Physiology* 2006, 33(9) 808–812.
31. Parasuraman S. Toxicological screening *J Pharmacol Pharmacother*. 2011; 2(2): 74–79.
32. Pizziketti RJ Pressman NS Geller EB, Cowan A., Adler MW. Rat coldwater tail flick: a novel analgesic test that distinguishes opoid agonist from mixed antagonists. European Journal of pharmacology 1985;119:23-29.
33. Sewell PS and Spencer PD. Modification of the antinociceptive activity of narcotic agonists and antagonists by intraventricular injection of biogenic amines in mice Brit. J. Pharmacol.1974; 51: 140 – 141.
34. File SE. The use of social interaction as a method for detecting anxiolytic activity of chlordiazepoxide-like drugs. Journal of Neuroscience Methods. 1980;2 (3):219–238.
35. Vestergaard P. Epilepsy, osteoporosis and fracture risk - a meta-analysis. *Acta Neurol Scand.* 2005; 112:277-86.
36. FRAX. The World Health Organization fracture risk assessment tool. *Available at www.shef.ac.uk/FRAX*. Accessed May 26, 2010.
37. Hohman EE, Connie MW. A Grape-Enriched Diet Increases Bone Calcium Retention and Cortical Bone Properties in Ovariectomized Rats. *J Nutr* 2015;145:253–9
38. Herzog W, Powers K, Johnston K, Duvall M. A new paradigm for muscle contraction. *Frontiers in Physiology*. 2015; Vol. 6:174
39. Mansikka H, Pertovaara A. Influence of selective alpha 2-adrenergic agents on mustard oil-induced central hyperalgesia in rats. *Eur J Pharmacol*. 1995; 281(1):43-8.