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ATPases in the colon of rats fed a Nigerian like diet supplemented with folate and bitter leaf (*Vernonia amygdalina*)

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KEYWORDS

Folate and bitter leaf, *Vernonia Amygdalina*, ATPases, colon carcinogenesis

A B S T R A C T

This study examined the effect of a wholly compounded Nigerian-like diet supplemented with folic acid and bitter leaf (*Vernonia amygdalina)* on the activity of ATPases in early colon carcinogenesis. It was also aimed to determine if any of the observed effect(s) of folic acid and bitter leaf is/are potentiated before the onset of colon carcinogenesis or also occur during the process. One hundred and twenty eight Wistar albino rats of 120g ± 0.3g were used for this study and were divided into two groups. Animals in the first group were fed with the wholly compounded food supplemented with folic acid and bitter leaf before being exposed to Cycads for two weeks (the pre-treated rats) while animals in the second group were fed with Cycads for two weeks before they were exposed to the wholly compounded food supplemented with folic acid and bitter leaf (the post-treated rats). The animals in each group were sub-divided into two diet classes. Animals in one group were fed with wholly compounded normal diet (ND) which served as the control class while animals in the second group were fed with wholly compounded Nigerian-like diet (NLD) which was low in protein and fat but high in carbohydrate and fiber, The animals in each diet class were further distributed into four subgroups. In each subclass, one group received the diet alone, another group received the diet and folic acid, and another group received the diet and bitter leaf, while the fourth group received the diet, folic acid and bitter leaf. The animals were given food and water ad libitum and were force fed with folic acid, bitter leaf and cycads. Such that they consumed 0.5mg/kg body weight of folic acid, bitter leaf and Cycads. Exposing the colon of rats to bitter leaf and folic acid significantly ($P<0.05$) increased the activity of Na^+-K^+ ATPase but significantly ($P<0.05$) decreased magnesium ATPase activity and calcium ATPase activity. These results suggest that folic acid and bitter leaf (*Vernonia amygdalina)* may protect against colon cancer before and after its onset.

Introduction

Na^+/K^+ATPase, Mg^{2+}ATPase and Ca^{2+}ATPase are membrane bound enzymes responsible for the transport of sodium/potassium, magnesium and calcium ions across the cell membrane at the expense of ATP by hydrolysis (Sudhandiran, 2013). They have been implicated in the regulation of many cellular functions including cell volume regulation, regulating the osmotic balance of the cell and maintaining high concentration of intra-cellular ions. Numerous studies have now established that the influx of ions across the plasma membrane into the cell is a key trigger or regulator of cellular processes relevant to tumor progression, including proliferation, migration, and apoptosis (Monteith *et al*, 2012).

Cancer is considered to be a group of diseases of multiple causes which occurs when cells become abnormal and divide without control or order (American Cancer Society, 1990). The development of cancer is referred to as carcinogenesis and the carcinogenic processes involve multiple steps (Sugimura, 1992). Cancer is a leading cause of death worldwide and the incidence of cancer is on the increase in every country of the world (Jemal *et al*, 2010 ; Bray *et al*, 2013). This increase, coupled with the harsh side effects of some of the cancer chemotherapy, has led to the search for more natural biological products, especially those derived from plant products. One of such plants with proven anti-breast cancer activity is *Vernonia amygdalina* (Izevbigie, 2003).

Vernonia amygdalina (commonly called Bitter Leaf) is one of the edible vegetables in Nigeria and other parts of African sub region. It is a shrub or tree which grows to about five meters high especially around forest margins and grows wild under severe anthropogenic and environmental pressures (Khalafalla *et al*, 2009). The full binomial name is *Vernonia amygdalina Del* (Farombi and Nwoye, 2011). In Nigeria where the plant is found in abundance, it performs both medicinal and nutritive functions. It is reported to be a medicinal plant for diabetes and fever (Crellin *et al*, 1989). It is used in the prevention of malaria fever, elimination of worms, treatment of stomach upset, induction of fertility in barren women and treatment of diabetic mellitus (Igile *et al*, 1995).

A root infusion is taken in Nigeria as a worm expeller as well as for intestinal parasitic infections. Chimpanzees have been observed to ingest the leaves when suffering from parasitic infections (Huffman and Koshimizu 1993). *Vernonia amygdalina* have been used for ingivitis and toothache due to its proven antimicrobial activity (Ademola and Eloff, 2011). It has been found to be effective as blood purifier, uterus toner and helps also to prevent atherosclerosis (Nwanjo, 2005; Erasto *et al*, 2006). *Vernonia amygdalina* is well known for producing anticancer agent, vernodaline and vernolide (Khalafalla *et al*, 2009). Imaga and Bamigbetan, (2013) concluded in their study that aqueous extract of *Vernonia amygdalina* is safe for consumption as food or as herbal medicine without plausible toxicity to body organs and tissues.

In this present study the effect of a wholly compounded Nigerian-like diet supplemented with folic acid and bitter leaf (*Vernonia amygdalina)* on the activity of ATPases in early colon carcinogenesis was examined. It also examined if any of the observed effect(s) of folic acid and bitter leaf is/are potentiated before the onset of colon carcinogenesis or also occur during the process.

Materials and Methods

Experimental Animals

Wistar albino rats of 120 ± 0.3g purchased from the animal house of Ambrose Ali University, Ekpoma, Edo state, were used for this study. The rats were weighed and assigned comparable weights in all groups (± 0.3g). The animals were acclimatized with their respective diets for a period of one week. The cages housing the rats were kept in an environment with free supply of air and light. Water and feed were changed daily.

Feed

The leaves of the plant cycads Circinalis were obtained from Santua garden, Ugbowo, Benin City, Edo state. The leaves of bitter leaf (*Vernonia amygdalina*) were obtained from Ekpoma express market, Ekpoma, Edo state. Both leaves were identified at botany department, Ambrose Ali University, Ekpoma. The vitamin mix was from vitadol. The soya beans, white garri, sugar and palm oil were obtained from Ekpoma express market, Ekpoma, Edo state.While the folic acid was obtained from Jofel pharmacy, Ekpoma, Edo state.

The leaves of cycads and bitter leaf (*Vernonia amygdalina*) were washed and dried in the oven at 50^{o}C and then blended into powder. The soya beans were cooked for about five hours, dried in the oven at 60^{o}C and blended into powder. The other food components which were already in powder form were mixed in their various proportions as shown in table 1. The ND was prepared by mixing together 44g of blended soya beans, 32.5g of white garri, 3ml of palm oil, 2g of powdered vitamin mix, 13g of sugar, and 1.5g of fiber while the NLD was prepared by mixing together 27g of blended soya beans, 40g of white garri, 1ml of palm oil, 2g of powdered vitamin mix, 5g of sugar, and 10g of fiber. The normal diet (ND) was patterned after previously fed diets by Schuette and Richard (1986) in their study of the effects of diets high in fats and/or fiber on colonic absorption of dimethylhydrazine (DMH) in rats. The diet rich in carbohydrate and fiber was patterned after that of Anderson and Gustafson (1987) in their study of the hypolipidaemic effect of a high carbohydrate and high fat diet.

Isolation of Colon

At the end of the twelve weeks of feeding, all the rats were weighed and starved overnight. Subsequently, they were sacrificed after sedating with chloroform and then dissected accordingly. The colon (first 10cm of the proximal end of the large intestine) was recovered from each rat and flushed several times with ice cold normal saline (0.9% NaCl solution) until free of debris. It was inverted and the mucosa was removed by scrapping with a glass slide. The tissue and mucosa were kept separately in sample bottles (10mL) and stored at -4^{0}C for analysis.

Preparation of Sample

1g of colon tissue and mucosa of each rat was homogenized separately in 9ml of 0.9% Nacl solution for 10 seconds. The homogenate was subsequently centrifuged at 10,000rpm for 15minutes at 4^{o}C. The supernatant was used as source of sample for biochemical analysis.

Biochemical Assay

Estimation of ATPases Activity

Estimation of Calcium ATPase Activity

The reaction mixture contained 1.5ml of 160mM Tris HCl cofactor. 0.3mM ATPase

solution was added and incubated for 1minute. The reaction was started by the addition of 0.2ml of the enzyme extract (supernatant from homogenate). The final mixture was 2.0ml. The mixture was further incubated for 10 minutes, 0.1ml of supernatant was taken for inorganic (Pi) determination. 2ml of ammonium molybdate were added followed by 2ml of 0.2% ascorbic acid. The colour developed was read immediately (within 1 minute) using the spectrophotometer at 625nm. Protein determination was carried out using Lowry's method.

Specific Activity of ATPase

The activity of ATPase was estimated from the following expression.

$$\text{Specific activity} = \frac{\text{Pi} \times 1 \times 4 \times 1}{10 \times 31 \times 0.1 \times \text{mg protein}}$$

Pi = inorganic phosphate obtained from standard curve.
4 = Total volume of reaction mixture
0.1 = Mixture of dilution of enzyme extract that was taken for the estimation of Pi after precipitation.
31 = Molar extinction of phosphorus
10 = incubation time

Estimation of Magnesium ATPase Activity

The above protocol was repeated for each sample except that of Tris buffer PH 7.4 which contains only 5mM $MgCl_2$ as co-factor. The amount of inorganic phosphate (Pi) released in each case was extrapolated from the standard calibration curve. Protein determination was also carried out using the method of Lowry et al, (1951).

Estimation of Sodium-Potassium ATPase Activity

The sodium-potassium ATPase was gotten by subtracting the magnesium ATPase activity from total ATPase .

Estimation of Total ATPase Activity

1.5ml of 50mM tris HCl buffer containing the cofactors PH 7.4 and 0.3ml of 5mM ATP solution were added into a test tube and incubated for 1 minute. The reaction was started by addition of 0.2ml of the enzyme extract (Supernatant from homogenate). The final volume of the mixture was 2.0µl. The mixture was further incubated for 10 minutes and terminated by the addition of 2ml of 10% trichloroacetic acid. After centrifugation at 1000rpm for 10 minutes, the supernatant was collected and used for inorganic phosphate determination. The above process was repeated for every sample.

Inorganic Phosphate Determination (pi)

The supernatant (0.1ml) above was taken and used for inorganic phosphate determination. 2ml of ammonium molybdate were added followed by 2ml of 0.2% ascorbic acid. The colour developed was read immediately within 1 minute at 625nm.

Protein Content Estimation

0.1ml of the supernatant was added to the respective test tube except the blank and made up to 4.5ml with distilled water. 5ml of alkaline reagent were added and allowed to stand for 15 minutes at room temperature. 0.5ml of folin ciocalteau was then added to the reaction mixture and allowed to stand for 30 minutes at room temperature for the colour to develop. The absorbance was read at 750nm against reagent blank.

Statistical Analysis

Statistical analysis was carried out using computer SPSS software.

Results and Discussion

Results are as presented in figures 1-12. Results showed that bitter leaf and folic acid significantly ($P<0.05$) increased the activity of Na^+-K^+ATPase but significantly ($P<0.05$) decreased magnesium ATPase activity and calcium ATPase activity

Na^+-K^+ATPase was observed with the inclusion of folic acid in the diets of animals. Increased activity of Na^+-K^+ATPase was also observed with the inclusion of bitter leaf in the diets of animals. This could be due to the antioxidant property they possess. They are able to scavenge free radicals that would have caused damage to the cell membrane. This implies that more sodium ion enters in to the colonic lumen making the colonic cells more viable. This result agrees with previous reports by Josiah *et al*, (2012) and Atangwho *et al*, (2007).

Decreased activity of magnesium ATPase was observed with the inclusion of folic acid and bitter leaf separately in the diets of animals. This implies less influx of magnesium into the cell. Very many enzymes and ion transport require magnesium and it plays a role in fatty acid and phospholipid metabolism, thus affecting permeability and stability of membranes. Magnesium ATPase is closely tied to the energy generation of oxidative phosphorylation of the mitochondria. The reduce magnesium ATPase activity implies less dependence on the oxidative phosphorylation for energy in these animals. This argument cannot be over emphasized in light of study which relate high intake of magnesium to low cancer risk. Studies by Molena-Montes *et al*, (2012) show that increased intake of magnesium is associated with reduced risk of pancreatic cancer. As folic acid and the bitter leaf reduced magnesium ATPase, it would invariably reduce the level of magnesium that enters the colonic mucosa or the tissue.

Table.1 Composition of Experimental Diet

Dietary Component	**ND**	**NDB**	**NDF**	**NDBF**	**NLD**	**NLDB**	**NLDF**	**NLDBF**
Soya beans (protein) (g)	44	44	44	44	27	27	27	27
White garri (carbohydrate) (g)	32.5	32.5	32.5	32.5	40	40	40	40
Palm oil (ml)	3	3	3	3	1	1	1	1
Vitamin/salt (g)	2	2	2	2	2	2	2	2
Sugar/sucrose (g)	13	13	13	13	5	5	5	5
Fiber (g)	1.5	1.5	1.5	1.5	10	10	10	10
Bitter leaf (g)	-	0.5	-	0.5	-	0.5	-	0.5
Folic acid (g)	-	-	0.5	0.5	-	-	0.5	0.5
Cycads (g)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Figure.1 Sodium Potassium ATPase Activity in the Colonic Mucosa of Pre-Treated rats Fed with Cycads and Nigerian-like Folic Acid and Bitter Leaf Supplemented Diets

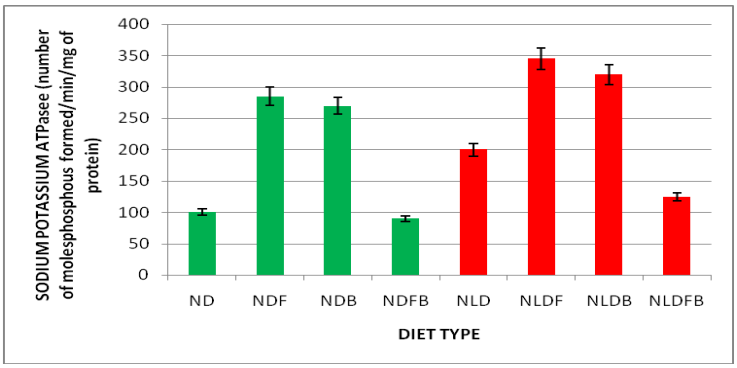


Figure.2 Sodium Potassium ATPase Activity in the Colonic Mucosa of Post-Treated Rats Fed with Cycads and Nigerian-like Folic Acid and Bitter Leaf Supplemented Diets

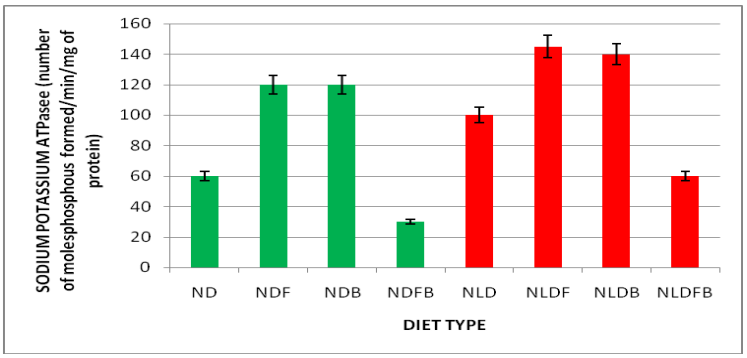


Figure.3 Sodium Potassium Activity ATPase in the Colonic Tissue of Pre-Treated Rats Fed with Cycads and Nigerian-like Folic Acid and Bitter Leaf Supplemented Diets

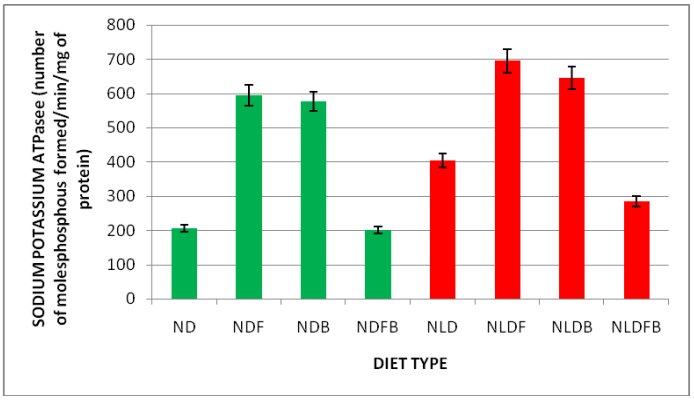


Figure.4 Sodium Potassium ATPase Activity in the Colonic Tissue of Post-Treated Rats Fed with Cycads and Nigerian-like Folic Acid and Bitter Leaf Supplemented Diets

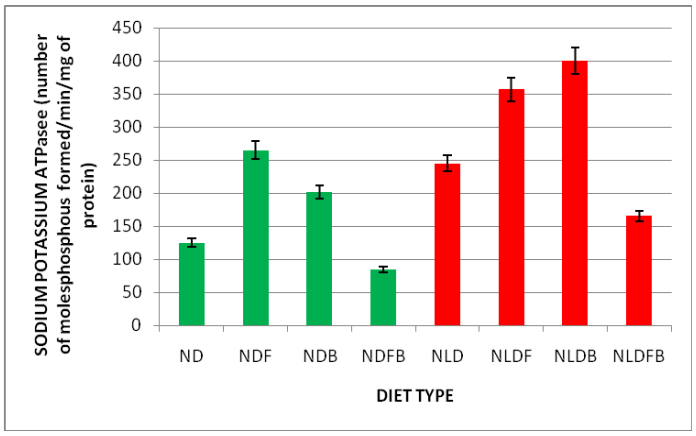


Figure.5 Magnesium ATPase Activity in the Colonic Mucosa of Pre- Treated Rats Fed with Cycads and Nigerian-like Folic Acid and Bitter Leaf Supplemented Diets

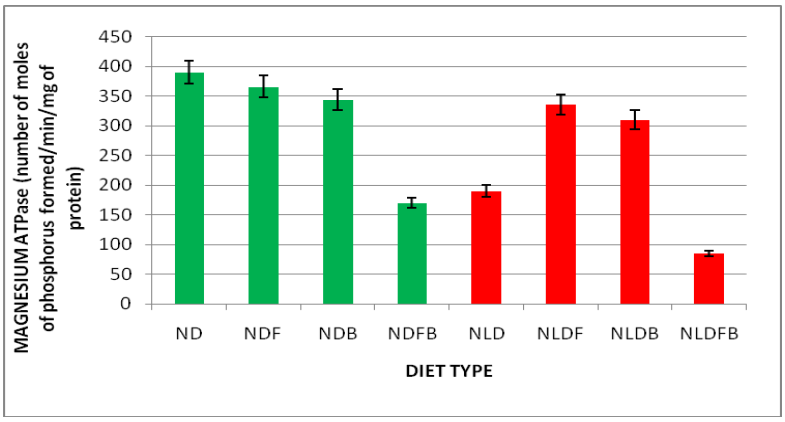


Figure.6 Magnesium ATPase Activity in the Colonic Mucosa of Post-Treated Rats Fed with Cycads and Nigerian-like Folic Acid and Bitter Leaf Supplemented Diets

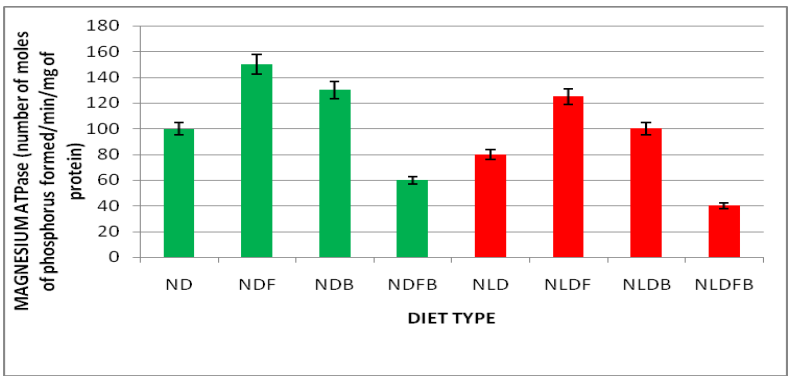


Figure.7 Magnesium ATPase Activity in the Colonic Tissue of Pre-treated rat Fed with Cycads and Nigerian-like Folic Acid and Bitter Leaf Supplemented Diets

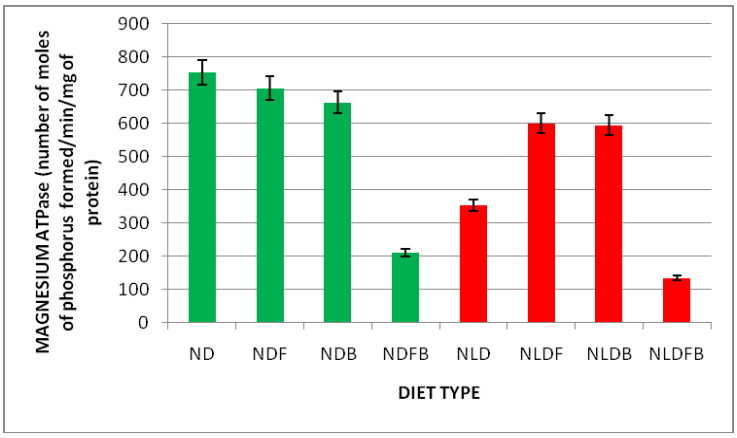


Figure.8 Magnesium ATPase Activity in the Colonic Tissue of Post-Treated Rats Fed with Cycads and Nigerian-like Folic Acid and Bitter Leaf Supplemented Diets

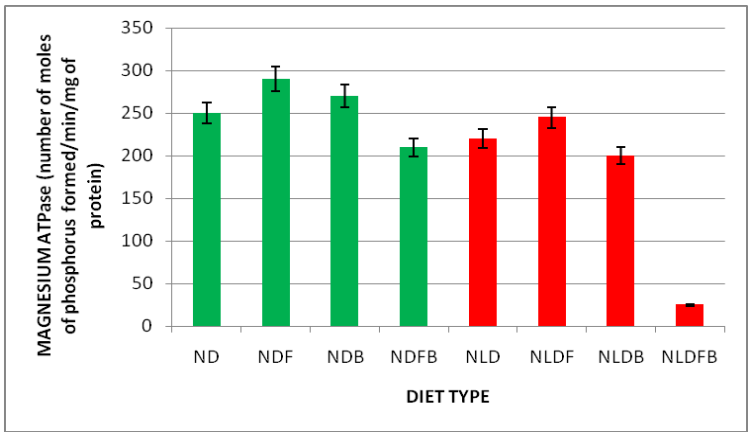


Figure.9 Calcium ATPase Activity in the Colonic Mucosa of Pre-treated Rats Fed with ycads and Nigerian-like folic Acid and Bitter Leaf Supplemented Diets

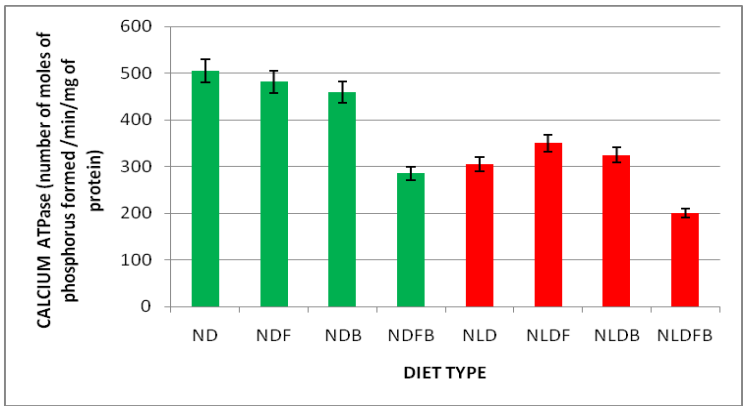


Figure.10 Calcium ATPase Activity in the Colonic Mucosa of Post-treated Rats Fed with Cycads and Nigerian-like Folic Acid and Bitter Leaf Supplemented Diets

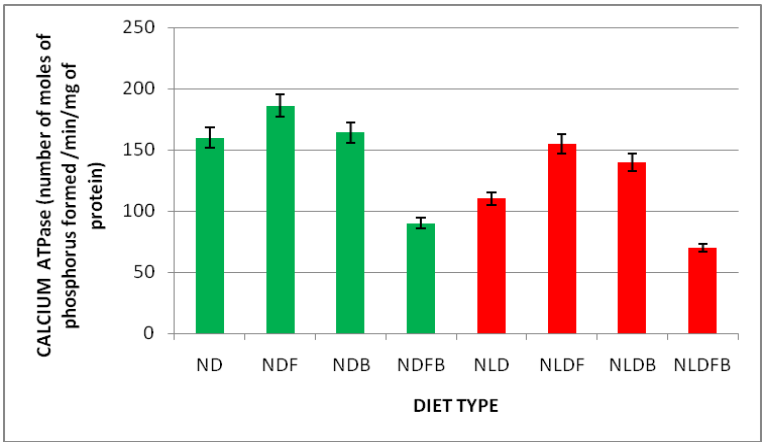


Figure.11 Calcium ATPase Activity in the Colonic Tissue of Pre-treated Rats Fed with Cycads and Nigerian-like Folic Acid and Bitter Leaf Supplemented Diets

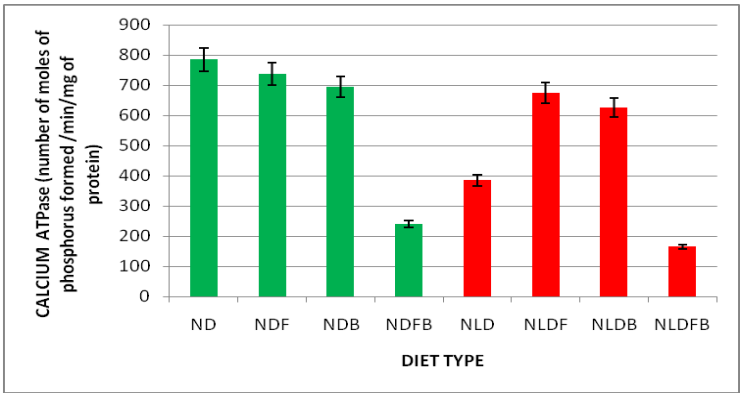
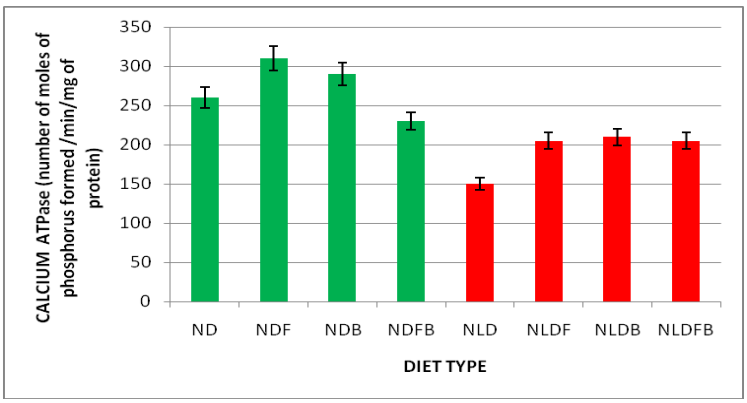


Figure.12 Calcium ATPase Activity in the Colonic Tissue of Post-Treated Rats Fed with Cycads and Nigerian-like Folic Acid and Bitter Leaf Supplemented Diets



The NLD is high in fibre. High dietary fiber has been reported to decrease dietary calcium absorption. The result of this work agrees with the study of Ashton et al, (2007) who reported that calcium was reduced in low-protein rats. In this study however whole tissue calcium ATPase was assessed. It would be interesting to carry out the effect of folic acid and bitter leaf on organelle calcium ATPases. In a recent study, Sarco/endoplasmic reticulum calcium ATPase (SERCA) enzymes play important roles in several signal transduction pathways that control proliferation, differentiation and apoptosis. A report has shown that SERCA2 expression is positively correlated with tumor node metastasis and grades of patients with colorectal cancer (Lu et al, 2014). The study reported similar findings in mice (Lu et al, 2014). Besides, SERCA2 expression was also increased in undifferentiated HT-29 cells as compared with that in differentiated HT-29 gal cells (Lu et al, 2014). Moreover, SERCA2 over expression promoted proliferation and migration of SW480 cells via activating MAPK and AKT signaling pathways, while silence of SERCA2 inhibited the proliferation and migration of SW480 cells (Lu et al, 2014).

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