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Assessment of the effects of ethanolic extracts of *Annona squamosa* leaves and stem on selected biochemical parameters

F. A. Sulaiman[1], A. M. O. AbdulRaheem[2], T. Garuba[3], F. A. Abubakar[1], F. A. Giwa[1], E. M. Sani[1] and F. H. Amokeoja[1]

[1]Department of Biochemistry, Faculty of Life Sciences, University of Ilorin.
[2]Department of Chemistry, Faculty of Physical Sciences, University of Ilorin.
[3]Department of Plant Biology, Faculty of Life Sciences, University of Ilorin.

[*]Author for correspondence: Tel: +2348051505889, +2348032127200
E-mail: Sulaiman.af@unilorin.edu.ng; faoziyat20022002@yahoo.com

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ABSTRACT: Plants are rich sources of secondary metabolites with interesting biological and medicinal activities. Compounds from plants continue to play a major role in primary health care as therapeutic remedies in many developing countries. Natural remedies from medicinal plants are found to be safe and effective. Many plant species have been used to treat various ailments including Annona squamosa, its various plant parts, stem, leaves, roots, etc has been used locally to manage various ailments, hence the need to assess its safety and possible effects on some endogenous biochemical markers. A completely randomized design (CRD) was used in this experiment.

The ethanolic extracts of leaves and stem of Annona squamosa were used in this research to investigate the effect on some selected biochemical parameters in healthy Wistar rats. Twenty experimental animals were randomly distributed into four (4) groups of five (5) animals each. Group A (Control) was administered appropriate volume (1ml) of distilled water, Group B was administered 50mg/kg bodyweight of Cyclophosphamide, Group C was administered 500mg/kg bodyweight of ethanolic extract of Annona squamosa leaf, Group D was administered 500mg/kg bodyweight of ethanolic extract of Annona squamosa stem. All of the doses were administered orally daily for 21 days. There was an increase in serum Alanine amino transaminase (ALT), Aspartate Amino Transaminase (AST) and Alkaline Phophatase (ALP) activity in the serum of the cyclophosphamide treated group and a subsequent decrease of these enzymes in the liver, kidney and heart. The groups administered with ethanolic extracts of Annona squamosa leaves and stem compared favourably with the control group in ALT, AST, and ALP activity in the serum, kidney, liver, and heart. The study showed that there was a significant rise in High-density Lipoprotein(HDL) concentration and albumin concentration and a decrease in Triglyceride concentration, bilirubin concentration, urea, and creatinine concentration in the groups administered 500mg/kg bodyweight of Annona squamosa leaves and stem.

Keywords: Ethanolic Extracts, Annona squamosa Leaves and Stem, Biochemical Parameters

Introduction

Natural remedies from medicinal plants are found to be safe and effective. Many plant species have been used to treat various ailments (Aamir et al., 2013 and Sulaiman et al., 2017). Even today compounds from plants continue to play a major role in primary health care as therapeutic remedies in many developing countries (Vanitha et al., 2011). Plants are a rich source of secondary metabolites with interesting biological activities (Chavan et al., 2010 and Deepika et al., 2011; Sulaiman et al., 2017). In general, these secondary metabolites are important sources with a variety of structural arrangements and properties (Vanitha et al., 2011).

The importance of conventional medicines in solving the best of health problem solutions is invaluable in a global pharmaceutical market. Natural products have been a significant source of marketable medicines and drug source (Aamir et al., 2013). Annona squamosa (plate 1) is commonly known as Custard apple, Sugar apple or Sweet sop in English, In Northern Nigeria, it is called fasadabur in Hausa, in Yoruba, it is called abo or arere, in Igbo, it is called mbugo-ago, Pommier cannelle in French, Sharifa in Hindi, Sitaphal in Telugu and Sitaphalam in Tamil (Pandey and Barve, 2011). It is distributed in tropical and subtropical trees and shrubs. They range from 10 to 20 ft (3-6 m) in height with irregular branches and zigzag twigs) and It is grown mostly in the Southern part of Nigeria (Folorunsho and Olorode, 2006).

The extract of Annona squamosa is highly rich in phenols, reducing sugar, flavones, glycosides, saponins, steroids, alkaloids, proteins, and tannins. The plant is traditionally used for the treatment of epilepsy, dysentery, cardiac problem, worm infection, constipation, hemorrhage, antibacterial infection, dysuria, fever, and ulcer. It also has antifertility, antitumor, and abortifacient properties (Biba et al., 2014). The earlier studies on Annona squamosal showed that this plant is a potent source of a wide variety of secondary metabolites belonging to several categories (Biba et al., 2014). Reports over the years have shown the antiperoxidative (Panda and Kar, 2007), Analgesic and anti-inflammatory activities (Chavan et al., 2010), antioxidant and antilipidemic activities (Rajesh et al., 2008), antitumor activity (Haijun et al., 2009, hepatoprotective activity (Mohammed et al., 2011), hypoglycemic and antidiabetic activities (Mujeeb et al., 2011) and genotoxic effect (Paramjit et al., 2009) of Annonna squamosal, but very little is known about its toxicological effect. Thus, this study focused on assessing the biochemical alterations that may occur as a result of the oral administration of Annona squamosa ethanolic extracts in a non-diseased and healthy state of Wistar rats.

Materials and Methods

Plant Material

Fresh leaves and stem of Annona squamosa, were collected and botanically authenticated at the Herbarium unit of the Plant Biology, University of Ilorin, Ilorin, Kwara State, Nigeria, where voucher specimens were deposited. The voucher number is UILH/004/1167.

Preparation of Extracts

The extract was prepared as described in a previous study (Sumithra et al., 2014). The dried Annona squamosa (leaves and stem) were pulverized to powder. Annona squamosa leaves (163.80g), Annona squamosa stem (208.34g), were successfully extracted in ratio 1:5 of 80% ethanol for 24 hours. The extracts were filtered using Whatman filter paper No. 1 and the filtrate was concentrated on a water bath. The concentrates were exposed to air and residual solvent (ethanol) was allowed to evaporate for a week, at room temperature to obtain the final dry extracts.

Assay Kits and Reagents

Alkaline Phosphatase (ALP), Aspartate Amino Transferase (AST), Alanine Amino Transferase (ALT), High-Density Lipoprotein (HDL), Triglycerides, Cholesterol, Bilirubin, Albumin, Protein, Creatinine, and Urea kits are products of Randox Laboratories Ltd. Ardmore Diamond Road Crumlin Co. Antrium United Kingdom. All other reagents used were of analytical grade and were prepared in a glass apparatus using distilled water and suitable solvents.

Animal Grouping

Twenty (20) experimental animals were randomly distributed into four (4) groups of five (5) animals each. 1ml of the extracts equivalent to 500mg/kg body weight was administered orally to the experimental animals for a period of Twenty one (21) days, except the control group which received 1ml of distilled water. The animal in group A (control) were administered appropriate volume (1ml) of distilled water, group B was administered 50mg/kg bodyweight of Cyclophosphamide, group C administered 500mg/kg bodyweight of ethanolic extract of Annona squamosa leaf and group D administered 500mg/kg bodyweight of ethanolic extract of Annona squamosa stem. A completely randomized design (CRD) was used in this experiment.

Statistical Analysis

Experimental data were presented as Means ± Standard error of the mean (SEM). Statistical analysis was implemented using GraphPad prism 6 and SPSS version 20.0. One way analysis of variance was used to compare variables among the different groups. The post-test analysis was done using the Duncan Multiple Range Test at $p<0.05$.

Results

Percentage Yield of Extracts

Table 1 shows the percentage yield of ethanolic extract of Annona squamosa leaf and stem. The higher yield was obtained in the Annona squamosa stem extract (11.2) when compared to that of the leaf extract (10.7).

Table 1: Percentage (%) yield of ethanolic extract of *Annona squamosa* leaf and stem

Samples	Before extraction (g)	After extraction (g)	Percentage Yield (%)
***Annonna squamosa* Leaf**	163.8	17.56	10.72039
***Annonna squamosa* Stem**	208.34	23.4	11.23164

Table 2: Weights of experimental animals over the period of experiment

Groups	Week 1	Week 4
Control	129.250 ± 2.175^{a}	166.250 ± 2.175^{b}
Cyclophosphamide	137.250 ± 4.327^{a}	173.750 ± 3.568^{b}
***Annona squamosa* leaf**	136.750 ± 2.889^{a}	174.250 ± 2.462^{b}
***Annona squamosal* stem**	131.250 ± 2.250^{a}	170.750 ± 2.719^{b}

Values are expressed as means of five (5) replicates ± S.E.M. Values carrying different superscripts across the group are significantly different)

The means above were for 5 rats per group taking after two weeks of acclimatization (week one) and the last week (week four) just before they were sacrificed. A consistent and significant increase in weight was recorded in all the experimental groups over 4 weeks of the experiment.

Average weight of organs isolated from each rat after the experiment

Table 3 shows the average weight of organs isolated from five experimental animals per group, after the experiment. There was no significant difference in the weight of the organs of the test groups when compared to the control

Table 3: Average weight of organs isolated after the experiment

Organs	Control (g)	Cyclophosphamide (g)	*Annonna squamosa* Leaf (g)	*Annonna squamosa* stem (g)
Liver	4.100 ±0.216	4.270 ± 0.261	4.316±0.311	4.213±0.121
Kidney	0.942±0.601	0.920 ± 0.130	0.812±0.087	0.795±0.069
Heart	0.460±0.0158	0.480 ± 0.300	0.468±0.057	0.448±0.032

Values are expressed as means of five (5) replicates ± S.E.M. Values carrying different superscripts across the group are significantly different 5) .

Organ to body weight ratio

Table 4 shows the effect of the administration of ethanolic extract of Annona squamosa leaf and stem on the Organ-Body weight ratio. There was no significant difference ($p<0.05$) in the Organ-Body weight ratio across all groups when compared to the control.

Table 4: Organ-Body Weight ratio

Groups	Liver	Heart	Kidney
Control	0.0239 ± 0.0011	0.0027 ± 0.0001	0.0055 ± 0.0003
Cyclophosphamide	0.0245 ± 0.0005	0.0027 ± 0.0001	0.0052 ± 0.0003
***Annona squamosa* leaf**	0.0242 ± 0.0023	0.0024 ± 0.0001	0.0051 ± 0.0006
***Annonna squamosa* stem**	0.0249 ± 0.0009	0.0026 ± 0.0001	0.0047 ± 0.0004

Values are expressed as means of five (5) replicates ± S.E.M. Values carrying different superscripts down the group are significantly different ($p<0.05$) .

Enzyme activities

Aspartate Aminotransferase.

There was a significant decrease ($p<0.05$) in AST activity in the liver (Figure 8) and kidney (Figure 9) of the group treated with cyclophosphamide as compared to the control and other test groups, the same trend was also recorded in the heart (Figure 10). Also, a corresponding increase ($p>0.05$) in serum AST activity was recorded in the group administered the reference drug as compared to other test groups.

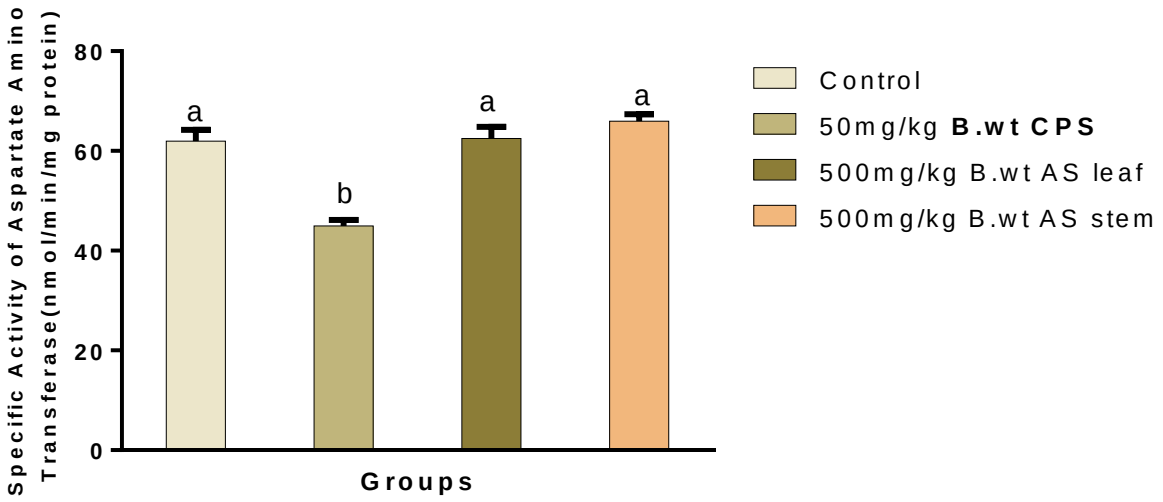

Figure 1: Aspartate Aminotransferase (AST) activity in the liver of animals administered ethanolic extract of *Annonna squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different ($p<0.05$).

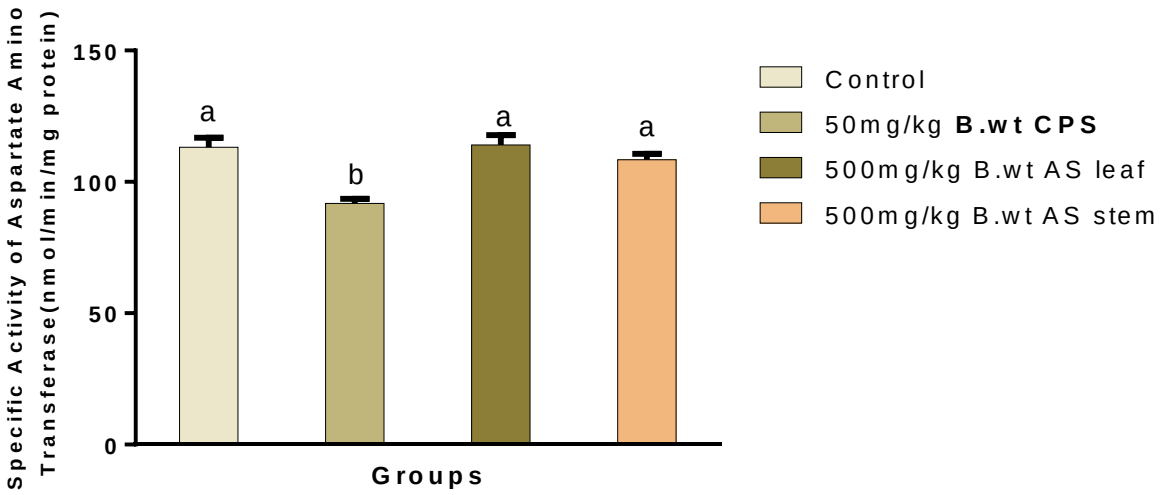

Figure 2: Aspartate Aminotransferase (AST) activity in the kidney of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different ($p<0.05$).

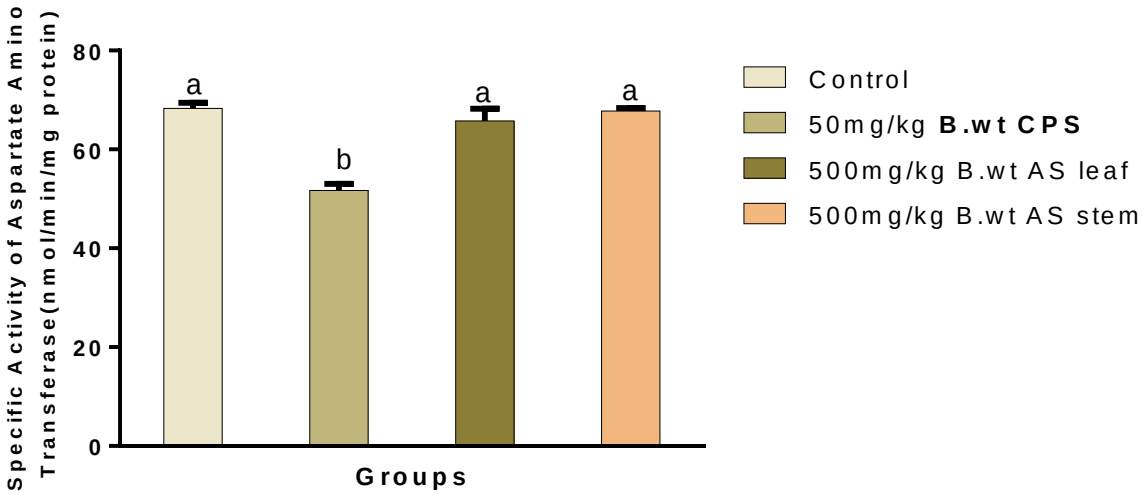

Figure 3: Aspartate Aminotransferase (AST) activity in the heart of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different ($p<0.05$).

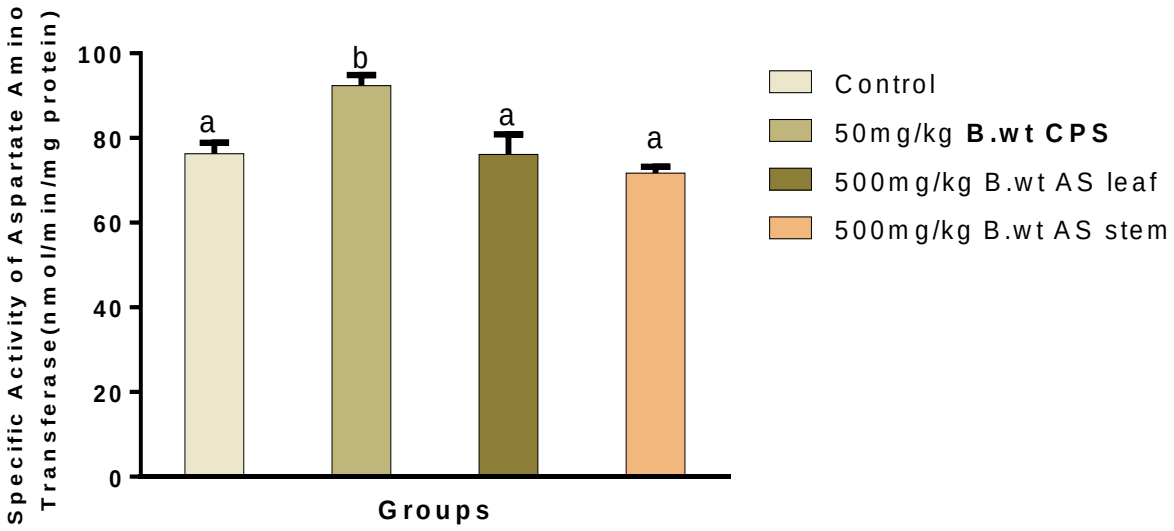

Figure 4: Aspartate Aminotransferase (AST) activity in the serum of animals administered ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different ($p<0.05$).

Alanine Aminotransferase Activity

The specific activity of ALT decreased significantly ($p<0.05$) in the liver, kidney, and heart of the group administered 50mg/kg body weight cyclophosphamide as compared to the control and other test groups (figure 12,13, and 14 respectively). However, there was a significant increase recorded in ALT activity in the serum of the cyclophosphamide group as compared to all other test groups.

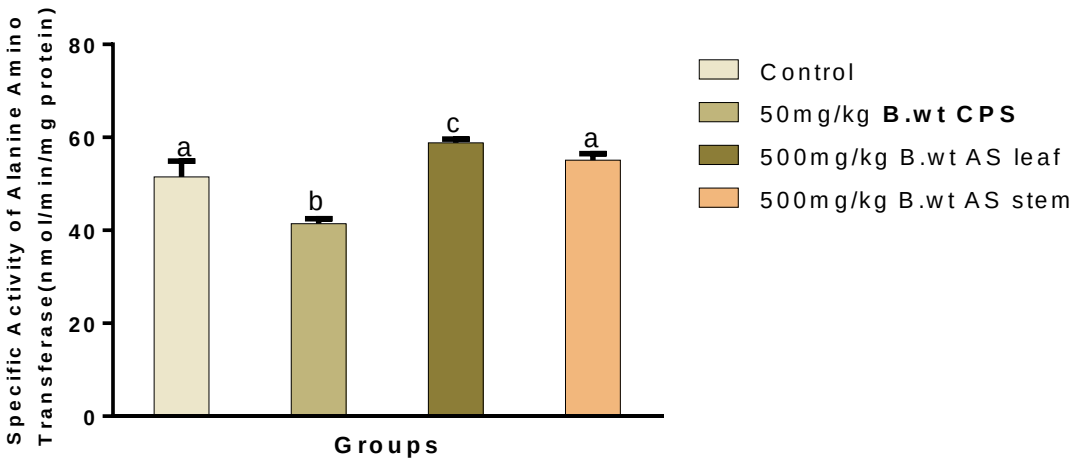

Figure 5: Alanine Aminotransferase (ALT) activity in the liver of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different ($p<0.05$).

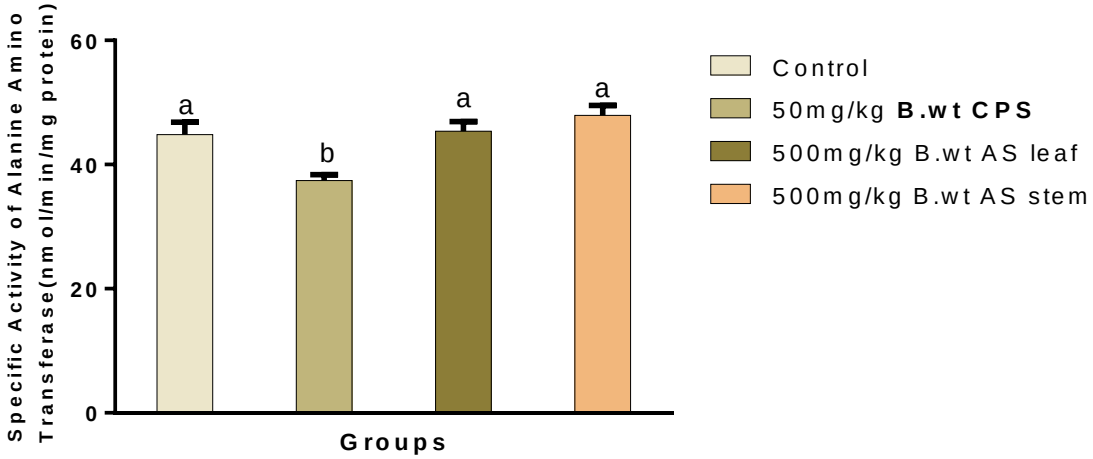

Figure 6: Alanine Aminotransferase (ALT) activity in the kidney of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different ($p>0.05$).

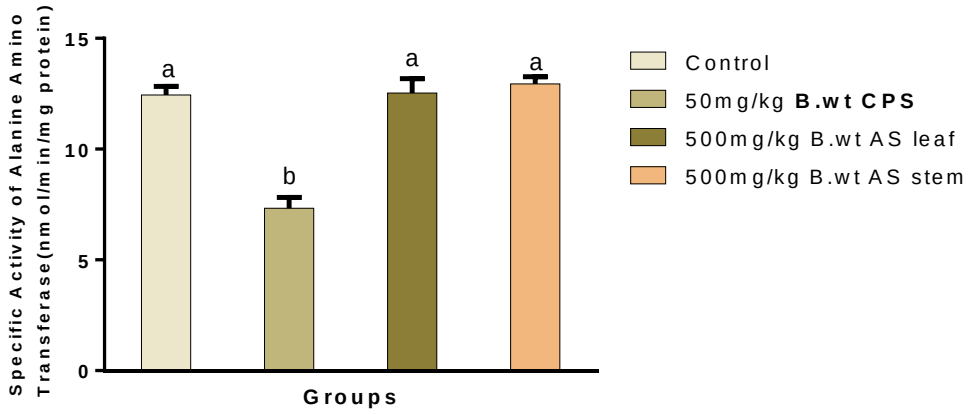

Figure 7: Alanine Aminotransferase (ALT) activity in the heart of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different ($p>0.05$)

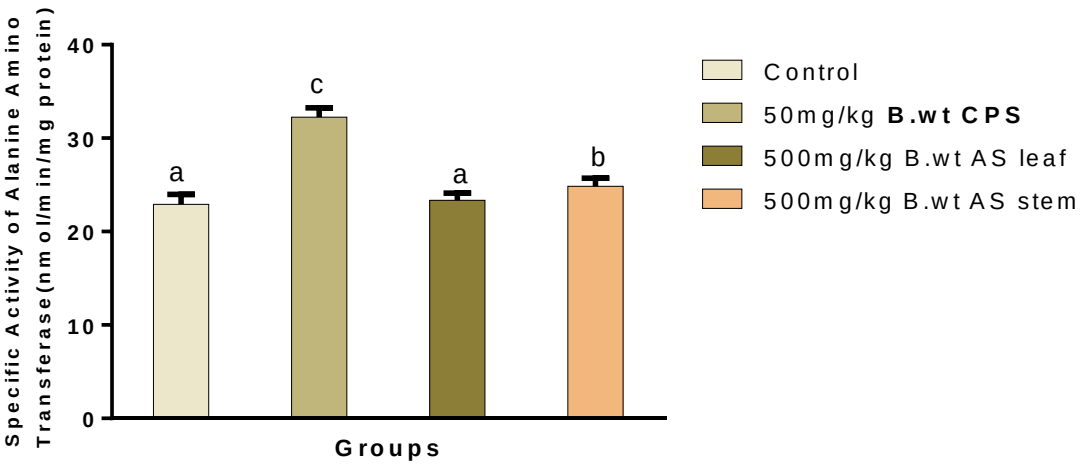

Figure 8: Alanine Aminotransferase (ALT) activity in the serum of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

Alkaline phosphatase activity

Alkaline phosphatase activity was recorded and was found to reduce significantly in the liver, kidney, and heart of the group administered 50mg/kg body cyclophosphamide with a corresponding increase in serum ALP activity as compared to the control and other test groups as shown in Figures 16, 17, 18 and 19 respectively.

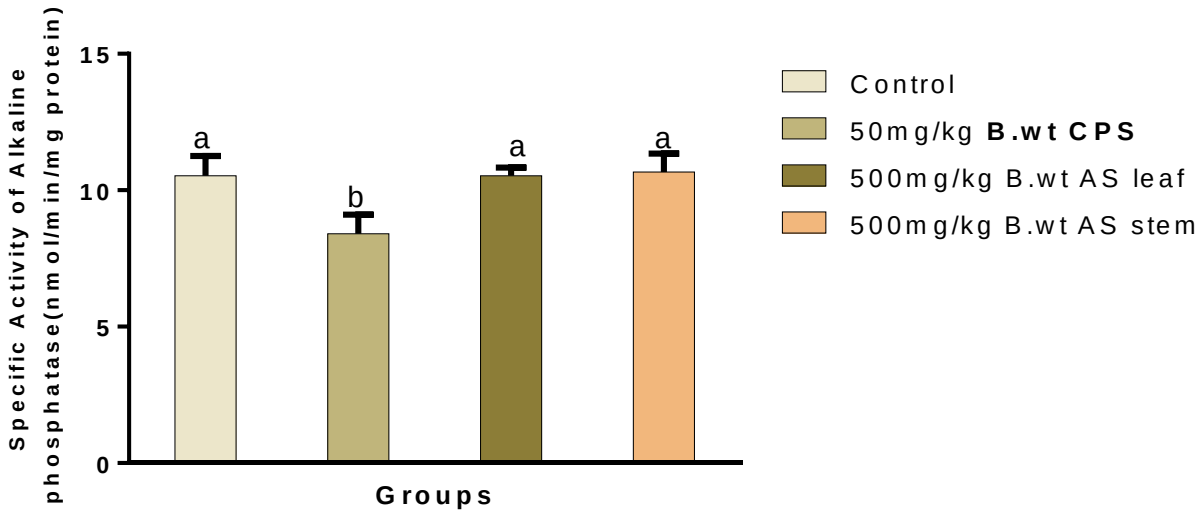

Figure 9: Alkaline phosphatase (ALP) activity in the liver of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

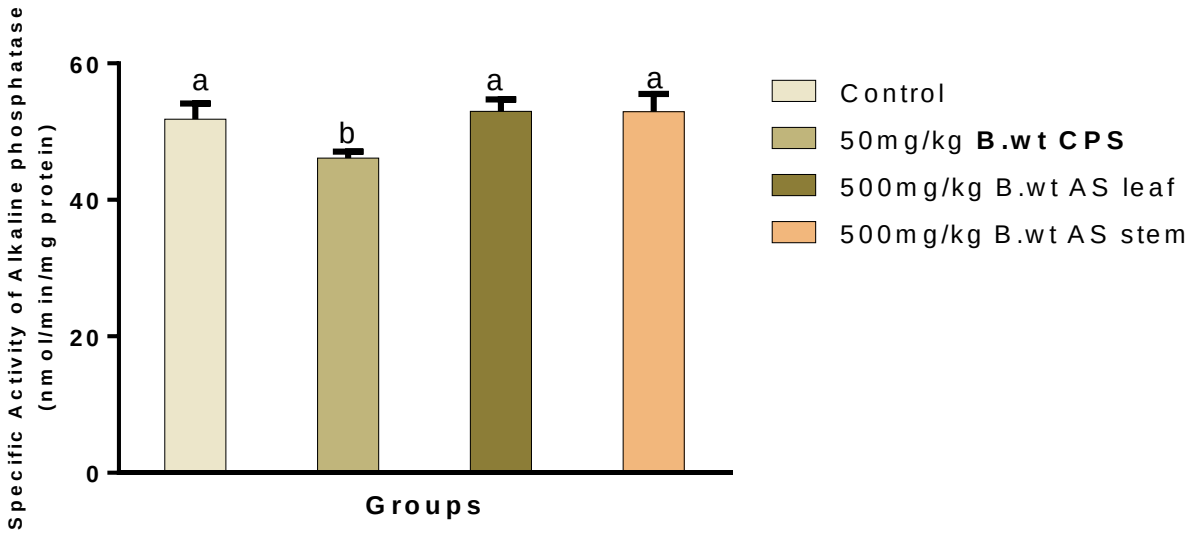

Figure 10: Alkaline phosphatase (ALP) activity in the kidney of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

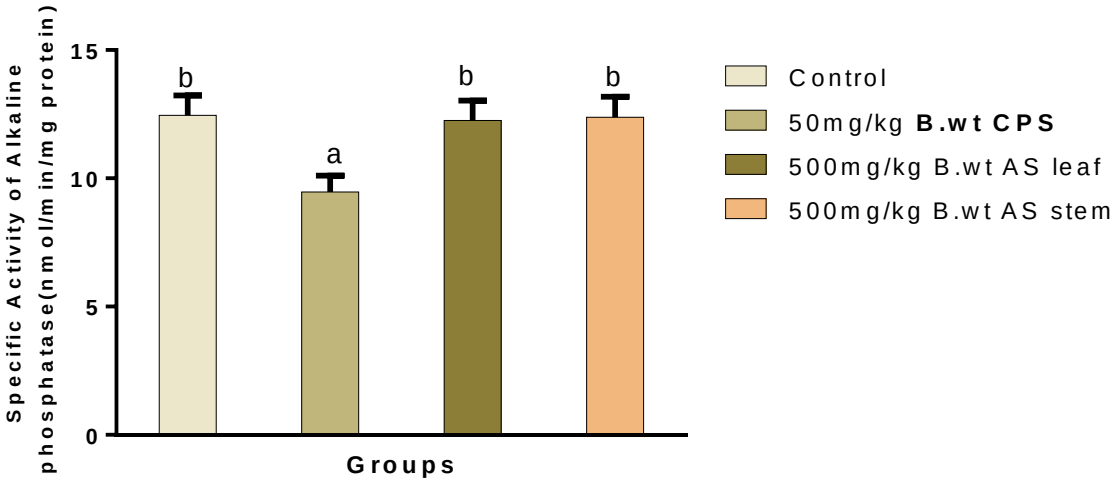

Figure 11: Alkaline phosphatase (ALP) activity in the heart of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

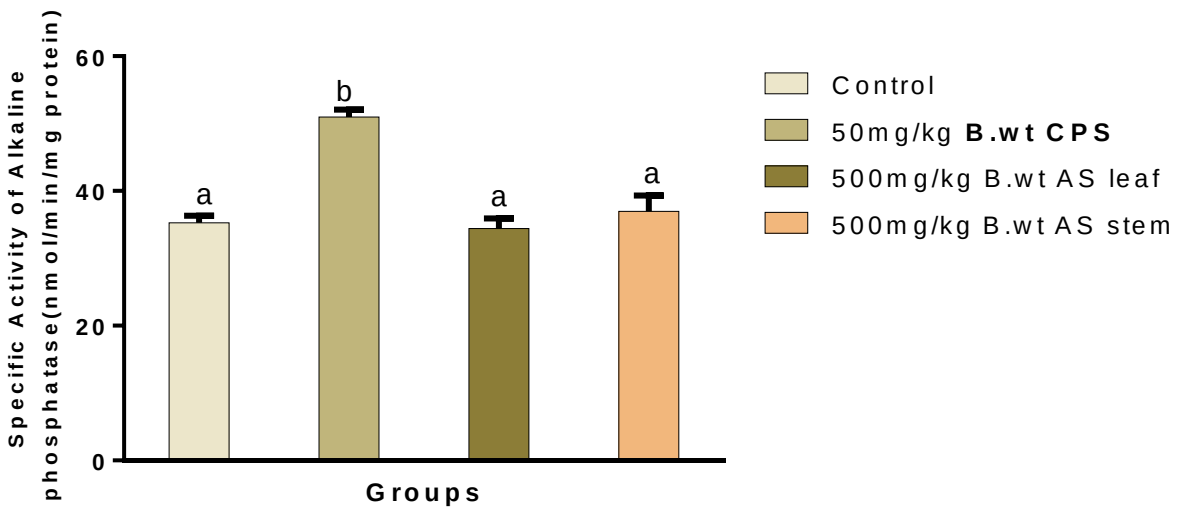

Figure 12: Alkaline phosphatase (ALP) activity in the serum of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

Heart function indices

There was a significant increase (p>0.05) in cholesterol and triacylglycerol concentration in the serum of the group administered 50mg/kg body weight cyclophosphamide as compared to the control and another test group (figure 20 and 21). However, the concentration of HDL reduced significantly (p<0.05) in the reference drug group as compared to other test groups (figure 22).

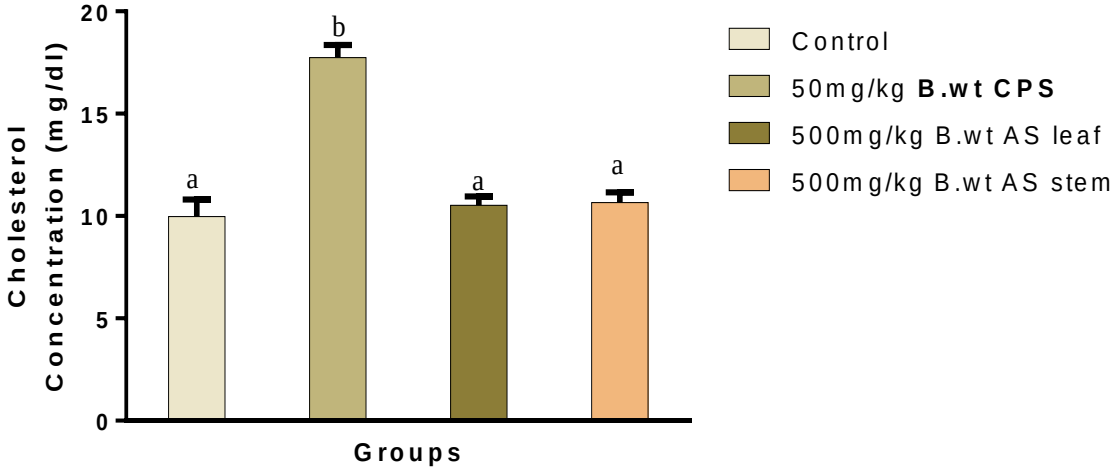

Figure 13: Cholesterol concentration in the serum of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

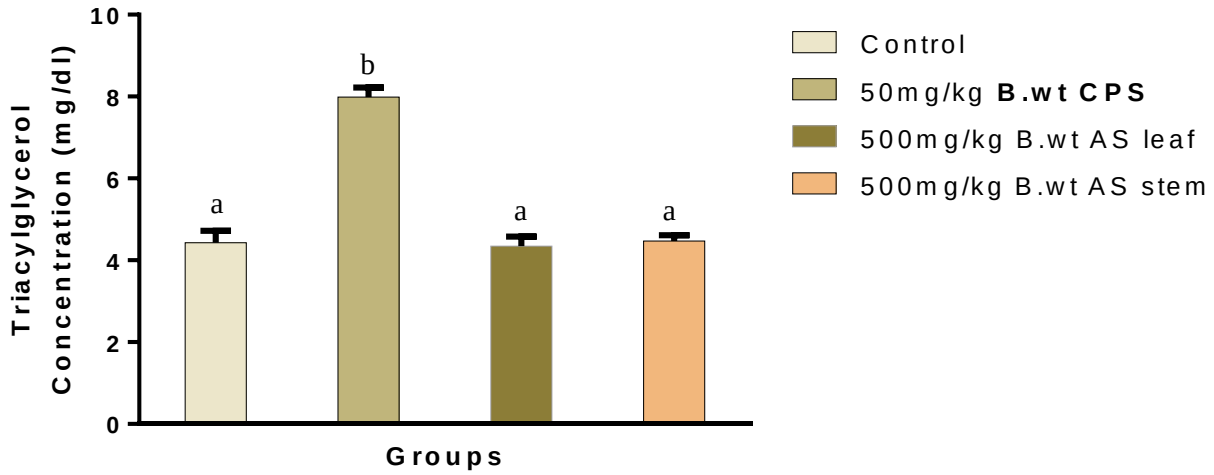

Figure 14: Triacylglycerol concentration in the serum of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

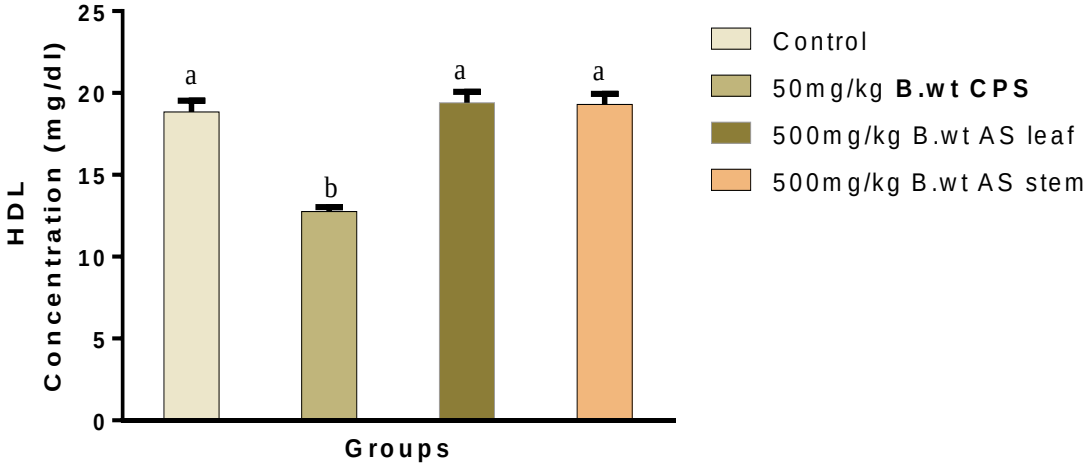

Figure 15: HDL concentration in the serum of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

Liver Function Indices

Total and direct bilirubin concentration as shown in Figures 23 and 24 increased significantly in the group administered 50mg/kg body weight cyclophosphamide as compared to the control and other test groups. Albumin concentration was also recorded and found to reduce significantly in the cyclophosphamide group when compared to other test groups (figure 25).

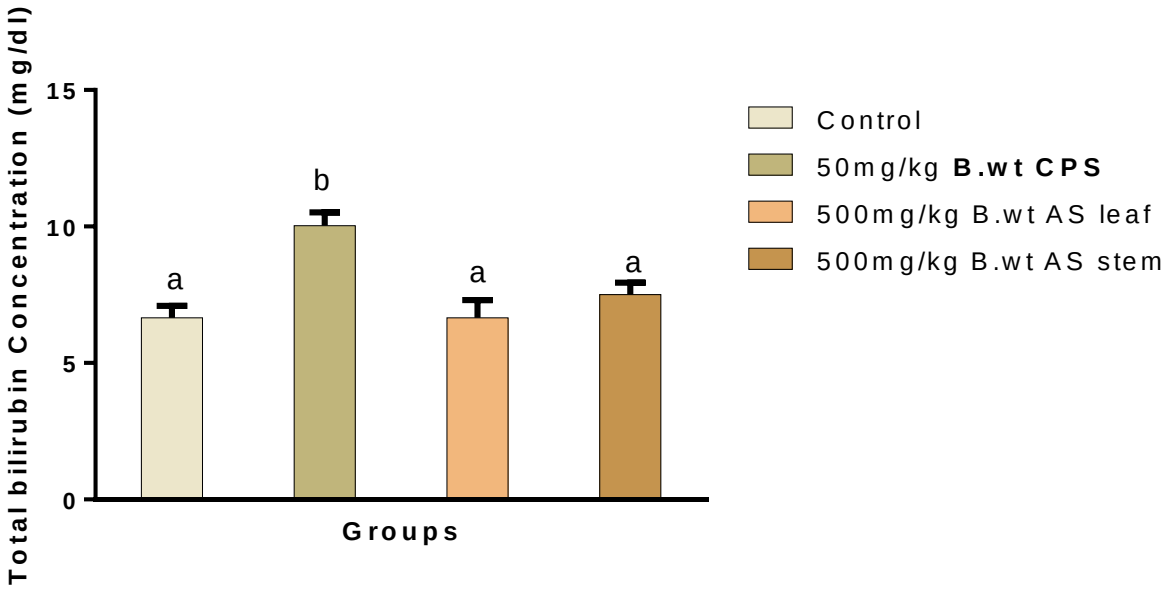

Figure 16: Total bilirubin concentration in the serum of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

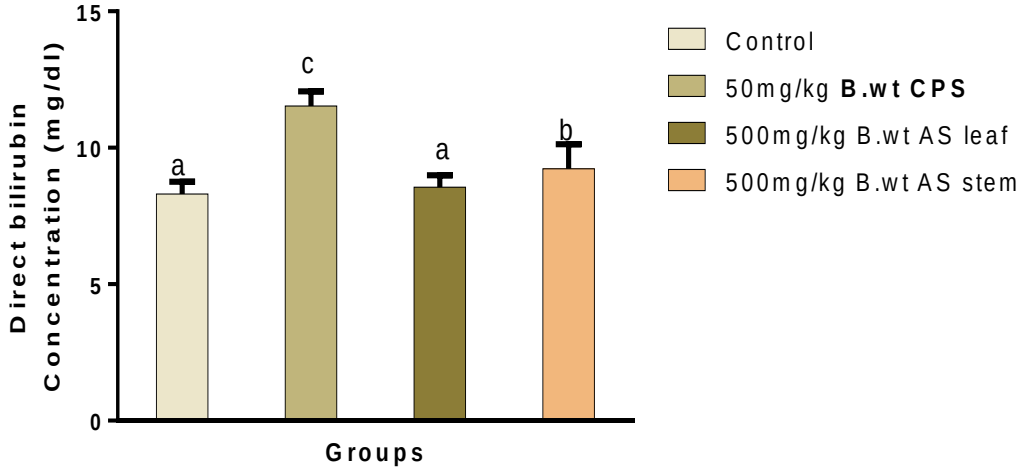

Figure 17: Direct bilirubin concentration in the serum of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

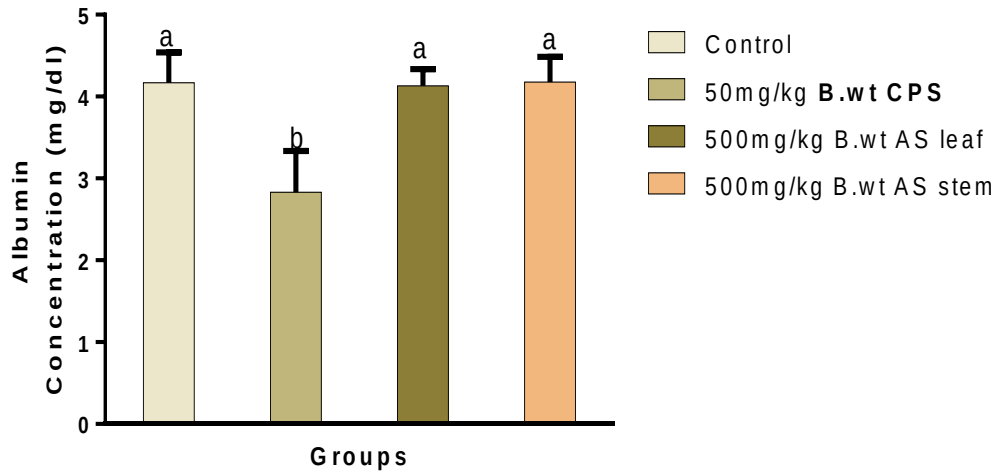

Figure 18: Albumin concentration in serum of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

Kidney function indices

The concentration of urea and creatinine increased significantly (p>0.05) in the group administered 50mg/kg body weight cyclophosphamide as compared to the control and other test groups (Figures 26 and 27).

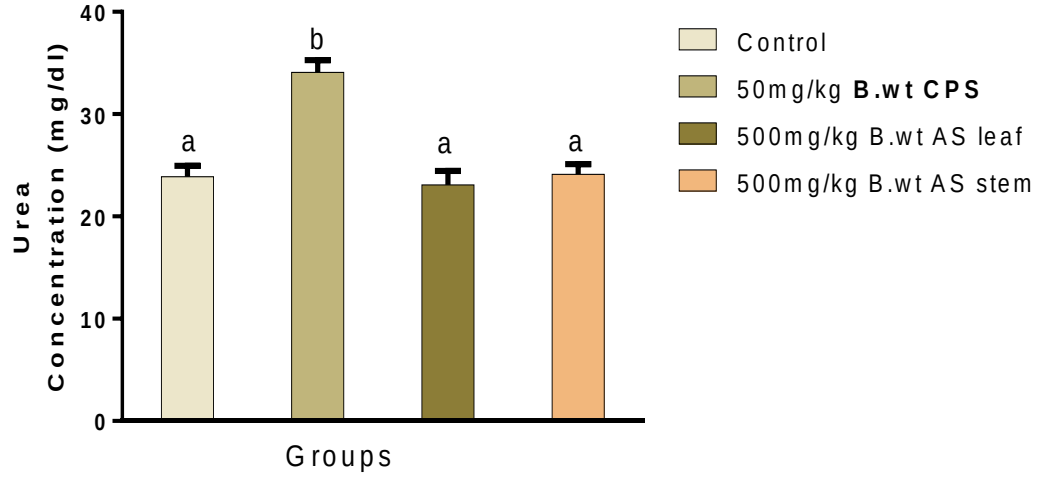

Figure 19: Urea concentration in the serum of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

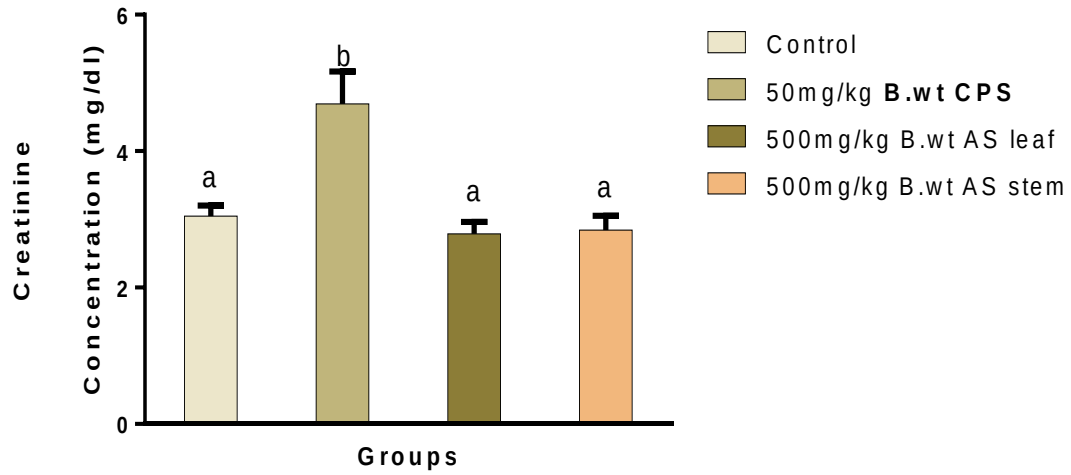

Figure 20: Creatinine concentration in the serum of animals administered Ethanolic extract of *Annona squamosa* leaf and stem. Values are expressed as means of five (5) replicates ± S.E.M. Bars carrying different superscript for each parameter are significantly different (p>0.05).

Discussion

The increase in weight of experimental rats recorded in this study, over a period of four weeks suggests that the ethanolic extract of Annona Squamosa is a good promoter of growth and will most likely contribute to the health of the patients when used locally to manage an ailment that may normally tamper with regular weight or growth.

The non-significant changes in weights recorded for the organs isolated from the experimental rats implied the ethanolic extract of Annona Squamosa is not hepatotoxic, not nephrotoxic neither did it pose any threat to cardiac health. This trend was also recorded in the organ body ratio of the experimental rats used in this study.

Most enzymes are present in cells at much higher concentrations than in plasma. Some occur predominantly in cells of certain tissues, where they may be located in different cellular compartments such as the cytoplasm or the mitochondria. 'Normal' plasma enzyme levels reflect the balance between the rate of synthesis and release into plasma during cell turnover and the rate of clearance from the circulation. The measurement of the activities of the marker enzymes in tissues and body fluid plays a significant and important role in diagnosis, disease investigation, and in the assessment of drug or plant extract for safety/ toxicity risk (Malomo, 2000; Yakubu, 2006; Sulaiman and Ekanem, 2009). The rise in serum ALT, AST, ALP activity in the serum of the cyclophosphamide treated group and a subsequent decrease of these enzymes in the liver, kidney, and heart suggests cellular membrane damage caused by the cytotoxic effect of cyclophosphamide as compared to the A. squamosa test group which maintained the integrity of the cell membrane.

Bilirubin and Albumin are indicators of liver functioning capacity (Xie et al., 2014). Bilirubin is formed by the breakdown of haemoglobin in the spleen, liver, and bone marrow. High levels of conjugated bilirubin in the serum mean the bile is not properly excreted therefore an obstruction may be present in the gall bladder or bile duct. A high level of unconjugated bilirubin means too much haemoglobin is being destroyed or that the liver is not actively treating the haemoglobin it receives. The high concentration of total and direct bilirubin obtained in the cyclophosphamide treated group as compared to the control and other test groups might be as a result of an obstruction in the gall bladder or inability of the liver to process the bilirubin taken up from the blood.

Albumin on the other hand helps to maintain water balance in the serum and also helps to transport a wide range of ligands, e.g. fatty acids, bilirubin, calcium, as well as hormones, such as tyrosine. Low levels of albumin in the serum can result in conditions like analbuminaemia; impaired albumin synthesis in the liver, and kidney disease amongst others. A high level of albumin however has little diagnostic relevance except perhaps in dehydration (Grant et al., 1987). The significant rise

in albumin concentration in all groups except for the cyclophosphamide treated group shows that the synthesis ability of the liver is intact which implies that water balance and hormone transportation in the serum was not distorted during the period of study. The cytotoxic effect of cyclophosphamide on liver cells could be the cause of the low albumin concentration observed in this study.

Plasma creatinine and urea concentrations are kidney function indices (Saad et al., 2006). Creatinine is a waste product of muscle turn over i.e. it is the breakdown product of creatinine phosphate released from skeletal muscle at a steady rate. It is filtered by the renal glomerulus. Generally, it is more sensitive and it is a specific test for renal function than the blood urea nitrogen (BUN). Urea is a waste product produced from the breakdown of protein. Blood urea is removed from the body via the urine, so the BUN level increases when glomerular filtration in the kidney is adversely affected. An increase in urea and creatinine concentration recorded in the cyclophosphamide treatment group is an indication of nephrotoxicity which might be due to the loss of function of the glomerulus.

Hypertriglyceridemia and hypercholesterolemia in combination with abnormally low concentrations of HDL cholesterol (high-density lipoprotein cholesterol) is one of the most common atherogenic profiles of lipid metabolism of high prevalence seen in several populations (Enas and Mehta, 1995; Vijaya Padma et al., 2012). Hyperlipidemia are reported as the major risk factors in lifestyle-related diseases such as atherosclerosis and related cardiovascular complications including cerebral paralysis and myocardial infarction (Chovaneikova and Simek, 2001). The high concentration of triacylglycerol, cholesterol and a subsequent decrease in HDL concentration recorded in the group treated with cyclophosphamide as compared to other groups shows the administration of the drug might pose a potential risk factor to the heart as a result of the accumulation of triacylglycerol and cholesterol in the arterial wall, this can lead to complications related to the heart.

Conclusion

In this research, this plant was able to maintain cellular membrane integrity of tissues of interest as compared to the standard drug used (cyclophosphamide) which has been reported in previous researches, to produce some cytotoxic effect on the cellular membrane.

The lipid profile and toxicological effects of the plant, Annona squamosa also compared favourably with that of the control group in the safety parameters assessed and is thus considered safe for consumption and for use as alternative medicine in the management of ailments as is currently being used locally in Nigeria.

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