

Evaluation of the Toxicological Effects of Ethanolic Extracts of *Annona squamosa* and *Piper nigrum* Plants on Rat

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Abstract

The purpose of this research was to evaluate the overall safety or toxicological effects of ethanolic extracts of *Annona squamosa* (leaves and stems) and *Piper nigrum* (seeds and stems) in healthy Wistar rats. Dried powdered leaves and stem of *Annona squamosa* and *Piper nigrum*'s seed and stem were extracted in ratio 1:5 of 80% ethanol for 24 hours. Thirty male healthy Wistar rats with average weight of 120-150g were randomly distributed into six groups (A-F) of five animals each. 1ml of the extracts equivalent to 500mg/kg body weight were administered to the experimental animals (group C-F) while 1ml of Cyclophosphamide equivalent to 50mg/kg body weight was administered to the reference group (group B) for a period of twenty one (21) days after one week of acclimatization, the control group on the other hand (group A) were administered 1ml of distilled water. The serum and organs (liver, kidney and heart) were collected for lipid profile, liver function indices and kidney function indices. Significant increase ($P < 0.05$) in the serum AST, ALT, ALP level of the group administered with 50mg/kg body weight of Cyclophosphamide and a subsequent decrease of the enzymes level in the liver, kidney and heart was recorded. In addition, there was a significant increase ($p > 0.05$) in the total cholesterol, triacylglycerol, total and direct bilirubin, creatinine and urea concentration with a gradual reduction in the serum HDL and albumin concentration in the reference drug group as compared to control group and the test groups. However, this shows the reduced toxicity of *Annona squamosa* and *Piper nigrum* at 500mg/kg body weight, which is an indication that the plants are safe for human consumption.

Keywords: *Annona squamosa*, *Piper nigrum*, Ethanolic extracts, Stem

Introduction

Medicinal plants are various plants thought by many to possess medicinal properties, but few of these plants or their phytochemical constituents have been proven by rigorous science to have medicinal effects (1). According to WHO survey, 80% populations living in the developing countries rely exclusively on traditional medicine for their primary health care needs of which most involve the use of plant extracts (2,3). *Annona squamosa* and *Piper nigrum* plants have medicinal properties and their pharmacological and therapeutic properties have been established.

The qualitative phytochemical analysis of *Annona squamosa* (4, 5) and *Piper nigrum* (1,6) extracts confirm the presence of various phytochemicals. Phytoconstituents of these plants have been reported for their anti-inflammatory (8, 9, 10), antidiarrheal (11), antimicrobial (12, 13, 14, 15, 16, 17), antioxidant (18, 19, 20, 21, 22, 23, 24) and insecticidal activities (25a), which makes them better and safer than orthodox medicines that usually have some side effects on vital organs (4, 5, 6, 7). However many of these plants are used locally without evaluating their toxicological effects on some vital organs which might cause cellular membrane damage. These two plants fall in this category; they are also used locally to manage cancer but detailed toxicological studies are yet to be conducted on them, hence the target of this research.

Materials and Methods

Dried leaves and stem of *Annona squamosa* and *Piper nigrum*'s seed and stem, were collected and botanically authenticated at the Herbarium unit of the Plant Biology Department, University of Ilorin, Kwara State, Nigeria, where voucher specimens were deposited. The voucher number are UILH/004/1167 and UILH/002/1203 respectively.

Preparation of Extracts

The extracts were prepared as described in a previous study (25b). The dried *Piper nigrum* (seed and stem) were pulverized to powder. *Piper nigrum* seed (85.67g) and *Piper nigrum* stem (70.59g) were successfully extracted in ratio 1:5 of 80% ethanol for 24 hours. Similarly, the dried *Annona squamosa* (leaves and stem) were pulverized to powder. *Annona squamosa* leaves (163.80g), *Annona squamosa* stem (208.34g), were successfully

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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 at room temperature to obtain the final dry extracts.

Animal Groupings: Thirty male healthy Wistar rats with average weight of 120-150g were randomly distributed into six groups (A-F) of five animals each. 1ml of the extracts equivalent to 500mg/kg body weight were administered to the experimental animals (group C-F) while 1ml of Cyclophosphamide equivalent to 50mg/kg body weight was administered to the Cyclophosphamide treated group (group B) and the control group (group A) received 1ml of distilled water. Administration to all groups was done for a period of Twenty one (21) days after first one week of acclimatization. The serum and organs (liver, kidney and heart) were collected for biochemical analysis (Enzyme assay, lipid profile, liver function indices and kidney function indices).

Enzyme Assay

Aspartate Aminotransferase Activity (AST)

The method described by Reitman and Frankel (26) was used in the assay of the activity of aspartate aminotransferase.

Alanine Aminotransferase Activity (ALT)

The method described by Reitman and Frankel (26) was used to assay for the activity of alanine aminotransferase.

Alkaline phosphatase activity (ALP)

The method described by Wright et al. (27) was employed. Test tubes were labeled according to the animal groupings. To each test tube was pipetted 0.01mls test sample followed by 0.05mls of the reconstituted reagent. This was mixed thoroughly and absorbance was taken immediately at a wavelength of 405 nm as well as after 1, 2, and 3 mins. Enzyme activity was calculated using the formula: $U/I = 2760 \times \Delta A_{405} \text{ nm/min}$ Where ΔA = Change in absorbance.

Liver Function Indices

Serum Albumin

The procedure described by Doumas and colleagues (28) was used for the determination of albumin in the serum of the animals. 3.0 ml of reagent 2 was added to test tubes containing 0.01 ml of serum (appropriately diluted). The blank and standard were constituted by replacing the serum with 0.01 ml of distilled water and reagent 1 respectively. The solution was incubated at 37°C for 10 minutes. Absorbance was read spectrophotometrically at 630 nm.

Calculation:

Albumin concentration (g/dl) = $\frac{A_{\text{sample}} \times \text{Concentration of standard}}{A_{\text{standard}}}$ Where Concentration of standard = 4.48 g/dL

Serum Bilirubin

Bilirubin was determined using the method described by Libor (29).

Direct Bilirubin: 0.2 ml of reagent 1, 0.05 ml of reagent 2 and 2.0 ml of 0.9% NaCl were added to 0.2 ml of the sample. The blank was constituted the same way as the test except 0.05 ml of reagent 2. The solution was mixed and incubated for 10 minutes at 25°C. The absorbance of the sample was read at 546 nm against the sample blank.

Total Bilirubin: 0.2 ml of reagent 1, 0.05 ml of reagent 2 and 1.0 ml of reagent 3 were added to 0.2ml of the sample. The blank was constituted the same way as the test except 0.05 ml of reagent 2. The solution was mixed and incubated for 10 minutes at 25°C. Then, 1.0 ml of reagent 4 was added to the solution (including the blank, this was mixed and incubated again for 15 minutes at 25°C. The absorbance of the sample was read at 578 nm against the sample blank.

Calculation: Total bilirubin (mg/dl) = $10.8 \times \text{ATB}$
Conjugated bilirubin (mg/dl) = $14.4 \times \text{ADB}$

Where; ATB = Absorbance of total bilirubin and ADB = Absorbance of direct bilirubin

Triglycerides

The triglycerides are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from Hydrogen Peroxide, 4- aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase

Procedure: 500µl of reagent 1 was added to 5µl of the sample. The blank and standard were constituted the same way as the test except adding 5µl of Distilled water and 5µl of the standard respectively to each 500µl of reagent 1. The solution were mixed and incubated for 10 minutes at 25°C. The absorbance of the sample was read at 578 nm against the sample blank.

Kidney Function Indices

Serum Urea

The method used for the determination of urea in the serum of the animals was as described by Veniamin and Vakirtzi (30).

Serum Creatinine

This was done using the method described by Bartels and Bohmer (31).

Statistical Analysis

Data were expressed as the means $\pm$ SEM of 5 determinations. Statistical analysis was performed using graph pad. The data were considered statistically significant at $P < 0.05$.

Results

Enzyme activities

Aspartate Aminotransferase

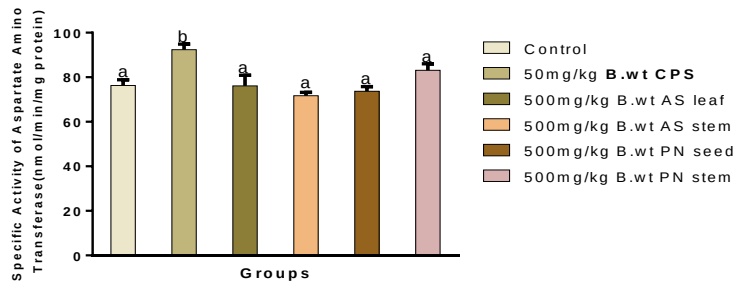


Figure 1: Aspartate Aminotransferase (AST) activity in the serum of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

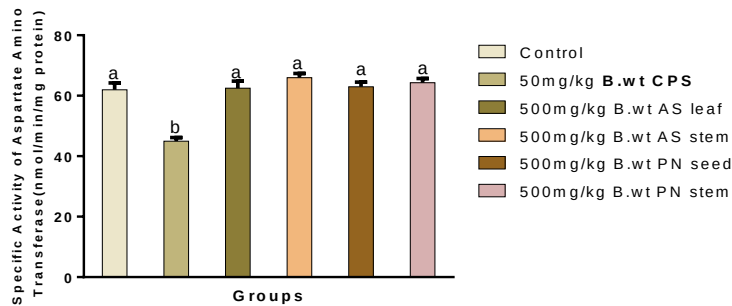


Figure 2: Aspartate Aminotransferase (AST) activity in the liver of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

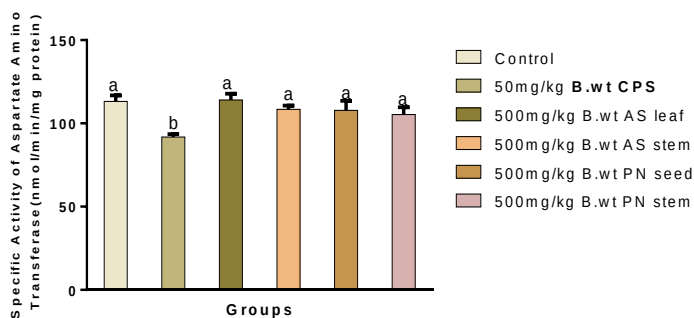


Figure 3: Aspartate Aminotransferase (AST) activity in the kidney of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

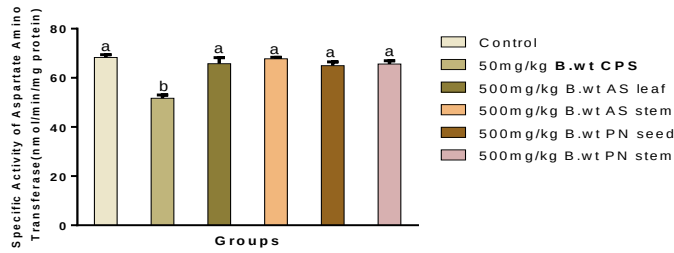


Figure 4: Aspartate Aminotransferase (AST) activity in the heart of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

Alanine Aminotransferase Activities

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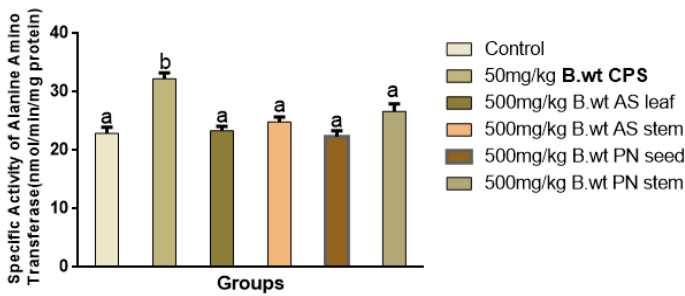


Figure 5: Alanine Aminotransferase (ALT) activity in the serum of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

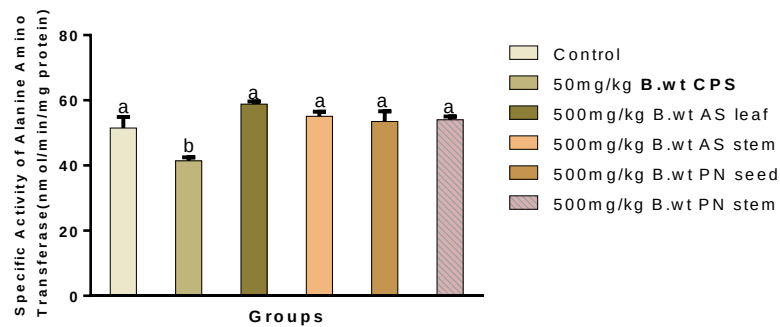


Figure 6: Alanine Aminotransferase (ALT) activity in the liver of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

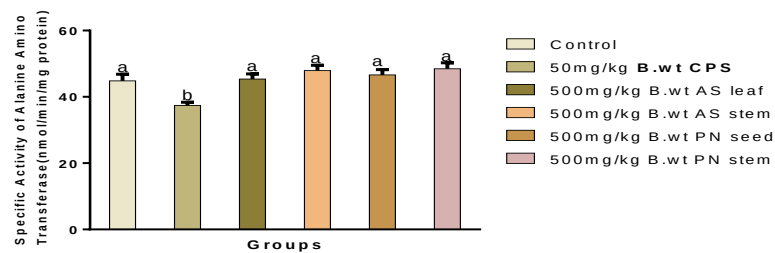


Figure 7: Alanine Aminotransferase (ALT) activity in the kidney of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

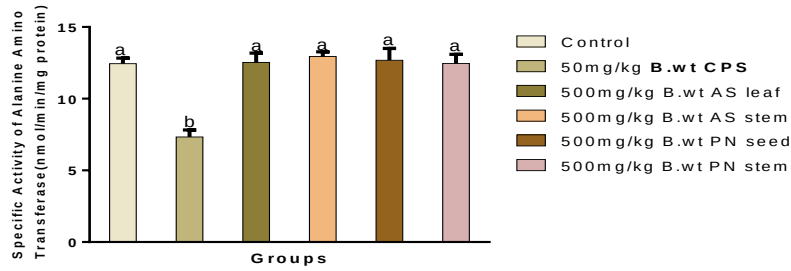


Figure 8: Alanine Aminotransferase (ALT) activity in the heart of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

Alkaline phosphatase Activities

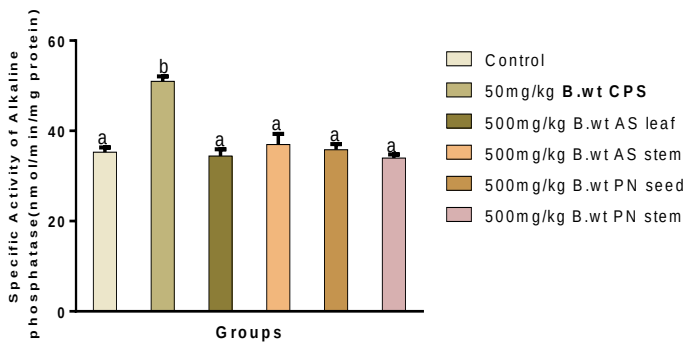


Figure 9: Alkaline phosphatase (ALP) activity in the serum of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

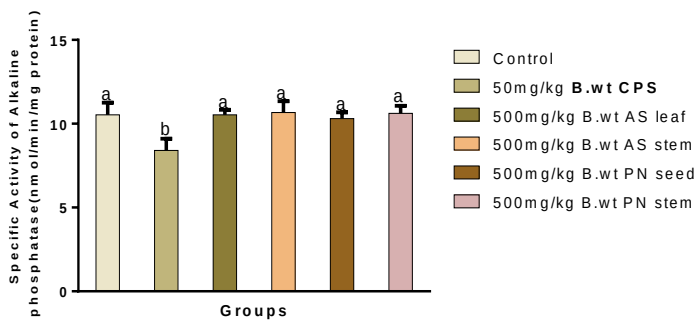


Figure 10: Alkaline phosphatase (ALP) activity in the liver of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

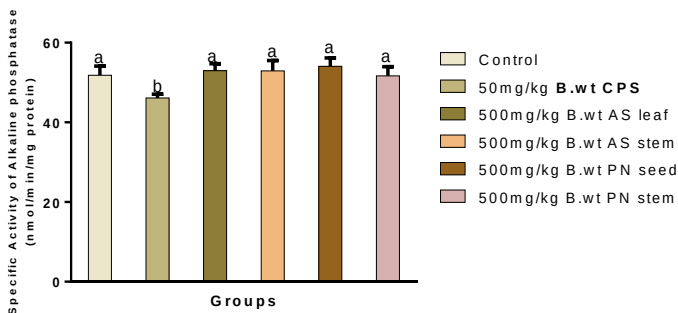


Figure 11: Alkaline phosphatase (ALP) activity in the kidney of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

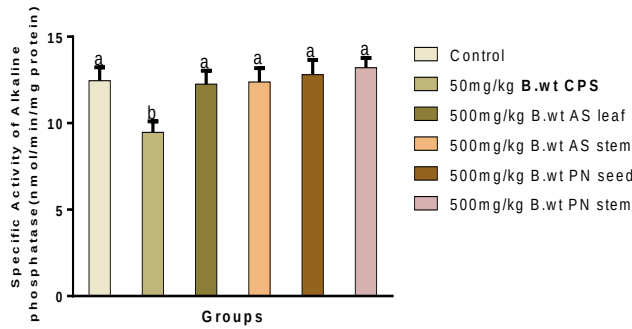


Figure 12: Alkaline phosphatase (ALP) activity in the heart of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

Lipid Profile

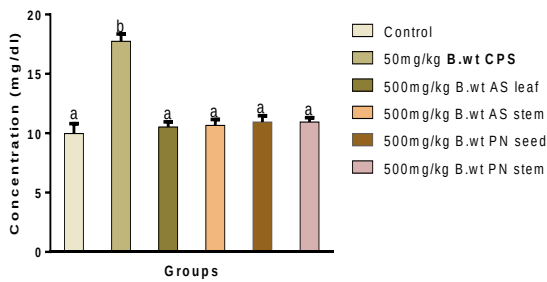


Figure 13: Cholesterol concentration in the serum of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

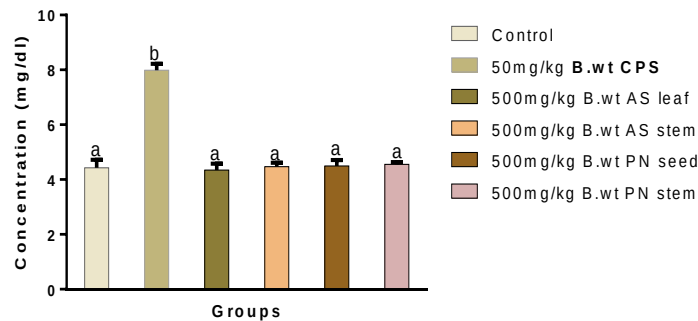


Figure 14: Triacylglycerol concentration in the serum of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

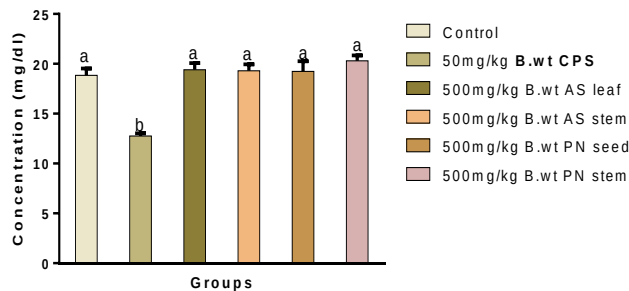


Figure 15: HDL concentration in the serum of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

Liver Function Indices

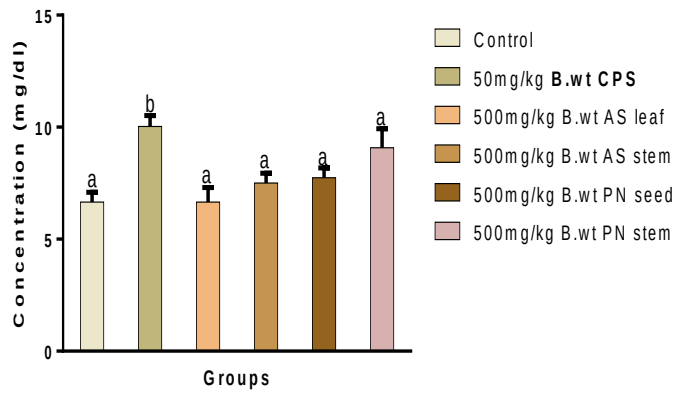


Figure 16: Total bilirubin concentration in the serum of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

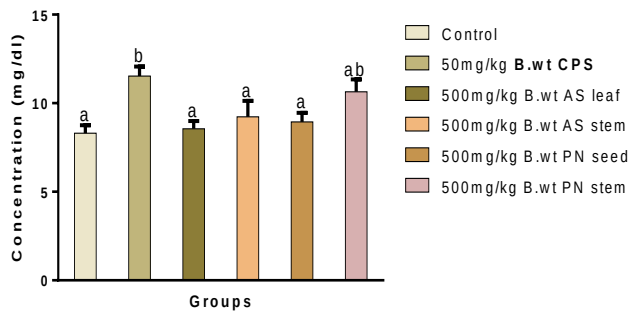


Figure 17: Direct bilirubin concentration in the serum of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

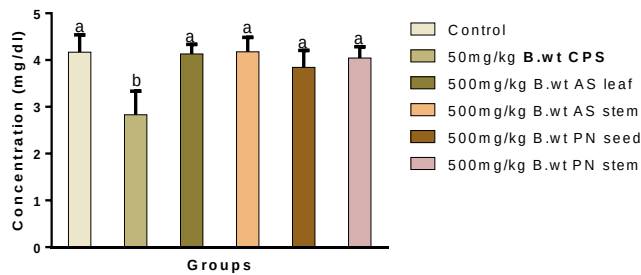


Figure 18: Albumin concentration in the serum of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

Kidney function indices

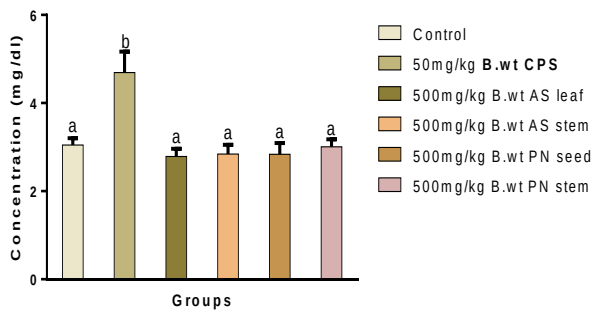


Figure 19: Creatinine concentration in the serum of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

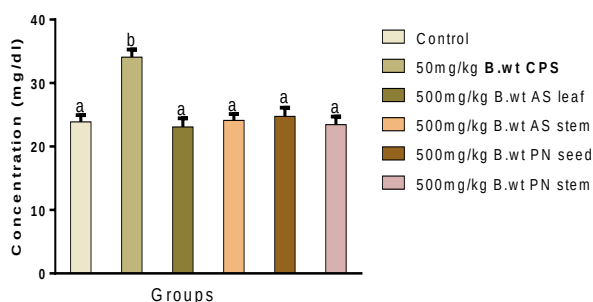


Figure 20: Urea concentration in the serum of animals administered with extracts of *Annona squamosa* and *Piper nigrum*

Discussion

Cyclophosphamide is a cancer drug (33), they are DNA alkylating agents. These anticancer pro-drugs are metabolized by two alternative cytochrome P450 (P450) pathways, drug activation by 4-hydroxylation and drug inactivation by N-dechloroethylation, which generates the neurotoxic and nephrotoxic by product chloroacetaldehyde (32). However, these activated metabolites also gain entry to normal body tissues, where they may induce host toxicity such as hepatotoxicity and nephrotoxicity (33).

There has been a growing awareness in recent years that dietary non-nutrient compounds can have important effects as chemopreventive agents (34, 35), and considerable work on the cancer chemopreventive effects of such compounds in animal models which has been undertaken (32). A number of common medicinal plants have good antioxidant properties and therefore may act as chemoprotector and radioprotector (34, 35). The antioxidant, hepatoprotective and anticancer properties of *A. squamosa* and *P. nigrum* have very well been established (32, 36) but not its toxicological effect.

From the result obtained there was significant rise in the serum AST, ALT, ALP level of the group treated with Cyclophosphamide and a subsequent decrease of the enzymes level in the liver, kidney and heart. This rise in the serum AST, ALT, ALP level may suggest cellular membrane damage caused by the cytotoxic effect of Cyclophosphamide as compared to the *Annona squamosa* and *Piper nigrum* extracts which maintained the integrity of the cell membrane.

While a significant increase ($p > 0.05$) was recorded in the total cholesterol, triacylglycerol, total bilirubin, direct bilirubin, creatinine and urea concentration in the serum of the group administered 50mg/kg body weight cyclophosphamide as compared to the control and other test groups. However, the concentration of HDL and albumin reduced significantly ($p < 0.05$) in the reference drug group as compared to control group and the groups administered 500mg/kg body weight of the extracts were observed. This shows enhanced protective activity (hepatoprotective, nephroprotective and cardioprotective effect) and reduced toxicity of *Annona squamosa* and *Piper nigrum* at 500mg/kg body weight, which is an indication that the plants are safe for human consumption.

Enzyme Activities

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 play a significant and important role in diagnosis, disease investigation and in the assessment of drug or plant extract for safety/ toxicity risk (37, 38, 39).

From the result obtained there was significant rise in the serum AST, ALT, ALP level of the group treated with Cyclophosphamide and a subsequent decrease of the enzymes level in the liver, kidney and heart. This rise in the serum AST, ALT, ALP level may suggest cellular membrane damage caused by the cytotoxic effect of Cyclophosphamide as compared to the *Annona squamosa* and *Piper nigrum* extracts which maintained the integrity of the cell membrane.

Lipid Profile

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 (20, 35). Hyperlipidemia is reported as the major risk factor in life style related diseases such atherosclerosis and related cardiovascular complications including cerebral paralysis and myocardial infarction (40, 41).

The extracts reduced the triacylglycerol and the total cholesterol with a gradual increase in the HDL cholesterol level as compared to the group treated with Cyclophosphamide. The high concentration of triacylglycerol,

cholesterol and a subsequent decrease in HDL concentration observed in the group treated with cyclophosphamide as compared to other groups shows the administration of the drug might have posed a potential risk factor to the heart as a result of accumulation of triacylglycerol and cholesterol in arterial wall, this can lead to complications related to the heart.

Liver Function Indices

Bilirubin and Albumin are indicators of liver functioning capacity (28, 29, 40). Bilirubin is formed by the breakdown of haemoglobin in the spleen, liver and bone marrow. High levels of conjugated bilirubin means the bile is not properly excreted therefore an obstruction may be present in the gall bladder or bile duct. High levels of unconjugated bilirubin mean too much haemoglobin is being destroyed or that the liver is not actively treating the haemoglobin it receives.

There was a high serum concentration of the total and direct bilirubin in the group treated with Cyclophosphamide as compared to the groups treated with the extracts. This high concentration of the total and direct bilirubin obtained in Cyclophosphamide treated group as compared to the control and other test groups may suggest an obstruction in the gall bladder or inability of the liver to process Haemoglobin 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; the reduction may also be as a result of impaired albumin synthesis in the liver, and kidney disease amongst others. High levels of albumin however have little diagnostic relevance except perhaps in dehydration (28).

A rise in the albumin concentration in all the groups except for the Cyclophosphamide treated group was observed. The significant rise in albumin concentration in all groups except for the cyclophosphamide treated group may signify 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 for the low albumin concentration observed in this study.

Kidney Function Indices

Plasma creatinine and urea concentrations are kidney function indices (31). Creatinine is a waste product of muscle turn over i.e. it is the breakdown product of creatine phosphate released from skeletal muscle at a steady rate. It is filtered by the renal glomerulus. Generally it is more sensitive and 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 BUN level increases when glomerular filtration in the kidney is adversely affected.

Increase in urea and creatinine concentration in the Cyclophosphamide treated group as compared to the groups treated with the extracts was observed. The significant increase in the cyclophosphamide treatment group may be an indication of nephrotoxicity which might be due to the loss of function of the glomerulus.

Conclusion

Annona squamosa and *Piper nigrum* are tropical fruit and spice respectively on which extensive phytochemical and bioactive investigations have been implemented. From the detailed literatures survey done, they were found to contain anticancer and antioxidant activities and are considered as chemo-protector and radio-protector due to the presence of alkaloid, flavonoid.

The result of this study shows enhanced protective activity (hepato-protective, nephron-protective and cardio-protective effects) and reduced toxicity of *Annona squamosa* and *Piper nigrum* at 500mg/kg body weight, which is an indication that the plants are safe for human consumption as it is currently being used locally.

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References

- 1). Ganesh P, Suresh Kumar R and Saranraj P. Phytochemical analysis and antibacterial activity of Pepper (*Piper nigrum* L.) against some human pathogens. *Central European Journal of Experimental Biology*, 3 (2):36-41. 2014.
- 2). Neha P and Dushyant. Phytochemical and Pharmacological Review on *Annona squamosa* Linn. *International Journal of Research in Pharmaceutical and Biomedical Sciences*; 2 (4), 1404-1412. 2011
- 3). Sandhya B, Thomas S, Isbael R. Complementary and alternative medicines; 3:110-114. 2006.
- 4). Anshuman Bhattacharya. Phytochemical and pharmacological evaluation of the seed of *Annona squamosa* Lin., *Int J of Pharmacy and Pharmeetical Sci*; 4(3)9294. 2012
- 5). Bhakuni DS, Tewari S, Dhar MM. Aporphine alkaloids of *Annona squamosa*. *Phytochemistry*; 11: 1819-1822. 1972.

- 6). Doucette CD, Hilchie AL, Liwski R, Hoskin DW. Piperine, a dietary phytochemical, inhibits angiogenesis. See comment in PubMed Commons below *J NutrBiochem*; 24: 231-239. 2013.
- 7). Forgacs P, Descondois JF, Provost R, Tiberghien et Touche A. Un Nouvel Heteroside Nitre Extrait D' *Annona squamosa*. *Phytochemistry*; 19: 1251-125. 1980.
- 8). Alitonou GA, Tchobo FP, Sessou P, Avlessi F, Menut. Chemical composition, antiradical and anti-inflammatory activities of four Annonaceae from Benin. *Int J Pharm Chem Biol Sc;i* 3:9 14-923 2013.
- 9). Bang JS, Oh da H, Choi HM, Sur BJ, Lim SJ. Anti-inflammatory and antiarthritic effects of piperine in human interleukin 1 β -stimulated fibroblast-like synoviocytes and in rat arthritis models. See comment in PubMed Commons below *Arthritis Res Ther* 11: R49. 2009.
- 10). Chavan MJ, Wakte PS, Shinde DB. Analgesic and anti-inflammatory activity of Caryophyllene oxide from *Annona squamosa* L. bark. *Phytomedicine* 17,149–151. 2010.
- 11). Shamkuwar PB, Shahi SR, Jadhav ST. Evaluation of antidiarrhoeal effect of Black pepper (*Piper nigrum* L). *Asian Journal of Plant Science and Research* 2:48-53. 2012.
- 12). Dorman HJD and SG Deans. "Antimicrobial agents from plants: antibacterial activity of plant volatile oils". *Journal of Applied Microbiology* 88 (2): 308–16. 2000.
- 13). Jayshree D Patel and Vipin Kumar. *Annona squamosa* L. Phytochemical analysis and Antimicrobial Screening, *Journal of Pharmacy Research*, 1(1), 34-38. 2008.
- 14). Khan M, Siddiqui M. Antimicrobial activity of *Piper* fruits. *Nat prod Rad* 6:111-113 2007.
- 15). Kulkarni VR and Chandrashekar C. Evaluation of Antimicrobial activity of *Annona squamosa* L., *J of Pharm and Biomedical Res*; 1(1):12-17. 2011.
- 16). Mukhlesur Rahman M, Shahnaj Parvin, Ekramul Haque M, Ekramul Islam M, Mohammad A. Mosaddik B. Antimicrobial and cytotoxic constituents from the seeds of *Annona squamosa* . *Fitoterapia*; 76: 484–489. 2005.
- 17). Patel D J, Kumar V. *Annona squamosa* L. Phytochemical analysis and Antimicrobial Screening. *J. of Pharmacy Res*;1(1): 34-38. 2008.
- 18). Agbor GA, Akinfiresoye L, Sortino J, Johnson R, Vinson JA. *Piper* species protect cardiac, hepatic and renal antioxidant status of atherogenic diet fed hamsters. *Food Chem*: 134: 1354-1359. 2012.
- 19). Ahmad N, Fazal H, Abbasi BH, Rashid M, Mahmood T, Fatima N. Efficient regeneration and antioxidant potential in regenerated tissues of *Piper nigrum* L. *Plant Cell, Tissue and Organ Culture. Plasma Res*; 102:129-134. 2010.
- 20). Gupta R. K, Achyut Narayan Kesari, Sandhya Diwakarc, Ameetabh Tyagia, Vibha Tandon, Ramesh Chandraa, Geeta Watal. *In vivo* evaluation of anti-oxidant and anti-lipidemic potential of *Annona squamosa* aqueous extract in Type 2 diabetic models. *Journal of Ethnopharmacology* 118, 21–25. 2008.
- 21). Nahak G and Sahu R.K. Phytochemical Evaluation and Antioxidant activity of *Piper cubeba* and *Piper nigrum*. *Journal of Applied Pharmaceutical Science*; 01 (8):153-157. 2011.
- 22). Shahidi F. Antioxidant factors in plant foods and selected oilseeds. *BioFactors*; 13: 179-185. 2000.
- 23). Shirwaikar A, Rajendran K, Kumar C.D, Bodla R. *In vitro* antioxidant studies of *Annona squamosa* Linn. Leaves, *Indian J Exp Biol*; (42): 803-7. 2004.
- 24). Vijayakumar RS, Surya D, Nalini N. Antioxidant efficacy of black pepper (*Piper nigrum* L.) and piperine in rats with high fat diet induced oxidative stress. *Redox Rep* 9: 105-110. 2004.
- 25a). Scott I M, Jensen H R, Philogene B J R, Arnason J T. A review of *Piper* spp. (Piperaceae) phytochemistry, insecticidal activity and mode of action. *Phytochemistry Reviews*, 7, 65-75. 2008.
- 25b). Sumithra P, Gricilda SF, Vimala G, Sathya J, Sankar V, Saraswathi R, Jayapriya G: Anti- cancer activity of *Annona squamosa* and *Manilkara zapotab* flower extract against MCF-7 cell line. *Der Pharmacia Sinica*; 5 (6) 98-104. 2014.
- 26). Reitman, S. and Frankel, S. A colorimetric method for determination of serum glutamate-oxaloacetate and pyruvate transaminase. *American Journal of Clinical Pathology* 28: 56-59. 1957
- 27). Wright P. J, Leathwood P. D, and Plummer, DT. Enzymes in rat urine. Alkaline phosphatase. *Enzymologia* 42: 317-327. 1972
- 28). Doumas BT, Watson WA, Biggs HG:. Albumin standards and measurement of serum albumin with bromocresol green. *Clinical Chimica Acta* 31: 87-92. 1971.
- 29). Libor NL: Impact of Serum Bilirubin on Human Diseases. *Pediatrics* 115: 1411-1412. Mayne, P. D. *Clinical Chemistry in Diagnosis and Treatment*. 6th Edition. Arnold International students' Edition, Pp. 196-205. 2005.
- 30). Veniamin, M. P. and Varkirtzi, C. Chemical basis of the carbamidodi-acetyl micro method for estimation of urea, cirulline and carbamyl derivatives. *Clinical Chemistry* 163: 3-6. 1970.
- 31). Bartels, H. and Bohmer, M. Kinetic determination of creatinine concentration. *Clinica Chimica Acta* 37: 193-197. 1972.

- 32). Agarwal S, Yadav RN, Garg G, Kumar M: Renal cell carcinoma with level 2 IVC thrombus. *BMJ* 10: 2018.
- 33). Chong-Sheng C, Lin JT, Goss K, Waxman DJ: Activation of the Anticancer Prodrugs Cyclophosphamide and Ifosfamide: Identification of Cytochrome P450 2B enzymes and site-specific mutants with improved enzyme kinetics. *Molecular Pharmacology* 65(5): 1278-85. 2004.
- 34). Manoharan S, Balakrishnan S, Menon V, Alias L, Reena A. Chemopreventive efficacy of curcumin and piperine during 7,12-dimethylbenz[a] anthracene-induced hamster buccal pouch carcinogenesis. *Singapore Med J* 50:139-46. 2009.
- 35). Selvendiran K, Sakthisekaran D. Chemo-preventive effect of piperine on modulating lipid peroxidation and membrane bound enzymes in benzo(a) pyrene induced lung carcinogenesis. See comment in PubMed Commons below *Biomed Pharmacother* 58: 264-267. 2004
- 36). Zoheir AD and Aftab A. A Review on Therapeutic Potential of *Piper nigrum L.* (Black Pepper): The King of Spices. *Medicinal & Aromatic Plants*; 3 (3): 2-6. 2014.
- 37). Adeyemi, OS & Sulaiman, FA. Biochemical and morphological changes in *Trypanosoma brucei brucei*-infected rats treated with homidium chloride and diminazene aceturate. *Journal of Basic and Clinical Physiology and Pharmacology*. (23)4:179-183. Doi: 10.1515/jbcpp-2012-0018. Available online at <http://www.degruyter.com/view/j/jbcpp.2012.23.issue-4/jbcpp-2012-0018/jbcpp-2012-0018.xml> (ISI/SCOPUS) 2012.
- 38). Sulaiman AF, Oloyede HOB, Akanji MA, Akinyele TJ & Dosunmu KO: GC-MS analysis of bioactive fractions of *Terminalia avicennoides* and *Bombax buopodezense* bark and lipid profile of *Trypanosoma brucei* infected Wistar rats. *African Scientist*. A NISEB Publication. 17:4 pg. 307-324. Available online at <http://www.niseb.org/afs>. 2017
- 39). Sulaiman AF, Adeyemi OS, Akanji MA et al.: Biochemical and morphological alterations caused by silver nanoparticles in wistar rats. *Journal of Acute Medicine* 5 (4) 96 -102. [http://www.e-jacme.com/article/S2211-5587\(15\)00094-1/pdf](http://www.e-jacme.com/article/S2211-5587(15)00094-1/pdf) (ISI/SCOPUS) 2015.
- 40). Busher JB. Serum Albumin and Globulin. *Clinical Methods: The History, Physical, and Laboratory Examinations*. 3rd edition. Boston: Butterworths; 1990. Chapter 101. 1990.
- 41). Saravanan G, Ponmurugan P. Ameliorative potential of S-allylcysteine: Effects on lipid profile and changes in tissue fatty acid composition in experimental diabetes. *Exp Toxicol Pathol*; 64: 639-44. 2012.
- 42). Jacobson TA, Ito MK, Maki KC. National Lipid Association recommendations for patient-centered management of dyslipidemia: Part 1-executive summary. *J Clin Lipidol*; 8(5):473-488. 2014.