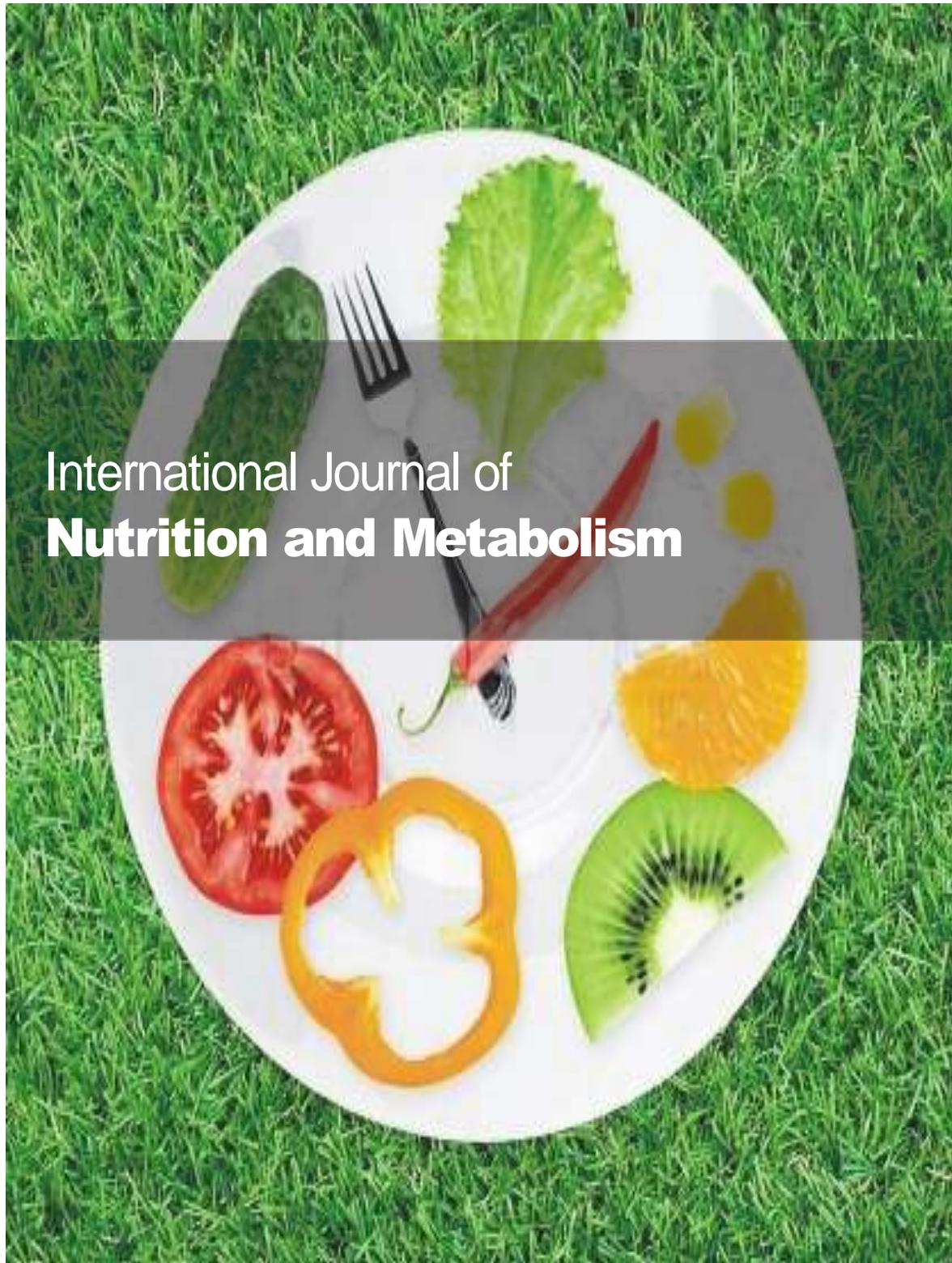


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Protective effect of vitamin C against theobromine induced hepatorenal and cardio toxicity in male albino Wistar rats

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Utibe Evans Bassey, Edet Okon Akpanyung, Dennis Uju Nwaokonko and Burch Takim Ndifon

Full Length Research Paper

Protective effect of vitamin C against theobromine induced hepatorenal and cardio toxicity in male albino Wistar rats

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The protective effect of vitamin C against theobromine induced toxicity in male albino Wistar rats was investigated. Twenty-five (25) male Wistar rats weighing between 140 – 160 g were divided into 5 groups with 5 rats in each group. Group 1 served as the control. Group 2 received 700 mg/kg body weight of theobromine daily for 4 days. Group 3 was administered 100 mg/kg body weight of Vitamin C daily for 21 days. Group 4 was intoxicated with 700 mg/kg of theobromine daily for 4 days before treatment with 100 mg/kg of Vitamin C for 21 days while Group 5 received 700 mg/kg of theobromine daily for 4 days and was allowed to recover naturally for 21 days. Biochemical indices of liver, kidney function and lipid profile were assayed using serum. The liver, kidney and heart tissues were used for histological studies. Significant increase ($p < 0.05$) in serum enzyme activities and concentrations of urea, creatinine, total and LDL cholesterol as well as decreased HDL cholesterol concentration were observed in Group 2 compared to the control. Treatment with Vitamin C in Groups 3 and 4 significantly decreased ($p < 0.05$) the activities of the serum enzymes, concentrations of urea, creatinine, total and LDL cholesterol while the concentration of HDL cholesterol was significantly increased when compared to Group 2. Histological evaluation of the liver, kidney and heart sections revealed degenerated cytoarchitecture and inflammation of these tissues following theobromine intoxication. However, the toxic features were observed to resolve in Group 4 when vitamin C was administered while cytoarchitectural degeneration persisted in Group 5. In conclusion, theobromine induced liver, kidney and cardio toxicity with negative modulation of lipid profile while vitamin C ameliorated the toxic effect of theobromine in albino Wistar rats.

Key words: Vitamin C, theobromine, liver function, kidney function, lipid profile, cardiotoxicity.

INTRODUCTION

Theobromine (3,7-dihydro-3,7-Dimethyl-H-purine-2,6-dione) is a crystalline, colourless and odourless powder

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with a slightly bitter taste naturally present in cocoa bean and cocoa based products. It is also a metabolite of caffeine in mammals. It is found in chocolate, the leaves of tea plant and kola nut. It is classified as a xanthine alkaloid, others of which include theophylline and caffeine (Smit, 2011). Theobromine is slightly water-soluble (330 mg/L) with melting point of 357°C and chemical formula of $C_7H_8N_4O_2$. Theobromine is an isomer of theophylline and paraxanthine. It is categorized as a dimethyl xanthine (Craig and Nguyen, 1984; Lamb et al., 1997; William, 2000).

Studies have shown that large doses of theobromine (0.8-1.5 g) may cause sweating, trembling and severe headache (Tarka, 1982). Reactions to theobromine differ according to dose; it showed limited subjective effects at 250 mg and negative mood effects at higher doses (Tarka, 1982). Aneja and Gianfagna (2001) observed that theobromine increased heart rate in a dose-dependent manner. Oral toxicity of theobromine has been reported in experimental animals with LD_{50} values ranging from 300 to 1350 mg/kg body weight (Tarka, 1982). In comparison with other methylxanthines, theobromine has a weak action on the central nervous system and is a weak antagonist of adenosine receptors. Target organs of theobromine toxicity in rodents are the testes (Sertoli cells) and thymus. Dogs also showed cardiomyopathy upon prolonged exposure (Gans, 1984; Strachan and Bennett, 1994). Eteng et al. (1998) reported that theobromine induces cardiotoxicity in experimental animals as evidenced by increased serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Histological evaluation of the heart of the experimental animals further proved that theobromine induces damage to the heart. Adeyina et al. (2008) also observed an increase in the activities of serum alkaline phosphate (ALP) and aspartate aminotransferase (AST) in rabbits treated with theobromine. These authors attributed the increase in serum activities of ALP and AST to the fact that theobromine caused breakdown of the membrane architecture of the hepatocytes leading to the spillage of these enzymes into blood circulation.

Vitamin C (ascorbic acid or ascorbate) is a water-soluble vitamin that plays essential biological functions. It serves as co-factor for certain enzymes and is involved in biological processes like synthesis of collagen and neurotransmitter. Vitamin C also functions as an antioxidant. Vitamin C is also involved in the reduction of hydroxyl ($OH\cdot$), superoxide ($O_2\cdot^-$), alkoxyl radical ($RO\cdot$), peroxy radical ($RO_2\cdot$), hydroperoxyl radical ($HO_2\cdot$), hydrogen peroxide (H_2O_2), singlet oxygen (1O_2), hypochlorous acid (HOCl), peroxy nitrite (NOO \cdot) and nitric oxide (NO \cdot) radicals (Niki, 1991). Specifically, Vitamin C has been shown to scavenge hydroxyl radical, reducing it to water. Furthermore, superoxide radical has been shown to be reduced to hydrogen peroxide then to water by ascorbate (Stadtman, 1991).

The presence of theobromine in chocolate and other cocoa products with its subsequent high consumption and potential toxicity necessitates the evaluation of various options against its toxicity. In line with this objective, (Akpanyung et al 2018a). had reported the potential of ethanol leaf extract of *Vernonia amygdalina* to modulate the toxicity of theobromine. The present study was designed to evaluate the ability of Vitamin C to ameliorate theobromine-induced toxicity in male albino rats.

MATERIALS AND METHODS

Source of theobromine and vitamin C

Pure synthetic theobromine was obtained from Sigma Andrich, UK. Vitamin C was obtained from Uchris Pharmacy, Uyo, Akwa Ibom State, Nigeria. Stock solutions of theobromine and Vitamin C were prepared daily during the period of administration. Aqueous solution of vitamin C is easily oxidized to dehydroascorbic acid, then to diketogluconic, oxalic and gluconic acid (Steskova et al., 2006).

Experimental animals

Twenty-five male Wistar rats weighing between 140 – 160 g were obtained from the Animal House, Faculty of Basic Medical Sciences, University of Uyo, Uyo, Nigeria. The rats were housed in a well-ventilated room under standard laboratory condition of 12 h light/dark cycle. The rats were allowed to acclimatise for a period of two weeks and were fed with rat chow and clean drinking water *ad libitum*. Institutional approval for the study was obtained from the Research and Ethical Committee, College of Health Science, University of Uyo, Uyo, Nigeria and the handling of animals was based on the international accepted standards (National Research Council, 1985).

Experimental design

Twenty-five male Wistar rats were selected into five groups with 5 rats in each group. Group I served as control while Group 2 received 700 mg/kg bw of theobromine daily for 4 days. Group 3 received 100 mg/kg bw of vitamin C daily for 21 days while Group 4 was intoxicated with 700 mg/kg bw of theobromine daily for 4 days before administration of 100 mg/kg bw of vitamin C. Group 5 received 700 mg/kg bw of theobromine daily for 4 days and was allowed a recovery period of 21 days.

After the last administration, the animals were fasted overnight and sacrificed under chloroform anaesthesia. Blood sample was collected through cardiac puncture using sterile syringes and needles into labelled sample bottles. Blood was allowed to clot and serum was obtained through centrifugation at 3000 rpm for 15 min using a bench top centrifuge (MSE minor). The heart, liver and kidney of the rats were removed and preserved in 10% buffered formalin for histological studies (Bancroft and Gamble, 2002).

Estimation of biochemical parameters

The reagents for the assay of the various biochemical parameters were obtained from Randox Laboratories Ltd. The assays were carried out based on the principles and protocols described in the reagent manufacturer's manual and user guide. Serum enzyme activities (ALT, AST and ALP) were determined. Also measured

Table 1. Effect of Vitamin C on Some Liver Enzyme Activities of Albino Wistar Rats Intoxicated with Theobromine.

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Group 1 – control	18.60 ± 0.51	97.00 ± 1.00	75.20 ± 1.59
Group 2 – theobromine	26.20 ± 1.11 ^a	121.00 ± 1.18 ^a	86.80 ± 2.22 ^a
Group 3 - vitamin C	15.80 ± 0.66 ^{a,b}	102.80 ± 2.58 ^b	58.00 ± 0.71 ^{a,b}
Group 4 - Theobromine before vitamin C	16.60 ± 0.93 ^b	85.00 ± 1.70 ^{a,b}	70.00 ± 0.71 ^{a,b}
Group 5 - theobromine then allowed For 21 days without treatment	21.60 ± 0.51 ^{a,b}	105.80 ± 1.69 ^{a,b}	57.00 ± 1.30 ^{a,b}

Data is presented as Mean ± SEM. a = significantly different when compared to Group 1 at P < 0.05; b = significantly different when compared to Group 2 at P < 0.05.

were the serum concentrations of urea and creatinine, electrolytes (Na⁺, K⁺, Cl⁻ and HCO₃⁻) and lipid profile (TC, TG, HDL-C). LDL-C and VLDL-C were calculated using Friedewald formula (Friedewald et al., 1972).

Histopathological Studies

The liver, kidney and heart sections were passed through the processes of fixation, dehydration, clearing, infiltration, embedding, sectioning and staining with haematoxylin and eosin (H and E) for examination under a light microscope. Photomicrographs of some of the tissue sections were taken using a digital camera fitted to the light microscope at a magnification of x100 (Bancroft and Gamble, 2002).

Statistical analysis

The data obtained were expressed as mean ± standard error of mean (SEM). One-Way analysis of variance (ANOVA) was used for comparison and results were subjected to post hoc test using Tukey multiple comparison. Test values of p < 0.05 were considered significant (Yockey, 2011).

RESULTS

Effect of vitamin C on some liver enzyme activities of albino Wistar rats intoxicated with theobromine

The effect of Vitamin C on liver enzyme activity of theobromine intoxicated albino Wistar rats is presented in Table 1. Theobromine is observed to induce elevation of ALT, AST and ALP activities in Group 2 compared with control. Administration of Vitamin C after theobromine to Group 4, significantly decreased (p<0.05) the activities of ALT, AST and ALP when compared to Group 2. Significant decrease in ALT and AST activities was observed in Group 5 in which the animals were allowed to stay for 14 days without any treatment after theobromine intoxication. The decrease in liver enzyme activities in Group 5 was to a lesser extent compared to the decrease observed in Group 4. Histological evaluation of the liver tissues (Figure 1) revealed enlarged and congested central vein as well as degeneration of cellular architecture in Group 2. Restoration of normal liver

cytoarchitecture was observed in Vitamin C treated group while inflammation of cellular features and mild degeneration of cytoarchitecture of the liver tissues were still observed in Group 5.

Effect of vitamin C on kidney function and histology in albino Wistar rats intoxicated with theobromine

Table 2 shows that administration of theobromine significantly elevated (p<0.05) the urea and creatinine concentrations in Group 2 while administration of Vitamin C after theobromine (Group 4) significantly reduced (p<0.05) the concentration of urea and creatinine when compared to Group 2. The concentrations of urea and creatinine in Group 5 were significantly high (p<0.05) when compared to Group 1 and not significantly different (p>0.05) from Group 2. The electrolyte concentrations were not significantly different when compared to the control. Photomicrographs of the kidney sections of albino rats with theobromine induced toxicity treated with Vitamin C (Figure 2) show degenerated glomeruli and renal tubular inflammation were observed in kidney tissues of animals in Group 2 following theobromine intoxication, However, normal cellular features were seen in Group 4 treated with vitamin C. However, glomeruli degeneration and renal tubular degeneration persisted in the kidney sections of Group 5 animals.

Effect of vitamin C on lipid profile and histology of the heart in albino Wistar rats intoxicated with theobromine

Table 3 presents the effect of vitamin C on lipid profile of albino rats intoxicated with theobromine. The concentrations of total cholesterol and low-density lipoprotein cholesterol in theobromine intoxicated group (Group 2) were significantly elevated (p<0.05) when compared to the control while the high-density lipoprotein was significantly decreased compared to Group 1. Administration of vitamin C (Group 4) significantly increased the concentration of HDL-cholesterol and

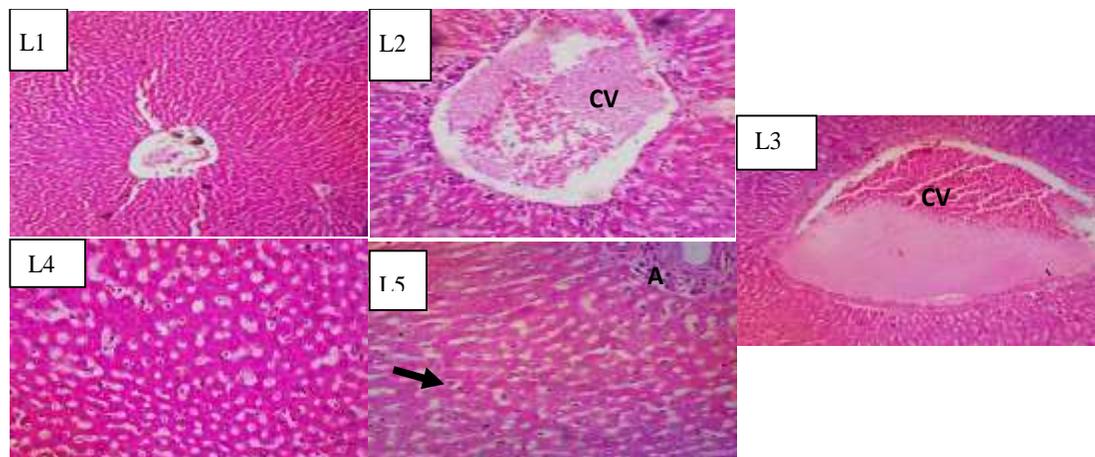


Figure 1. Photomicrograph of liver tissue of albino rats in Group 1 with normal cytoarchitecture of the liver (L1); Group 2 showed enlarged congested central vein (CV) and degeneration of cellular architecture (L2); Group 3 having normal Liver architecture with Hepatocytes (arrow) and slightly widened congested central vein (L3); Group 4 showing sparsely distributed hepatocytes (L4); Group 5 showing mononuclear inflammatory infiltrates (arrow) and widened sinusoid (L5). H and E technique; Magnification = x100.

Table 2. Effect of vitamin C on kidney function of Albino Wistar rats intoxicated with theobromine

Groups	Creatinine (µmol/L)	Urea (mmol/L)	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)
Group 1 – Control	48.86 ± 1.84	2.50 ± 0.17	132.66 ± 0.90	6.84 ± 0.46	97.18 ± 1.30
Group 2 – theobromine	56.60 ± 1.54 ^a	3.56 ± 0.21 ^a	133.94 ± 1.4	6.49 ± 0.37	95.34 ± 1.70
Group 3 - vitamin C	45.46 ± 1.31 ^b	2.78 ± 0.24 ^b	140.76 ± 0.59 ^{a,b}	6.41 ± 0.85	97.90 ± 1.10
Group 4 - theobromine before vitamin C	45.00 ± 1.31 ^b	2.50 ± 0.24 ^b	140.80 ± 1.60 ^{a,b}	6.31 ± 0.20	99.80 ± 0.81 ^b
Group 5 - theobromine then allowed for 21 days without treatment	59.36 ± 1.53 ^a	3.26 ± 0.19 ^a	135.68 ± 0.65	5.77 ± 0.16 ^a	97.36 ± 0.63

Data is presented as Mean ± SEM. a = significantly different when compared to Group 1 at P < 0.05; b = significantly different when compared to Group 2 at P < 0.05.

decreased the concentration of total cholesterol and LDL-cholesterol when compared to Group 2. There was no significant difference ($p > 0.05$) in the triglyceride concentrations in the treated group compared to the control. Significantly high and low concentrations ($p < 0.05$) of LDL-cholesterol and HDL-cholesterol respectively were observed in Group 5. The histology of the heart tissue of theobromine intoxicated group (Group 2) revealed haemorrhage permeating the pericardium and degeneration of cellular features were observed. Normal cytoarchitecture of the heart tissues were observed in vitamin C treated group while pericardium inflammation was observed in Group 5. The photomicrographs are presented in Figure 3.

DISCUSSION

The present study evaluated the effect of vitamin C on

theobromine induced toxicity to the liver, heart and kidney in male albino Wistar rats by determination of some biochemical indices and histological studies.

The liver is a vital organ that plays crucial role in the metabolic clearance of toxic substances (Campbell, 2006). It has been reported that most toxicants undergo first pass metabolism in the liver before entry into general systemic circulation thereby making the liver highly susceptible to toxic injury by chemical substances (Pandit et al., 2012). Toxic injury to the liver compromises integrity of the cell membrane of the hepatocytes resulting in the leakage of cytosolic content, including marker enzymes such as ALT, AST and ALP into systemic circulation. Subsequently, there is remarkable increase in the activities of these liver enzymes in the blood (Jaeschke et al., 2012; McGill, 2016).

In the present study, the administration of theobromine induced a significant increase ($p < 0.05$) in the activities of ALT, AST and ALP (Table 1) compared with the control.

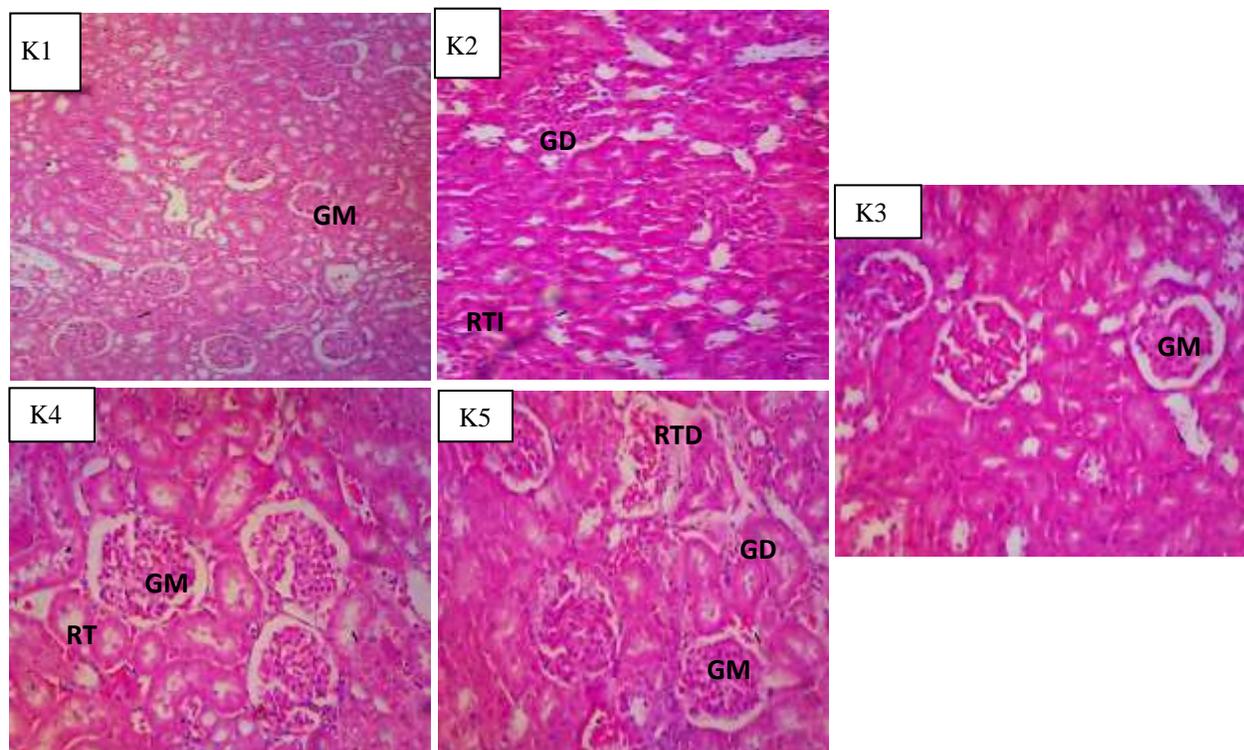


Figure 2. Photomicrographs of kidney of albino rats in Group 1 showed normal renal tissue cyto-architecture with distinct glomeruli and bowman's capsule (K1); Group 2 revealed degenerated glomeruli (GD) and renal tubular inflammation (RTI) (K2); Group 3 showing normal cellular architecture of the kidney (K3); Group 4 reveals kidney cytoarchitecture with normal renal tubules (RT) and glomeruli (GM) (K4). Group 5 reveals glomeruli degeneration and renal tubular degeneration (K5). H and E technique; Magnification = x100.

Table 3. Effect of vitamin C on lipid profile of albino Wistar rats intoxicated with theobromine.

Groups	TCHOL (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	VLDL-C (mmol/L)
Group 1 – control	2.34 ± 0.04	1.38 ± 0.03	1.15 ± 0.04	0.57 ± 0.08	0.63 ± 0.01
Group 2 – theobromine	3.05 ± 0.29 ^a	1.24 ± 0.12	0.91 ± 0.03 ^a	1.58 ± 0.29 ^a	0.56 ± 0.06
Group 3 - vitamin C	2.30 ± 0.05 ^b	1.25 ± 0.11	1.12 ± 0.04 ^b	0.61 ± 0.13 ^b	0.57 ± 0.05
Group 4 - theobromine before Vitamin C	2.31 ± 0.14 ^b	1.13 ± 0.13	1.17 ± 0.11 ^{bc}	0.63 ± 0.10 ^b	0.51 ± 0.06
Group 5 - theobromine then allowed for 21 days without treatment	2.62 ± 0.10 ^b	0.96 ± 0.05 ^a	0.87 ± 0.02 ^{ac}	1.27 ± 0.15 ^{ac}	0.48 ± 0.04

Data is presented as Mean ± SEM. a = significantly different when compared to Group 1 at P < 0.05; b = significantly different when compared to Group 2 at P < 0.05.

This is suggestive of hepatotoxicity induced by theobromine in the experimental animals. Other authors had earlier reported that theobromine is hepatotoxic (Eteng et al., 1998; Adeyina et al., 2008; Akpanyung et al., 2018a). Conversely, the administration of vitamin C to the theobromine intoxicated rats caused a significant decrease ($p < 0.05$) in the activities of these liver enzymes. Vitamin C is an established antioxidant which has been shown to scavenge free radicals with resultant reduction

in reactive oxygen and nitrogen species in the blood (Iqbal et al., 2004; Adikwu and Deo, 2013).

Histological evaluation of the liver of animals exposed to theobromine revealed distorted cellular morphology, inflammation and necrosis. These observations are characteristics of liver damage induced by other chemical substances (Akpanyung et al., 2015). Interestingly, the administration of vitamin C was found to restore the damaged cells to normal. However, these abnormal

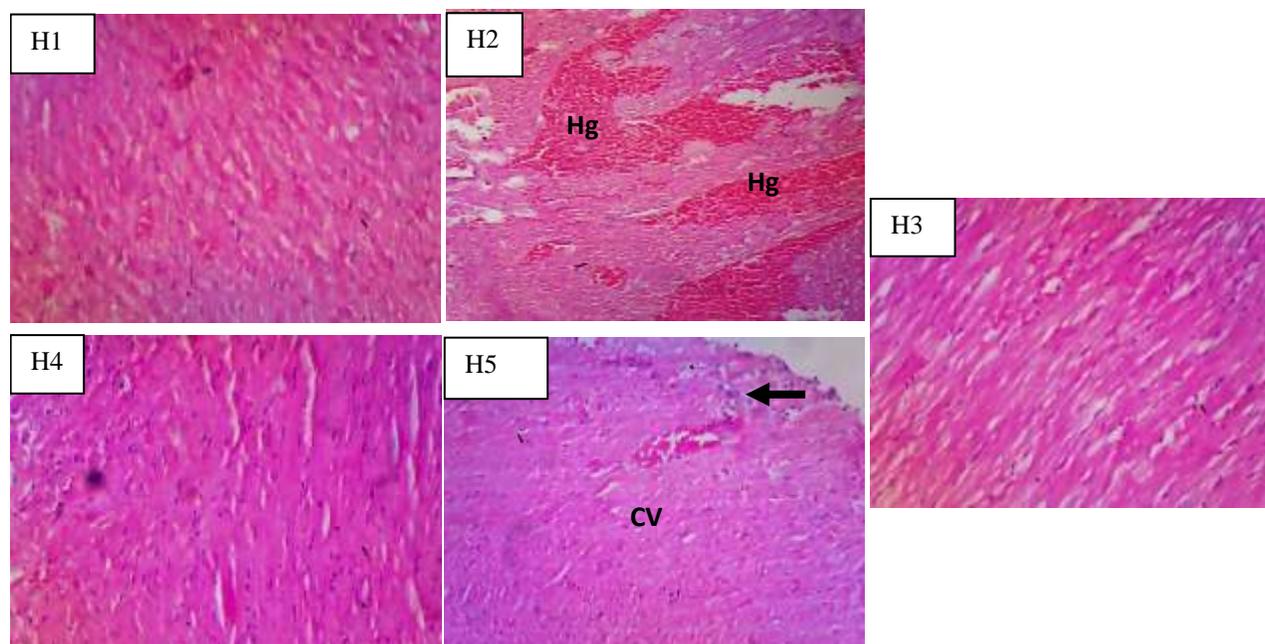


Figure 3. Photomicrograph of heart tissue of albino rats in Group 1 with normal cytoarchitecture of the heart (H1): Group 2 shows heart tissue with haemorrhage (Hg) permeating the myocardium and degeneration of cellular architecture (H2): Group 3 reveal normal histology of the heart (H3): Group 4 reveal normal histology of the heart (H4): Group 5 showing congested blood vessels (CV) and pericardium inflammation (arrow) (H5). H and E technique; Magnification = x100.

cellular degenerative features were retained in Group 5 which was left without treatment with Vitamin C. Thus, the result of the histological studies is supportive of the biochemical observations made in this study.

The kidney is also very susceptible to damage by chemical substances (Gheshlaghi, 2012). The kidneys are involved in the process of elimination of waste products of metabolism. They also selectively reabsorb specific substances and help in maintaining osmolarity of the system (Esteva-Font, 2012). Changes in glomerular hemodynamics, tubular cell toxicity and inflammation are possible mechanism of nephrotoxicity by chemical substances and drugs (Kim and Moon, 2012). Damage to the kidney often results in systemic retention of waste products as a consequence of the loss of filtration function of the kidneys (Prakash et al., 2003). Serum or plasma concentrations of urea, creatinine and electrolytes have been used as biomarkers for assessment of renal function (Ogedegbe, 2007). In the present study, creatinine and urea concentrations were significantly elevated following administration of theobromine (Table 2). Administration of vitamin C after exposure of the animals to theobromine significantly decreased ($p < 0.05$) the concentrations of urea and creatinine when compared to Group 2.

Vitamin C has been reported to be nephroprotective (Adeneye and Olagunju, 2009). The antioxidant potentials of vitamin C enable it to scavenge free radicals generated by chemical substances which would have damaged

specific cells in the kidney tissues (Markowitz and Perazella, 2005). Normal histology of the kidney tissue reveals distinct glomeruli and the distal and convoluted renal tubules (Figure 2: K1). In the present study, degeneration of the glomerulus and renal tubular inflammation were observed in the animals treated with theobromine (Figure 2: K2). The observed pathohistological changes are in line with the biochemical assay which shows accumulation of urea and creatinine following theobromine administration. These results are consistent with pathological studies on the histology of damaged kidney tissues reported by Tarladacalisir et al., (2008). Regeneration of cytoarchitectural features such as the Bowman's capsule, glomeruli and renal tubules were observed in the vitamin C treated groups especially in Group 4. (Figure 2: K4) Evidence of glomeruli and tubular degeneration were still present in Group 5 in which the animals were intoxicated with theobromine and then left without treatment with Vitamin C (Figure 2: K5).

Measurement of the lipoprotein concentrations is useful in evaluating dangers posed on the cardiovascular system by any substance (Barter et al., 2007). High density lipoprotein cholesterol is involved in the transport of cholesterol from tissues to the liver while low density lipoprotein cholesterol is reported to aid in the deposition of cholesterol in extrahepatic tissues (Trajkovska and Topuzovska, 2017). Consequently, elevation of LDL-C has a negative impact on the cardiovascular system and could result in cardiovascular events such as arrhythmia,

cardiac arrest and heart attack (Dayuan and Mehta, 2005). Elevation of LDL-C observed in the present study indicates that administration of theobromine might increase the risk of cardiovascular events (Akpanyung et al., 2018b).

The administration of vitamin C after theobromine intoxication resulted in a significant decrease ($p < 0.05$) in the concentrations of total cholesterol and low-density lipoprotein cholesterol. The HDL-C concentration was increased in vitamin C treated group. Simultaneous reduction in the concentration of LDL-C and increased HDL-C concentration in serum as observed in this study is a positive indicator of cardiovascular health (Sloop and Garber, 1997). It has been reported that vitamin C correlates negatively with total cholesterol, triglycerides and low-density lipoprotein cholesterol while it exhibits a positive correlation with high-density lipoprotein (Howard and Meyers, 1995). The HDL-C and LDL-C concentrations in Group 5 decrease and increase respectively when the animals were allowed to recover naturally. A decreased HDL-C and increased LDL-C concentrations is a risk factor for development of cardiovascular disease (Niroumand et al., 2016).

The histology of the heart sections of animals treated with theobromine revealed regions of haemorrhage indicating the toxicity of theobromine on the heart. Eteng et al., (1998) had earlier reported the cardiotoxicity of theobromine. Recent reports by Akpanyung et al. (2018a) have provided further evidence that theobromine is toxic to the heart. Normal histological features as observed in Group 1 were also visible in vitamin C treated groups. However, congested blood vessels and inflammation of the pericardium were still observed in Group 5 where vitamin C was not administered after treatment with theobromine

Conclusion

The present study has shown that theobromine is toxic to the liver, kidney and heart as demonstrated by measurement of some biochemical parameters and histological studies. Administration of Vitamin C was demonstrated to ameliorate the deleterious effects of theobromine.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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