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Research article

Toxicity assessment of sub-acute and sub-chronic oral administration and diuretic potential of aqueous extract of *Hibiscus sabdariffa* calyces

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ABSTRACT

Background: Food and herbal usage of *Hibiscus sabdariffa* (HS) is attaining improved global relevance and acceptance without recourse to its potential toxic effects. This study investigated the safety profile of acute, sub-acute, sub-chronic administrations and diuretic potential of aqueous extract of *Hibiscus sabdariffa* calyces (AEHSC).

Method: Acute oral toxicity, sub-acute and sub-chronic toxicity as well as diuretic studies were carried out on HS. A total of 20 Wistar rats were used for each toxicity study and assigned into four groups of five rats. The extract was administered as a single daily dose of 125, 250 and 500 mg/kg body weight (bwt) for 28 and 90 days respectively. To evaluate diuretic activity, 25 rats were divided into five groups of five rats and administered normal saline, hydrochlorothiazide 10 mg/kg, AEHSC 67.5, 125 and 250 mg/kg via the oral route. Urine sample was collected after 18 h, volume measured and concentration of electrolytes analyzed. The hematological and biochemical parameters were evaluated as well as the histopathology of kidney and liver.

Results: The acute oral toxicity was found to be >2000 mg/kg. AEHSC did not alter concentration of WBC, MCV, MCHC, lymphocyte as well as total and direct bilirubin in the sub-acute study. However, AEHSC significantly ($p < 0.05$) increased total protein, albumin, globulin, Na^+, Cl^-, HCO_3^- and platelet levels, while levels of uric acid, creatinine, K^+, RBC, Hb, total cholesterol, triglycerides, LDL-C, HDL-C and atherogenic index were decreased significantly ($p < 0.05$). In the sub-chronic study, AEHSC significantly ($p < 0.05$) increased the levels of globulin, urea, creatinine, MCH and atherogenic index. The concentrations of uric acid, WBC, platelets and HDL-C were significantly ($p < 0.05$) decreased. In both the sub-acute and sub-chronic studies, activities of ALP, ALT, AST, GGT and LDH in selected organs were altered without significant increase ($P < 0.05$) in activity of these enzymes in the serum. The AEHSC at all the doses showed remarkable diuretic activity during 18 h period comparable to hydrochlorothiazide. The extract also showed a non-dose-dependent increase in excretion of electrolytes. Histological analysis of sections of the liver and kidney for both sub-acute and sub-chronic studies showed normal histology comparable to the control group.

Conclusion: This study revealed AEHSC has some toxic effects in rats on sub-chronic administration. In addition, the extracts produced a significant diuretic activity. Hence, prolonged oral consumption of the extract may not be recommended.

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1. Introduction

Traditional medicine is an important part of developing African culture which has been practiced by ancients much before the advent of advanced orthodox medical practices. *Hibiscus sabdariffa* L. (Malvaceae) commonly known as Roselle is a plant found in many countries. The calyces is claimed to be effective as hepatoprotective (Lee et al., 2012) and anti-pyretic (Mahadevan et al., 2009), diuretic (Laikangbam and Damayanti Devi, 2012), anti-hypertensive (Shehata and El Menoufy, 2008), antimicrobial and anti-inflammatory source (Reanmongkol and Itharat, 2007). Phytochemical studies showed the presence of mucilage, gossypetin, calcium citrate, flavonoids and ascorbic acid (Mahadevan et al., 2009). Some pharmacological activities of *H. sabdariffa* (HS) include lowering of anti-atherosclerotic activity and serum lipids in cholesterol-fed rats and rabbits as well as protecting human erythrocytes against lipid peroxidation among others.

Previous report on the toxicity profile of HS showed no observed toxicity at 15 g/kg calyces (high dose) of aqueous and ethanol extracts in mice within 7 days after oral administration (Reanmongkol and Itharat, 2007). Akindahunsi and Olaleye (2003) reported that prolonged usage of the aqueous-methanolic extract of calyces at the dose of 250 mg/kg could cause liver injury in rats. Sireeratawong et al. (2013) also reported the safety HS (red species) aqueous calyces extract when administered as single oral dose of 5,000 mg/kg bwt after fourteen days. They also reported safety at doses between 50 and 200 mg/kg bwt of extract after oral administration every day for two hundred and seventy days.

Meanwhile, Orisakwe et al. (2004) reported induced testicular toxicity by AEHSC at doses ranging from 1.15 to 4.60 g/kg after 12 weeks of administration. Another research reported a gross reduction in weight, diarrhoea and finally death of the animals after albino rats were orally administered alcohol and water extracts of HS dried calyx for ninety days duration at a dose of 2,000 mg/kg (Fakeye et al., 2009). Despite the extensive usage of HS in traditional medicines and as a drink widely consumed in Nigeria, the sub-acute and sub-chronic toxicity study of the aqueous extract of the plant of Ilorin (Nigerian) origin has not been ascertained. Therefore, the aim of this study was to assess the safety in rats, of orally administered AEHSC harvested from African Centre for Herbal Research, Ilorin (ACHRI), Nigeria plantation.

2. Materials and methods

2.1. Materials

Analytical grade of hydrohlorothiazide, sodium chloride (NaCl) and formalin were used. Kits for assaying biochemical parameters were purchased from Randox Lab. Ltd, Antrim, UK.

2.2. Plant materials

HS dried calyces were harvested in November from ACHRI plantation situated at University of Ilorin, Ilorin and authenticated at the herbarium section of the Centre (Voucher specimen number ACHRI/H16/101). The plant material were transported in a bag. The dry calyces were further dried under the shade, pulverized using a mechanical blender and transferred into airtight containers for subsequent work.

2.3. Preparation of crude extracts

The pulverized calyces (808 g) of *HS* were soaked in 20% (w/v) cold distilled water for 24 h. The extract was filtered using muslin and then filtered again using Whatman No 1 filter paper. The resulting filtrate was concentrated over water bath at 40 °C (Kambizi et al., 2017) and weighed. The concentrated extract was then stored at +4 °C until required for analysis.

2.4. Animals

The University of Ilorin's Ethics Review Committee (UIERC) granted the ethics clearance approval for this study. Experiments were carried out in accordance to guidelines of International Animal Care and Use Committee (IACUC).

Six weeks old male Wistar rats (100–120g) used for the research, were obtained from the Animal House of Central Research Laboratory, University of Ilorin. The Wistar rats were given food and clean water and conditioned in the laboratory for one week.

2.5. Acute toxicity study (LD_{50})

The acute toxicity study was carried out according to the Organization of Economic Co-operation and Development (OECD) guideline for testing of chemicals (OECD, 2001). Three Wistar rats were placed in separate cages and labeled. The animals were allowed to acclimatize to the laboratory conditions for about 24 h. They were fasted for 12 h after which they were all given the aqueous extract at concentration of 300 and 2000 mg/kg via an oral cannula. The animals were given feed and water. Two hours after administration of the extract, the animals were observed for six (6) hours for signs of toxicity and mortality and then for 24 h. When no mortality was recorded after 24 h the experiment was repeated using another set of 3 rats. The signs of toxicity and mortality were observed during the first 6 h, then for 24 h. Daily observation for signs of toxicity was continued for 14 days.

2.6. Sub-acute toxicity study

The animals were weighed and divided into four (4) groups of five (5) animals each and fasted overnight. The aqueous extract (125, 250 and 500 mg/kg/bwt) were administered orally for 28 days. The control group received a dose of 0.5 mL of distilled water orally once a day for 28 days (OECD, 2002). The body weight changes were monitored throughout the experimental period on weekly basis. Similarly, the animals were observed for manifestation of toxicity and mortality (Pillai et al., 2011).

2.7. Sub-chronic toxicity study

The animals were also weighed and divided into four (4) groups of five (5) animals each. After fasting the rats overnight, the control group received a dose of 0.5 mL of distilled water orally once a day for 90 days while the animals in the other three groups were given different doses (125, 250 and 500 mg/kg) of the aqueous extract orally once a day for 90 days. The animals were also monitored throughout the experimental period on weekly basis. The animals were observed for manifestation of toxicity and mortality (WHO, 2000).

At the end of the observation periods, the animals were fasted overnight in the sub-chronic and sub-acute toxicity studies. Anaesthetics were administered successively to the animals in a jar saturated with dichloromethane vapour. Blood samples were collected via ocular puncture into ethylene diamine tetra acetic acid (EDTA) coated bottles for determination of hematological parameters and heparinized bottles for biochemical parameters. After the blood samples clot, it was centrifuged and the serum were collected and then used for biological parameters. The rats were sacrificed by decapitation with scissors and vital organs (kidney and liver) were taken for histology. The harvested organs (kidneys and liver) were fixed for 24 h in 10 % neutral-buffered formalin and processed into paraffin sections using a carousel-type automatic tissue processor where the tissue samples passed through the following processes: fixation, dehydration in increasing gradients of alcohol, clearing in xylene and impregnation in wax.

The processed tissue samples were embedded in paraffin and 5 microns thick sections were cut using the rotary microtome. The sections were subsequently stained with haematoxylin and eosin.

2.7.1. Haematoxylin and eosin staining method

The sections of the samples were dewaxed in xylene and then rehydrated through descending grades of alcohol and then brought to water. The sections were stained for 5 min in Harris Haematoxylin and washed in running water until they were "blue". They were then differentiated in 1% acid alcohol for 5 s and washed in tap water until they were "blue" again. Further "bluing" was done by dipping briefly in ammonia solution followed by washing in distilled water for 5 min. The sections were stained in Eosin for 10 min, washed in a running distilled water for 5 min and then dehydrated through increasing gradients of alcohol. They were cleared in xylene and mounted with coverslips using DPX mountant (mixture of distyrene, tricresyl phosphate and xylene). The stained slides were examined to assess evidence of acute or chronic damage in the tissues (Bancroft and Layton, 2012).

2.8. Hematological and biochemical estimations

Red blood corpuscles (RBC) count, white blood corpuscles (WBC) count, mean cell volume, hemoglobin (Hb), mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, monocyte, neutrophil, lymphocyte and platelet count of the control and extract-treated groups were determined and compared with control group using an Automatic Haemotology Analyzer (Model PC 607 Erma Inc., Japan).

The biochemical parameters in the plasma and liver homogenate were estimated using Erba test reagent kits on an Erba Smartlab Auto-Analyser. The biochemical parameters estimated in blood include glucose (GLU), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (CREA) and blood urea nitrogen (BUN). In the liver homogenate, glutathione (GSH), lactate dehydrogenase (LDH), `alkaline phosphatase (ALP), and gama glutamyl transferase (GGT), total protein (TP) were estimated.

2.9. Diuretic study

Twenty-five albino rats of both sexes were divided into five groups of five rats per group at random and each kept in separate standard cages with twelve hours light/dark cycle condition in the animal house. The animals were fed on commercial feed and distilled water *ad libitum*. The cages were cleaned every day, food and water was changed daily. The animals were fasted for twelve hours before the experiment but water was allowed.

Control was treated with 0.2 mL of normal saline and the positive control was treated with a standard drug, hydrochlorothiazide 10 mg/kg. Three treatment groups were administered with different doses of the extract. Oral route of administration was used.

- Group 1: Control 0.2 mL normal saline
- Group 2: Hydrochlorothiazide-standard 10 mg/kg
- Group 3: *H. sabdariffa* aq. extract 67.5 mg/kg
- Group 4: *H. sabdariffa* aq. extract 125 mg/kg
- Group 5: *H. sabdariffa* aq. extract 250 mg/kg

Diuretic activity was determined following the methods used by Lahlou et al. (2007) with slight modifications. Each rat was placed in a metabolic cage for 24 h prior to the commencement of the experiment for adaptation and then fasted overnight with free access to water. The animals were pretreated with 1 mL normal saline (0.9% NaCl) 90 min prior to the start of the experiment to obtain uniform water and salt load.

Immediately after administration of the extract, the rats were placed in the metabolic cage. Urine was collected and the volume measured 18 h after treatment. The urine was stored at -20 °C; tested for electrolyte (sodium, potassium and chloride ion) analyses and pH determined.

2.10. Statistical analysis

The results are presented as mean ± standard error of the mean of five animals. Analysis of variance (ANOVA) test using GraphPad Prism. A probability level of $p < 0.05$ was accepted statistically.

3. Results

3.1. Acute toxicity studies

AEHSC (2000 mg/kg) administered as a single oral dose produced death in one of three Wistar rats for the first test but on repeating this dose (confirmation) in another set of three rats, there was no signs of toxicity, no mortality, no change in general behaviour or other physiological activities. On single administration of a lower concentration of 300 mg/kg AEHSC, there was no death in any of the three Wistar rats used for the first test as well as the second set of animals used for confirmation. There was no toxicity exhibited, no behavioral changes or other activities related to it physiological and no mortality. Thus, according to OECD 2001 guideline the acute oral toxicity was stipulated to be higher than 2000 mg/kg. Caution: LD_{50} has to be confirmed in studies with a larger number of animals.

3.2. Sub-acute and sub-chronic toxicity

3.2.1. The effect of aqueous extract of H. sabdariffa on body weight of Wistar rats

There was no significant difference between the body weight of the control group and that of groups treated with 125, 250 and 500 mg/kg extract throughout the four weeks of oral administration of the aqueous extracts to the rats as shown in Figures 1 and 2 respectively.

There was a significant increase ($p < 0.05$) in the concentration of total protein after sub-acute administration of AEHSC to rats at dose levels 125 and 250 mg/kg bwt except at 500 mg/kg bwt where the levels of total protein significantly decreased ($p < 0.05$) when compared with the control rats. In contrast, sub-chronic administration of AEHSC at 500 mg/kg bwt significantly increased ($p < 0.05$) the concentration of total protein, while the 125 and 250 mg/kg bwt significantly decreased ($p < 0.05$) the total protein in treated rats when compared with the control (Table 1).

In addition, albumin concentration in treated rats was significantly increased ($p < 0.05$) in a dose-dependent manner by all doses of AEHSC, while the extract (125 mg/kg bwt) significantly decreased ($p < 0.05$) albumin concentration in rats after a 90-day administration of AEHSC, whereas there was no significant alteration ($p > 0.05$) in albumin level by 250 and 500 mg/kg bwt of AEHSC when compared with the control group (Table 1).

There was a significant increase ($p < 0.05$) in globulin concentration in rats administered with AEHSC at 125 and 250 mg/kg bwt for 28 days, while the 500 mg/kg bwt administration caused a significant decrease ($p < 0.05$) in the levels of globulin in the exposed rats when compared with the control group. Conversely, the highest dose (500 mg/kg bwt) of AEHSC resulted in a significant increase ($p < 0.05$) in globulin concentration after repeated sub-chronic exposure of rats, whereas the 250 mg/kg bwt of AEHSC significantly decreased ($p < 0.05$) the globulin concentration with no significant alteration ($p > 0.05$) in rats that received 125 mg/kg bwt of AEHSC when compared with the control (Table 1).

A 28-day oral administration of AEHSC at all treatment doses did not significantly change the levels of direct bilirubin in exposed rats. Meanwhile, only 250 mg/kg bwt of the extract significantly increased ($p < 0.05$) the direct bilirubin concentration in rats while the doses of 125 and 500 mg/kg bwt significantly decreased the direct bilirubin level in rats when compared with the control rats (Table 1).

In the 28 days oral administration of AEHSC study, only rats that received 500 mg/kg bwt of the extract had their total bilirubin concentration significantly reduced ($p < 0.05$), while those that were exposed to

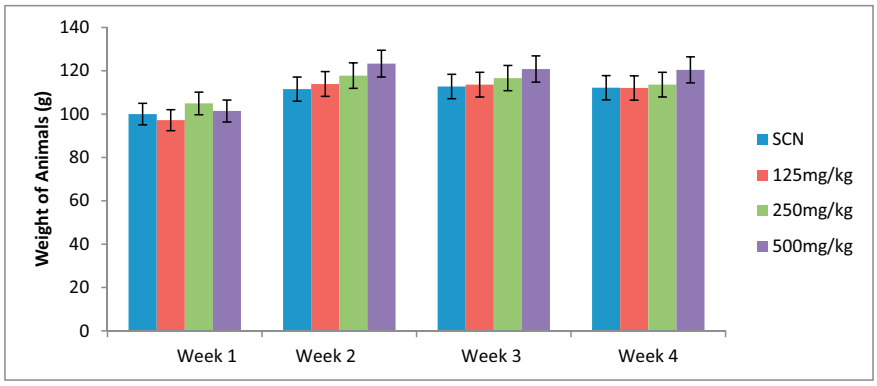

Figure 1. Effect of administering sub-acute dose of AEHSC on body weight of rats (n = 5, SCN = control).

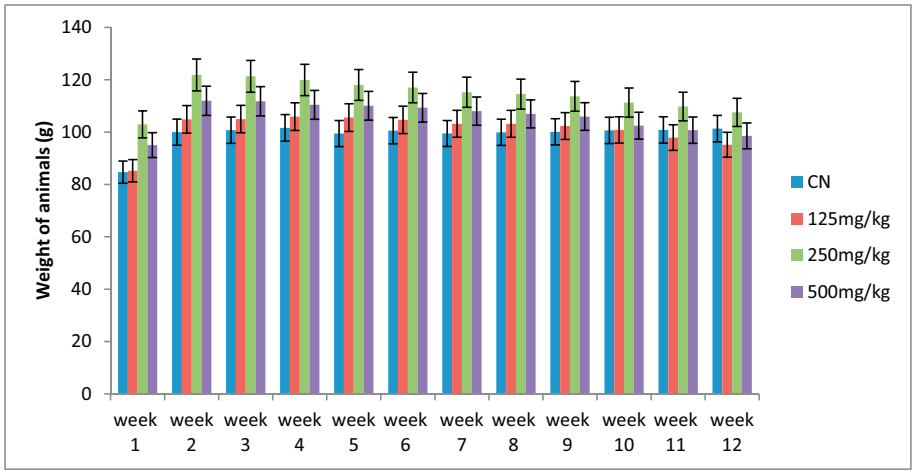

Figure 2. Effect of administering sub-chronic dose of AEHSC on body weight of rats (n = 5, CN=control).

Table 1. Effects of administering orally sub-acute and sub-chronic dose of AEHSC on selected liver function indices.

Study	Parameter (mg/dL)	Treatment (mg.kg bwt) AEHSC			
		Control	125	250	500
Sub-Acute	Total Protein	3.26 ± 0.14^{a}	3.99 ± 0.12^{b}	4.80 ± 0.09^{b}	2.76 ± 0.04^{b}
	Albumin	0.44 ± 0.01^{a}	0.69 ± 0.01^{b}	0.79 ± 0.02^{b}	1.08 ± 0.04^{b}
	Globulin	2.73 ± 0.11^{a}	3.23 ± 0.11^{b}	3.48 ± 0.16^{b}	1.78 ± 0.10^{c}
	Direct Bilirubin	2.44 ± 0.06^{a}	2.50 ± 0.04^{a}	2.55 ± 0.03^{a}	2.56 ± 0.05^{a}
	Total Bilirubin	3.48 ± 0.03^{a}	3.65 ± 0.02^{a}	3.77 ± 0.06^{a}	4.13 ± 0.18^{b}
Sub-Chronic	Total Protein	4.95 ± 0.36^{a}	4.77 ± 0.17^{a}	4.04 ± 0.29^{a}	6.32 ± 0.38^{b}
	Albumin	1.77 ± 0.05^{a}	1.29 ± 0.13^{b}	1.94 ± 0.07^{a}	2.00 ± 0.13^{a}
	Globulin	3.26 ± 0.12^{a}	3.48 ± 0.09^{a}	2.57 ± 0.07^{b}	4.65 ± 0.06^{b}
	Total Bilirubin	1.46 ± 0.07^{a}	1.48 ± 0.52^{a}	1.63 ± 0.08^{a}	1.45 ± 0.10^{a}
	Direct Bilirubin	1.37 ± 0.14^{a}	1.01 ± 0.06^{b}	1.53 ± 0.09^{b}	1.12 ± 0.11^{b}

Data are mean ± SEM, (n = 5). Values with superscripts alphabet that is different from the control for each parameter are significantly different ($p < 0.05$).

125 and 250 mg/kg bwt of AEHSC experienced no significant alteration ($p > 0.05$) in their total bilirubin concentration. Similarly, there was no change in all the extract doses of the total bilirubin in rats that were exposed to AEHSC for 90 days (Table 1).

Table 2 depict effects of sub-acute and sub-chronic administration of AEHSC on selected kidney function parameters. There was no significant difference ($p > 0.05$) in the urea concentration of rats exposed to AEHSC for 28 days when compared to the control, whereas rats that received 250 and 500 mg/kg bwt of the extract had a dose-dependent, significant increase ($p < 0.05$) in the concentration of urea when compared with the control. Administration of the three studied doses of AEHSC for 90 days significantly increased ($p < 0.05$) in a dose-dependent manner urea concentration of the exposed rats when compared to the control (Table 2).

The serum concentration of uric acid in rats was significantly reduced ($p < 0.05$) after a 28-day oral administration of AEHSC at dose levels of 250 mg/kg bwt, while the 125 mg/kg bwt of the extract increased though not significantly ($p > 0.05$) when compared to the control. In addition, administration of AEHSC for 90 days, significantly decreased ($p < 0.05$) serum concentration of uric acid when compared with the control.

Table 2. Effects of administering orally sub-acute and sub-chronic dose of AEHSC on selected kidney function indices in Wistar rats.

Study	Parameter		Treatment (mg/kg bwt) AEHSC			
			Control	125	250	500
Sub-acute	Urea (mg/dL)		55.06 ± 1.27^{a}	52.86 ± 0.76^{a}	81.19 ± 1.11^{b}	95.10 ± 0.79^{b}
	Uric acid (mg/dL)		23.87 ± 0.79^{a}	24.70 ± 0.49^{a}	20.47 ± 1.12^{b}	21.59 ± 1.26^{a}
	Creatinine (mg/dL)		24.47 ± 0.77^{a}	14.81 ± 0.91^{b}	19.14 ± 1.80^{b}	22.43 ± 0.81^{a}
	Electrolytes (mmol/L)	Na^{+}	132.25 ± 0.85^{a}	138.50 ± 1.19^{b}	133.00 ± 1.22^{a}	133.75 ± 0.75^{a}
		K^{+}	6.28 ± 0.40^{a}	7.00 ± 0.15^{a}	5.83 ± 0.39^{a}	5.55 ± 0.19^{a}
		Cl^{-}	99.50 ± 0.96^{a}	100.50 ± 1.71^{a}	100.75 ± 2.06^{a}	100.25 ± 1.31^{a}
		HCO_3^{-}	29.75 ± 0.85^{a}	34.25 ± 1.03^{b}	28.50 ± 0.65^{a}	35.75 ± 1.89^{b}
Sub-chronic	Urea (mg/dL)		48.16 ± 0.57^{a}	51.06 ± 0.71^{a}	64.61 ± 1.20^{c}	79.01 ± 1.73^{b}
	Uric acid (mg/dL)		24.70 ± 1.28^{a}	16.61 ± 1.06^{b}	16.48 ± 0.90^{b}	15.95 ± 0.78^{b}
	Creatinine (mg/dL)		3.39 ± 0.11^{a}	4.44 ± 0.15^{b}	7.32 ± 0.32^{b}	5.53 ± 0.14^{b}
	Electrolytes (mmol/L)	Na^{+}	138.33 ± 0.88^{a}	138.67 ± 2.03^{a}	136.33 ± 1.45^{a}	141.67 ± 4.33^{a}
		K^{+}	5.00 ± 0.12^{a}	5.10 ± 0.20^{a}	5.93 ± 0.19^{a}	5.17 ± 0.27^{a}
		Cl^{-}	101.00 ± 1.00^{a}	98.67 ± 1.20^{a}	100.00 ± 2.31^{a}	108.67 ± 2.33^{b}
		HCO_3^{-}	27.33 ± 0.88^{a}	29.00 ± 0.57^{a}	28.33 ± 1.20^{a}	25.33 ± 0.67^{a}

Data are mean ± SEM, (n = 5). Values with superscripts alphabet that is different from the control for each parameter are significantly different ($p < 0.05$).

Similarly, a 28- day exposure of rats to AEHSC, significantly decreased ($p < 0.05$), in a dose-dependent manner, the levels of creatinine in rats, but there was a non-dose-dependent increase ($p < 0.05$) in rats that received AEHSC at dose levels of 125, 250 and 500 mg/kg bwt when compared to the control as shown in Table 2.

Serum concentration of Na^{+} showed a significant increase ($p < 0.05$) in rats exposed to 125 mg/kg while a decrease was observed in those exposed to doses of 250 and 500 mg/kg bwt of AEHSC for 28 days, but a prolonged (90 days) administration of the extract to rats at all considered dose levels did not significantly alter ($p > 0.05$) Na^{+} in test groups when compared with the control. Repeated 28 and 90 days oral administration of AEHSC to rats at all dosages, showed no significantly difference ($p < 0.05$) of K^{+} levels when compared to the control as shown in Table 2.

In both 28- and 90-days exposure period, all tested doses did not significantly alter the serum level of Cl^{-} in rats except at 500 mg/kg bwt where a 90-day exposure of rats resulted in a significant increase ($p < 0.05$) in exposed rats, when compared to the control rats.

In addition, the 500 mg/kg bwt of AEHSC significantly increase ($p < 0.05$) the serum level of HCO_3^{-} after a 28-day exposure period, whereas the 125 and 250 mg/kg bwt of the extract did not significantly increase the serum concentration of HCO_3^{-} in exposed rats when compared to the control (Table 2).

There was no significant alterations in RBC, Hb, MCV, MCH, MCHC and LYM for the sub-acute and sub-chronic administration. For the 28-day study, there was no significant change in WBC but a significant decrease in the 90-day study with 125 mg/kg AEHSC compared to the control. There was a significant increase in PLT for the 28-day study but

Table 3. Effects of administering orally sub-acute and sub-chronic dose of AEHSC on selected haematological parameters in Wistar rats.

Study	Parameter	Treatment (mg/kg bwt) AEHSC			
		Control	125	250	500
Sub-acute	PCV (%)	47.20 ± 1.02^{a}	52.40 ± 0.76^{b}	56.90 ± 1.25^{b}	46.40 ± 1.13^{a}
	RBC(10^3/μL)	7.80 ± 0.06^{a}	7.42 ± 0.06^{a}	7.39 ± 0.15^{a}	7.49 ± 0.13^{a}
	Hb(g/dL)	11.83 ± 0.10^{a}	10.78 ± 0.05^{a}	11.93 ± 0.13^{a}	11.38 ± 0.08^{a}
	MCV (fL)	59.18 ± 1.21^{a}	60.55 ± 0.50^{a}	58.00 ± 0.41^{a}	61.80 ± 1.48^{a}
	MCH (pg)	14.58 ± 0.11^{a}	14.48 ± 0.15^{a}	14.60 ± 0.11^{a}	15.18 ± 0.09^{a}
	MCHC (g/dL)	25.53 ± 0.15^{a}	24.03 ± 0.35^{b}	24.30 ± 0.16^{a}	24.40 ± 0.35^{a}
	WBC(10^6/μL)	14.08 ± 0.27^{a}	14.65 ± 0.20^{a}	14.80 ± 0.17^{a}	16.15 ± 0.43^{a}
	LYM(%)	84.83 ± 0.55^{a}	73.50 ± 1.13^{b}	83.60 ± 0.98^{a}	83.70 ± 0.36^{a}
	PLT (10^3/μL)	980.00 ± 14.07^{a}	1133.25 ± 27.30^{b}	1060.25 ± 3.54^{b}	1047.50 ± 7.79^{b}
Sub-Chronic	PCV (%)	46.44 ± 0.73^{a}	49.12 ± 1.13^{a}	44.70 ± 0.53^{a}	47.94 ± 0.73^{a}
	RBC (10^3/μL)	6.60 ± 0.20^{a}	6.76 ± 0.04^{a}	5.88 ± 0.06^{a}	6.38 ± 0.28^{a}
	Hb(g/dL)	10.17 ± 0.33^{a}	11.47 ± 0.37^{a}	10.10 ± 0.25^{a}	10.60 ± 0.24^{a}
	MCV (fL)	70.53 ± 2.42^{a}	69.37 ± 1.41^{a}	73.93 ± 1.47^{a}	74.93 ± 1.18^{a}
	MCH (pg)	15.40 ± 0.31^{a}	16.17 ± 0.18^{a}	17.20 ± 0.46^{a}	16.70 ± 0.30^{a}
	MCHC (g/dL)	21.90 ± 0.78^{a}	23.37 ± 0.19^{a}	22.67 ± 0.30^{a}	22.27 ± 0.17^{a}
	WBC(10^6/μL)	17.73 ± 0.33^{a}	5.40 ± 0.10^{b}	10.07 ± 0.07^{b}	12.03 ± 0.67^{b}
	LYM (%)	95.83 ± 0.43^{a}	93.33 ± 1.41^{a}	96.10 ± 0.00^{a}	97.23 ± 0.33^{a}
	PLT (10^3/μL)	845.67 ± 20.33^{a}	881.00 ± 5.00^{a}	564.33 ± 5.70^{b}	602.33 ± 48.29^{b}

Data are mean ± SEM, (n = 5). Values with superscripts alphabet different from the control for each parameter are significantly different ($p < 0.05$). AEHSC: Aqueous Extract of *Hibiscus Sabdariffa* Calyces; bwt: body weight; PCV: Packed Cell Volume, RBC: Red Blood Cell, Hb: Haemoglobin, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration, WBC: White Blood Cell, LYM: Lymphocyte, PLT: Platelet.

not for the 90 day study when compared to the control (Table 3). There was a significant increase in PCV at dose of 125 and 250 mg/kg for the 28-day study while no significant change for the 90-day study at all doses (Table 3).

There was a significant dose dependent decrease in total cholesterol especially at 500 mg/kg for sub-acute toxicity while that of the sub-chronic, there was no significant difference at all doses when compared to the control (Table 4). There was also no significant difference in triglycerides at both 28- and 90 -day study when compared to the control. For both 28- and 90-day study, there was a significant decrease in HDL-C at all the doses when compared to the control. While there was no significant change in LDL-C at all the doses for the 90- day study there was a significant decrease only at dose of 500 mg/kg for the 28 day study. At doses of 125 and 250 mg/kg, there was a significant increase in atherogenic index for the 28 day study while the 90 day study did not exhibit significant difference to the control group.

Following the treatment for 28 day, there was no significant change in alkaline phosphatase at all doses in the serum but a significant increase at dose of 250 and 500 mg/kg in the liver and kidney while for the 90 day treatment, there was significant decrease in the kidney and liver when compared with the control. For the 28 day study, there was a significant decrease in Alanine Transaminase in the liver, and kidney while there was a significant increase in serum level when compared with the control. Also, for the 90 day treatment, there was a significant decrease in alanine transaminase the liver and an increase in the kidney. The 28 day treatment exhibited a general increase in aspartate transaminase in the liver, kidney and serum while the 90 day treatment exhibited a significant decrease of aspartate transaminase in the liver and serum but no significant change in the kidney when compared to control (Table 5).

For the 28- day treatment, the Gammaglutamyl Transferase and Lactate Dehydrogenase in liver and serum did not exhibit significantly change from the control, but the 90- day treatment exhibited significant increase compared to the control (Table 5).

3.3. Histopathology on the liver and kidney

Histological analysis of sections of the liver and kidney in the rats administered with AEHSC (125, 250 and 500 mg/kg bwt) showed essentially normal histology comparable to the rats in the control group. The liver tissues showed preserved architecture, cords of normal hepatocytes with no features of acute or chronic damage and portal tracts with no significant inflammation (Plate 1 a–d). The kidneys show preserved architecture comprising normal glomeruli, tubules and vessels. There are no features of glomerular damage or evidence of acute or chronic tubular damage (Plate 1 e and f).

3.4. Diuretic activity of AEHSC

The results of the evaluation carried out on AEHSC are shown in Tables 6 and 7. The diuretic hydrochlorothiazide increased the urine volume to 3.78 ± 0.63 while AEHSC at the dose of 67.5, 125 and 250 mg/kg bwt showed marked diuresis during 18 h period as compared to the control group. There was no observed significant difference in the urine pH (Table 6). Diuretic index showed that diuretic action of the test groups was comparable to the reference standard especially at doses of 250 and 67.5 mg/kg.

The dose of 250 mg/kg produced a slight increase in K^+ excretion compared with the control group. It also produced a significant increase in Cl^- and Na^+ excretion. The dose of 125 mg/kg produced no significant K^+ loss but produced significant Cl^- and Na^+ loss. The dose of 67.5 mg/kg showed very significant Na^+ and Cl^- excretion. No significant K^+ loss was observed. The inhibition of renal tubular reabsorption of electrolytes seen in the experiment into the blood stream promotes the formation of urine.

4. Discussion

The global acceptance of medicinal plants for treatment of many diseases is as a result of their pharmacologically important phytoconstituents which are believed to be natural thus, associated with little or no toxicity. Some of these phytochemicals could be in organic or inorganic forms and are quite beneficial to the biological system at specific quantity. These pharmacologically active compounds form basis for the treatment of various ailments (Wang et al., 2014), and are frequently abused because they are taken in high doses. The excessive use of these medicinal plants may cause toxicity in major tissues or organs. Thus, toxicity studies are important in establishing the safety or detrimental effects of medicinal plants regardless of the duration of its usage. The LD_{50} in this study is in line with that reported by Hopkins et al. (2013).

The liver (the primary organ for the metabolism, detoxification and distribution of foreign agents), the kidney (major excretory organ) (Gupta et al., 1994) as well as blood are important in the assessment of safety or toxicity of medicinal plants. The synthetic or excretory potential of the liver for total protein, albumin, globulin and bilirubin is reduced if the function of the organ is challenged. Serum total protein level may increase in cases of dehydration and infections (causing increase in immunoglobin concentration), while a decrease may result due to impaired protein synthesis, over hydration, malabsorption, liver disease and hypogammaglobulinaemia (Saidu et al., 2007). In this study, the significant alteration of total protein levels in rats exposed to sub-acute oral doses may suggest that the synthetic function of the liver was affected. Albumin, a transport protein, is the most abundant plasma proteins synthesized by the liver. A reduction in albumin and globulin

Table 4. Effects of sub-acute and sub-chronic administration of AEHSC on lipid profile of Wistar rats.

Study	Parameter (mg/dL)	Treatment (mg/kg bwt) AEHSC			
		Control	125	250	500
Sub-acute	Total Cholesterol	4.26 ± 0.05[a]	6.48 ± 0.22[b]	4.35 ± 0.09[a]	2.02 ± 0.22[b]
	Triglycerides	4.04 ± 0.04[a]	4.31 ± 0.07[a]	4.20 ± 0.05[a]	3.75 ± 0.09[a]
	HDL-C	2.70 ± 0.15[a]	1.66 ± 0.01[b]	1.29 ± 0.09[b]	1.09 ± 0.02[b]
	LDL-C	2.40 ± 0.34[a]	4.22 ± 0.05[b]	2.86 ± 0.20[a]	1.43 ± 0.04[b]
	Atherogenic Index	1.30 ± 0.09[a]	2.59 ± 0.00[b]	2.18 ± 0.02[b]	1.15 ± 0.09[a]
Sub-Chronic	Total Cholesterol	4.51 ± 0.20[a]	4.49 ± 0.09[a]	4.32 ± 0.03[a]	4.58 ± 0.17[a]
	Triglycerides	3.17 ± 0.21[a]	3.16 ± 0.06[a]	3.41 ± 0.25[a]	4.11 ± 0.06[a]
	HDL-C	2.01 ± 0.12[a]	1.44 ± 0.27[b]	1.48 ± 0.06[b]	1.45 ± 0.11[b]
	LDL-C	2.35 ± 0.09[a]	2.21 ± 0.10[a]	2.42 ± 0.10[a]	2.50 ± 0.12[a]
	Atherogenic Index	1.32 ± 0.03[a]	1.40 ± 0.06[a]	1.63 ± 0.07[a]	1.81 ± 0.07[a]

Data are mean ± SEM, (n = 5). Values with superscripts alphabet that is different from the control for each parameter are significantly different ($p < 0.05$). AEHSC: Aqueous Extract of *Hibiscus Sabdariffa* Calyces, bwt: body weight; HDL-C: High Density Lipoprotein-Cholesterol; LDL-C: Low Density Lipoprotein-Cholesterol.

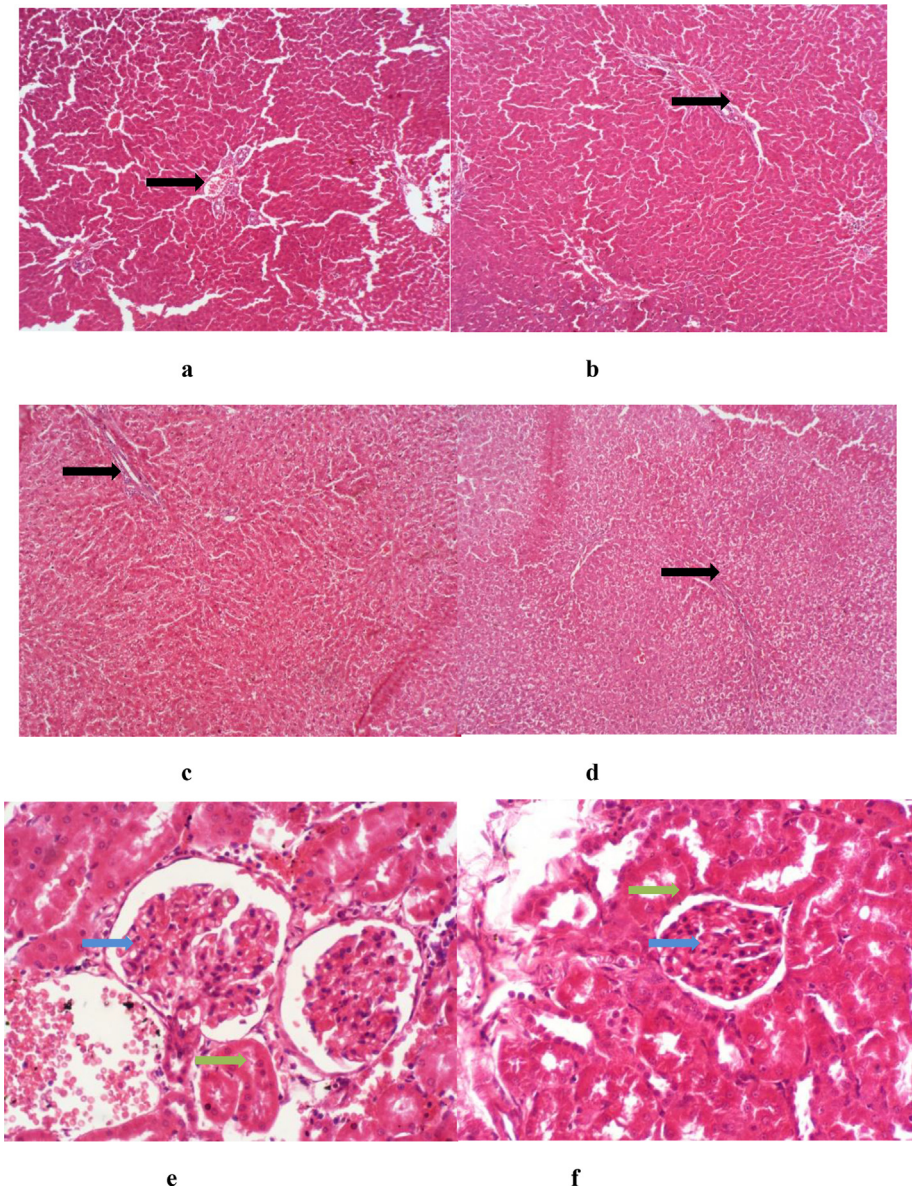

Plate 1. Histologic sections of liver and kidney following daily treatment with AEHSC at 250 and 500 mg/kg body weight respectively for 28 and 90 days. [Liver after 28 days (a & b) and 90 days (c and d) days); kidney after 28 days (e) and 90 days (f). (Black arrows show normal hepatocytes and portal tracts; blue arrows show normal glomeruli; green arrows show normal tubules.] H/E x 100.

serum concentration is apparent in liver damage and diseases. Increase in the serum levels of albumin as evident in this study is a signal of no liver damage or disease thus, normal synthetic ability of the liver.

Bilirubin (total and direct) is an important parameter for the assessment of the excretory function of the liver and haemolytic anaemia (Saidu et al., 2007). Bilirubin is produced upon breakdown of hemoglobin in the liver, spleen, and bone marrow. An increase in tissue or serum bilirubin level occurs through increased breakdown of RBC (hemolysis) or in the case of hepatitis or bile duct obstruction (liver damage) (Chitra et al., 2015). In the current work, AEHSC increased total bilirubin levels of the rats administered with 500 mg/kg bwt after a sub-acute exposure which may be suggestive of haemolytic anaemia in the animals. Also, decrease in direct bilirubin by sub-chronic doses of AEHSC may suggest that the liver excretory function is being compromised.

Kidney function was assessed using important predictive biomarkers such as urea, uric acid, creatinine as well as serum electrolytes. Creatinine and urea are non-protein nitrogenous end products of protein metabolism that must be removed continually. Hence, elevation of these kidney function indices is indicative of kidney dysfunction which is caused primarily by injury (Akindele et al., 2014). An increase in the serum concentration of creatinine in rats after sub-chronic exposure may suggest that the extract decreased glomerulus filtration rate which is a parameter for assessing kidney function. During deamination, NH_3 is removed from the blood by conversion into urea and an increase may be as a result of high glomerular filtration (Olaniyan et al., 2016). Elevation in urea concentrations with sub-acute and sub-chronic administration of AEHSC suggests efficient kidney function produced by the extract for removal of NH_3. Excess uric acid is caused by defects in the metabolism of purine nucleotides. Hyperuricaemia has a biochemical link with gout which may in turn result to arthritis (Saidu et al., 2007). Increase in serum concentrations of uric acid in rats exposed to various doses of sub-acute administration of AEHSC is an indication that AEHSC may predispose the animals to arthritis. Estimating levels of electrolytes (Na^+, K^+, Cl^- and HCO_3^-) in serum may be important in assessment of renal function since the outcome of regulatory mechanism of osmotic balance and ionic charges can be determined by the levels of electrolytes in the

Table 5. Effects of 28- and 90-day administration of AEHSC on activities of selected enzymes in Wistar rats.

Study	Enzyme	Organ/Tissue	Treatment			
			Control	125 mg/kg bwt AEHSC	250 mg/kg bwt AEHSC	500 mg/kg bwt AEHSC
Sub-acute	Alkaline Phosphatase (U/L)	Liver	9.81 ± 0.47^{a}	9.19 ± 0.48^{a}	11.87 ± 0.13^{b}	16.36 ± 0.57^{b}
		Kidney	4.75 ± 0.02^{a}	2.73 ± 0.18^{b}	4.55 ± 0.00^{a}	10.06 ± 0.37^{b}
		Serum	6.50 ± 0.67^{a}	6.12 ± 0.53^{a}	6.95 ± 0.33^{a}	3.69 ± 0.28^{b}
	Alanine Transaminase (U/L)	Liver	10.03 ± 0.49^{a}	7.61 ± 0.12^{b}	9.18 ± 0.33^{a}	7.57 ± 0.62^{b}
		Serum	7.48 ± 0.03^{a}	8.43 ± 0.56^{a}	7.48 ± 0.03^{a}	8.69 ± 0.17^{a}
	Aspartate Transaminase (U/L)	Liver	31.37 ± 0.92^{a}	27.49 ± 0.95^{b}	30.16 ± 0.48^{a}	35.95 ± 1.42^{b}
		Serum	6.34 ± 0.01^{a}	3.93 ± 0.08^{b}	3.08 ± 0.07^{b}	13.38 ± 1.00^{b}
	Gammaglutamyl Transferase (U/L)	Liver	6.15 ± 0.73^{a}	6.42 ± 0.70^{a}	5.31 ± 0.10^{a}	7.46 ± 0.53^{a}
		Serum	2.15 ± 0.12^{a}	2.37 ± 0.08^{a}	2.35 ± 0.15^{a}	2.93 ± 0.24^{a}
	Lactate Dehydrogenase (U/L)	Liver	8.53 ± 0.37^{a}	8.86 ± 0.10^{a}	9.54 ± 0.06^{a}	8.80 ± 0.04^{a}
		Serum	6.72 ± 0.42^{a}	3.53 ± 0.26^{b}	5.56 ± 0.10^{a}	4.94 ± 0.31^{b}
Sub-Chronic	Alkaline Phosphatase (U/L)	Liver	6.59 ± 0.69^{a}	6.42 ± 0.03^{a}	7.79 ± 0.51^{a}	3.77 ± 0.25^{b}
		Kidney	9.43 ± 0.35^{a}	8.46 ± 0.14^{a}	6.42 ± 0.34^{b}	6.28 ± 0.58^{b}
		Serum	5.12 ± 0.17^{a}	5.01 ± 0.41^{a}	4.96 ± 0.08^{a}	2.01 ± 0.15^{b}
	Alanine Transaminase (U/L)	Liver	5.59 ± 0.25^{a}	3.51 ± 0.16^{b}	3.78 ± 0.42^{b}	3.38 ± 0.18^{b}
		Serum	3.84 ± 0.18^{a}	3.22 ± 0.10^{a}	4.52 ± 0.42^{a}	3.70 ± 0.35^{a}
	Aspartate Transaminase (U/L)	Liver	9.86 ± 0.25^{a}	5.78 ± 0.26^{b}	6.99 ± 0.09^{b}	8.53 ± 0.23^{a}
		Serum	6.75 ± 0.44^{a}	6.16 ± 0.30^{a}	8.49 ± 0.14^{b}	4.66 ± 0.30^{b}
	Gammaglutamyl Transferase (U/L)	Liver	3.48 ± 0.09^{a}	2.54 ± 0.13^{b}	5.83 ± 0.04^{b}	7.06 ± 0.30^{b}
		Serum	2.80 ± 0.01^{a}	2.20 ± 0.05^{a}	2.85 ± 0.16^{a}	2.21 ± 0.07^{a}
	Lactate Dehydrogenase (U/L)	Liver	6.33 ± 0.11^{a}	8.45 ± 0.18^{b}	9.37 ± 0.25^{b}	9.91 ± 0.23^{b}
		Serum	3.36 ± 0.09^{a}	3.21 ± 0.11^{a}	3.87 ± 0.27^{a}	4.68 ± 0.17^{b}

Data are mean ± SEM, (n = 5). Values with superscripts alphabet that is different from the control for each parameter are significantly different ($p < 0.05$).

Table 6. Effect of AEHSC on urine volume, diuretic index and pH.

Parameter	Treatment				
	0.2 mL (control)	10 mg/kg HCT	67.5 mg/kg AEHSC	125 mg/kg AEHSC	250 mg/kg AEHSC
Urine Volume (mL)	1.53 ± 0.63^{a}	3.78 ± 0.81^{b}	3.88 ± 0.89^{b}	3.02 ± 0.70^{b}	3.98 ± 2.11^{b}
Diuretic Index	-	2.47	2.53	1.97	2.60
pH	8 ± 0.28^{a}	8.25 ± 0.25^{a}	8.25 ± 0.22^{a}	8.37 ± 0.11^{a}	7.83 ± 0.34^{a}

Data are mean ± SEM, (n = 5). Values with superscripts alphabet that is different from the control for each parameter are significantly different ($p < 0.05$). HCT: hydrochlorothiazide.

blood (Eccles, 1993). Sodium is the major cation of the extracellular fluid where it regulates acid-base equilibrium and protects the body against excessive fluid loss. Potassium is the major intracellular cation with similar role to those of sodium. However, imbalances in these ions may be due to renal failure or renal tubular acidosis or alkalosis (Eccles, 1993; Holmes, 1993). In this study, the serum electrolytes of rats exposed to both sub-chronic and sub-acute doses of the extract were altered which is a pointer that AEHSC may have significant influence on the electrolyte, acid-base balance and water, at these doses. This is suggestive that kidney function is being compromised as a result of renal damage or failure although there were no histological evidences of glomerular or tubular damages.

Enzymes such as ALP, AST, ALT, GGT and LDH are good indicators of liver function and important biomarkers for predicting toxicity (Hilaly et al., 2004). These enzymes have high activities in specific organ with little activity in the serum. Consequently, blood levels of AST and ALT increase upon exposure of organs to toxic agents (Crook, 2006). Increase in serum concentrations of these enzymes may be due to leakage of the enzyme to the blood stream. ALT is a more sensitive serum marker enzyme for liver damage compared to AST because of its production within the cells of the liver while AST can be found in other tissues (Adeyemi et al., 2010). Therefore, these enzymes are relevant in assessing hepatic dysfunction and damage (hepatocellular necrosis) because of their leakage into blood stream (Hassoun and Stohs, 1995). Thus, in this study, the increased activities of these enzymes in organs without a concomitant increase in their serum activities strongly suggest that the sub-acute and sub-chronic administration of AEHSC is non-toxic and did not damage cells of the liver. These findings are consistent with the normal histological features seen in the liver sections examined. The increase in ALT and AST is in line with studies carried out by Akindahunsi and Olaleye (2003) and Ali et al. (2003).

Evaluation of lipids such as TC, TG, HDL-C, LDL-C as well as atherogenic index (AI) can provide vital information on predisposition of the heart to atherosclerosis and lipid metabolism as well as other associated coronary heart diseases (Yakubu et al., 2008; Wang et al., 2014). AI is a more precise tool for the prediction of cardiovascular risk as it considers ratio of a bad cholesterol (TC, TG, LDL-C) to the good one (HDL-C). In the present study, the sub-acute and sub-chronic administration of AEHSC influenced lipid metabolism in rats with the 500 mg/kg bwt dose of the extract and the sub-acute exposure having the least effect. The AI was reduced at the dose (500 mg/kg) thus decreasing the chance of cardiovascular risk. The decrease in LDL-C in this study is in line with that reported by Olatunji et al. (2005).

Evaluation of haematopoietic indices can be used to assess the deleterious effects of toxic agents (Agbaje et al., 2009). Therefore, change in the hematopoietic system is predictive of toxicity in animals (Olson et al., 2000). Alteration of concentrations of PCV, RBC, Hb, MCV, MCH and MCHC is important in the diagnosis of anaemia. The non-significant

Table 7. Effect of AEHSC on Sodium, Potassium and Chlorine excretion in urine.

Variable	Treatment				
	0.2 mL (control)	10 mg/kg HCT	67.5 mg/kg AEHSC	125 mg/kg AEHSC	250 mg/kg AEHSC
Sodium (ppm/100 g/18 h)	0.3 ± 0.3^a	2.025 ± 1.24^b	62.25 ± 16.96^b	13.57 ± 8.88^b	7.05 ± 4.25^b
Potassium (ppm/100 g/18 h)	6.64 ± 0.089^a	7.89 ± 0.02^b	6.72 ± 0.162^a	6.31 ± 0.168^a	8.12 ± 0.045^b
Chlorine (ppm/100 g/18 h)	21.57 ± 6.71^a	117.16 ± 7.90^b	120.24 ± 6.59^b	155.24 ± 4.45^b	121.75 ± 9.39^b

Data are mean ± SEM, (n=5). Values with superscripts alphabet that is different from the control for each parameter are significantly different ($p<0.05$). HCT: hydrochlorothiazide.

change in these parameters after sub-acute and sub-chronic administration of AEHSC suggests that the extract, after prolonged use, may not induce anaemia in rats. WBC and differentials such as lymphocytes (the main effectors cells of the immune system) are used as indicators of the system's response to toxic and exogenous substances including plants (Adedapo et al., 2004). In this study, significant reduction in WBC, after sub-chronic administration of AEHSC to rats, without alteration of lymphocytes may suggest that the extract only exerted mild challenge (leukopenia) on the immune system of the animals. Platelets are one of the factors for blood coagulation to prevent bleeding in cases of injury. Reduction in platelet levels can result in distortion of the coagulation process, the implication being excessive bleeding. Significant reduction in platelet concentration after sub-chronic administration of AEHSC to rats suggests that the animals may be predisposed to excessive bleeding in the event of injury. However, sub-acute administration of the extract had an opposite effect in animals suggesting its preventive effects of bleeding.

The diuretic activity observed agrees with findings reported by other researchers for experimental animals (Aguwa et al., 2004). Chloride ion excretion was markedly increased in all groups. This result is in contrast with other reports which mention that the urinary excretion of chloride remains unchanged (Aguwa et al., 2004). The diuretic effect of AEHSC was confirmed by increase in excretion of sodium, chloride, potassium and urine volume. The observed diuretic effect of AEHSC may be due to inhibition of tubular re-absorption of water and electrolytes.

5. Conclusion

This research has provided vital information on the alterations in biochemical parameters as tools for establishment of toxicity. It was evident from results in this research that the extract was relatively less toxic with sub-acute than sub-chronic administrations. Though, non-toxic effects of sub-chronic use of the extract for few biochemical parameters were also established. These observations suggest that the aqueous extract of *Hibiscus sabdariffa* calyces is orally safe during sub-acute administration while the sub-chronic usage should be done with caution. The increased urine volume and electrolyte excretion confirm the diuretic potential of the plant and it produced diuresis comparable to the reference standard hydrochlorothiazide.

Declarations

Author contribution statement

N.S. Nginga and A.L. Quadri: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

A.T. Kola-Mustapha and M.T. Bakare-Odunola: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

O. Atolani, E.O. Ajani and L. Kambizi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

R.O. Ayanniyi, M.O. Buhari and O.O. Folaranmi: Performed the experiments; Analyzed and interpreted the data.

T.O. Amusa, E.O. Ajani, A.T. Oladiji and P.E. Ebong: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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