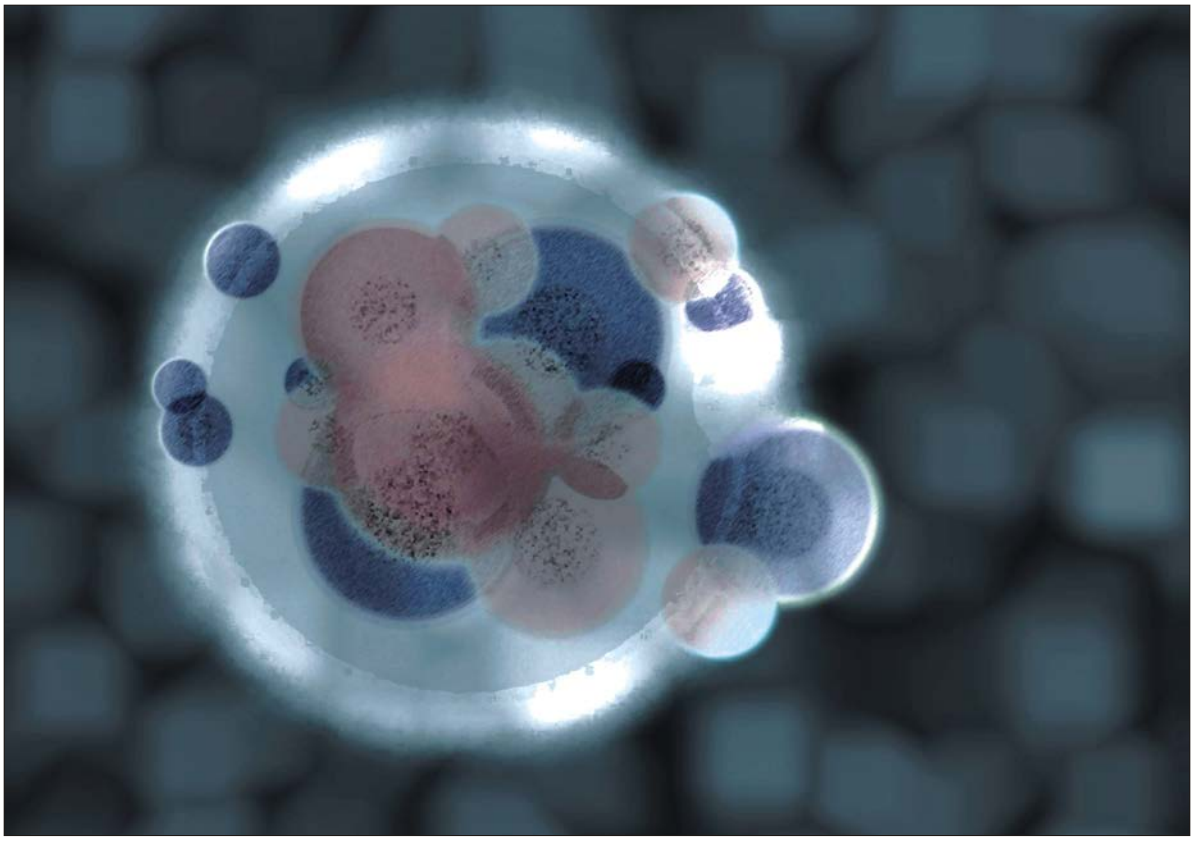

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# Protective Effect of Pretreatment of Rats with Calyx Extract of *Hibiscus sabdariffa* against Carbon Tetrachloride-induced Hematotoxicity

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ABSTRACT
Beneficial health impact of medicinal plants reported in scientific literature includes enhancement of antioxidant defense mechanism and amelioration of toxicities. The present study aimed to investigate the pre and post-treatment protective effects of methanolic extract of *Hibiscus sabdariffa* (MEHS) on $CCl_4$-induced hematotoxicity and alterations in lipid profile in rats. The 55 rats were randomly divided into 11 groups of 5 animals each. The pretreatment and post treatment groups were administered 200, 600 and 1000 mg $kg^{-1}$ doses of MEHS 10 days before and after oral administration of $CCl_4$ (2.5 mL $kg^{-1}$ b.wt.), respectively. Hematological parameters and serum lipids were analyzed by standard methods. The administration of $CCl_4$ significantly induced hematotoxicity as evidenced by decreased RBC, PCV and Hb with significantly increased WBC count ($p<0.05$). The pretreatment with MEHS and vitamin E significantly ($p<0.05$) increased the RBC, PVC and Hb concentration. Similarly, MEHS doses at 600 and 1000 mg $kg^{-1}$ and vitamin E 9.6 mg $kg^{-1}$ b.wt., significantly decreased WBC ($p<0.05$). In the post-treatment, there were beneficial significant changes in all treatments for PCV, at 1000 mg $kg^{-1}$ MEHS for Hb and at vitamin E administration for Hb and WBC count. However, there were no significant differences observed in both treatment approaches for serum lipid concentrations compared to $CCl_4$-treated rats ($p>0.05$). The data from this study suggest the prophylactic protective role of MEHS against hematotoxicity in carbon tetrachloride challenge.

**Key words:** Hematotoxicity, *Hibiscus sabdariffa*, lipid profile, vitamin E, carbon tetrachloride

## INTRODUCTION

Human exposure to toxic agents in both occupational and environmental settings is a global public health challenge. Environmental chemicals and drugs including acetaminophen and carbon tetrachloride have been reported in scientific literature for deleterious effects on the liver, kidneys, brain and hematopoiesis (Renugadevi and Prabu, 2010; Sharifudin *et al*., 2013; Rizwan *et al*., 2014). Traditionally, health concerns in exposed human populations have revolved around the association of environmental chemicals with anemia, bone disease, emphysema, diabetes, atherosclerosis, hypertension, cancer and inflammatory disorders (Ellinger *et al*., 2011; Anetor, 2012; Layachi and Kechrid, 2012). Carbon tetrachloride is used as an organic solvent in oil products, insecticide, resin, wax and organic diluent in rubber products and in cooling equipments; it is used as stain remover for furniture and carpets also in extraction of plants (Altug *et al*., 2007). Although, there are many anecdotal reports linking a number of chemicals to hematotoxicity, a chemically-induced toxicity of the blood, carbon tetrachloride is an important chemical associated with hepatotoxicity, nephrotoxicity and

hematotoxicity. For example, experimental liver damage produced by carbon tetrachloride has been extensively studied and the profile of damage after single administration has been well established (Dahiru *et al*., 2003). Effects such as fatty degeneration, fibrosis, hepatocellular apoptosis, carcinogenicity, microcytic hypochromic anemia, thrombocytopenia and lymphopenia in the blood as evidenced by the reduction in the PCV, RBC and platelets with increases in WBC differentials have been associated with $CCl_4$ toxicity in rats (Saba *et al*., 2010). Early studies have documented evidences to indicate the mechanism underlying $CCl_4$ toxicity and that well-established mechanism involves free radical and related reactive species generation. Carbon tetrachloride is activated into reactive metabolites through the action of the cytochrome P450 enzyme system mainly located in the liver in more abundant amount than in any other organ such as lung, kidney or intestine (Adesanoye and Farombi, 2010). The $CCl_4$ is activated by CYP2E1, CYP2B1 or CYP2B2, CYP2A and possibly CYP3A (Adesanoye and Farombi, 2010; Weber *et al*., 2003) to its intermediate trichloromethyl radical ($CCl_3^*$) which can react with oxygen to produce the trichloromethyl peroxy radical ($CCl_3OO^*$), a highly reactive specie in the presence of oxygen (Weber *et al*., 2003; Knockaert *et al*., 2012). A consensus has emerged that $CCl_4$ toxicity is a multifactorial process involving the generation of $CCl_4$-derived free radicals, lipid peroxidation, covalent binding to macromolecules, loss of calcium homeostasis, nucleic acid hypomethylation and inflammatory cytokines (Knockaert *et al*., 2012). After propagation of the peroxidation process, polyunsaturated lipids are finally degraded in small molecules such as malondialdehyde (MDA) or 4-hydroxynonenal (HNE), which are highly reactive aldehydes that can form protein and DNA adducts (Kadiiska *et al*., 2005). The oxidative scenario generates further Reactive Oxygen Species (ROS) to reduce endogenous antioxidant defense status leading to an imbalance called oxidative stress which has been implicated in the etiology of various chronic degenerative diseases.

However, toxicity of many chemicals may be prevented or ameliorated by traditional medicinal plants, as they have been severally reported to have multiple biological activities including free radical scavenging activity (Fakurazi *et al*., 2012). Phytochemical investigations on crude extracts of medicinal plants reported the presence of bioactive antioxidant compounds for myriads of nutritional and medicinal benefits (Mansour *et al*., 2014) and this has contributed immensely to the ongoing growing recognition and use of medicinal plants as complimentary or alternative remedies (Kumbhare *et al*., 2012). Thus, plants that scavenge or inhibit the formation of ROS and oxidative stress related disorders may have relevance to public health. Among the natural medicinal plants, *Hibiscus sabdariffa* is one widely known and consumed in Asia and Africa as a food, herbal drinks, beverages, flavoring agent in food industry and as a herbal medicine for health benefits. In folk medicine, it has been used to treat hypertension, inflammatory disease and cancer (Kuriyan *et al*., 2010). Various studies in male rats have demonstrated the hepatoprotective, nephroprotective, anticholesterolemic, antidiabetic and anti-obesity activities of different parts of *Hibiscus sabdariffa* (Da-Costa-Rocha *et al*., 2014). The flowers of *Hibiscus sabdariffa* contain anthocyanins, flavonoids, protocatechuic acid and polyphenols (Kuriyan *et al*., 2010) and studies have highlighted the role of these phytochemicals that may act as antioxidants to ameliorate oxidative damage to normal tissue (Adeyemi *et al*., 2014).

To our knowledge, the anti-hematotoxic potential of *Hibiscus sabdariffa* against $CCl_4$-induced hematotoxicity in rats has not been commonly reported. Hence, the present study focused on anti-hematotoxic potential of methanolic extract of *Hibiscus sabdariffa* calyx in $CCl_4$-induced hematotoxicity.

## MATERIALS AND METHODS

**Animals and assay kits:** The 55 albino rats of Wistar strain weighing between 150 and 200 g obtained from the experimental animal house, Department of Physiology, College of Medicine, University of Nigeria, Enugu Campus were used for this study. They were maintained on standard pellet diet and tap water *ad libitum* and kept in cages with wood chip beddings under a 12 h light/dark cycle and room temperature 24-26°C. Rats were acclimatized for 1 week prior to experiment and received humane care according to the criteria outlined and approved by the Animal Ethics and Care Committee, University of Nigeria. The lipid assay kits were supplied by Randox Laboratory Ltd. (Co. Antrim, UK). Carbon tetrachloride purchased from British Drug House (Poole, Dorset, UK) was used in this experiment. All other chemicals were of analytical grade and were prepared in all glass-distilled water.

**Plant extract:** The dry calyces of *Hibiscus sabdariffa* L. were purchased at Ogbette commercial market, Enugu State, Nigeria. Mr C. Okoli, a Botanist at Renaissance University, Ugbawka, Enugu State identified the calyces as *Hibiscus sabdarrifa* Linn. The calyces were cleaned with distilled water. The 100 g was ground with manual grinder and soaked in 80% methanol for 48 h. The ground calyces were further extracted with 5 changes of 80% methanol. The pooled extract was filtered with Whatman no. 42 filter paper and the filtrate concentrated in an oven at 40°C and stored at -20°C until required (Olatunji *et al*., 2005a). The percentage extract yield was around 3% of the dry weight of starting material. For the experiment described below, the extract was re-suspended in distilled water to the required concentration and administered to the rats by oral gavage.

**Animal treatment:** The 55 male albino rats were randomly distributed into 11 groups (n = 5) in wire mesh cages

on a 12 h light/dark cycle. Group 1, 2 and 3 were normal, $CCl_4$ (orally, 2.5 mL $kg^{-1}$ b.wt.) and MEHS (1000 mg $kg^{-1}$ b.wt.,) controls, respectively. Groups 4, 5 and 6 served as pretreatment groups and administered 200, 600 and 1000 mg $kg^{-1}$ b.wt., of MEHS, respectively, once daily by oral gavage for 10 days before single oral administration of 2.5 mL $kg^{-1}$ b.wt., of $CCl_4$. Group 7 was pretreated with vitamin E (9.6 mg $kg^{-1}$). Animals in group 8, 9 and 10 received 2.5 mL $kg^{-1}$ $CCl_4$ before administration of MEHS (post-treatment) with similar doses and period (Loki and Rajamohan, 2003). Group 11 was post-treated with vitamin E (9.6 mg $kg^{-1}$). All animals had free access to water and standard rat pellets throughout the experimental period. Animal welfare and experimental procedures were performed according to the approved protocols of the Institutional Animal Ethics and Care Committee, University of Nigeria, Nnsuka, Nigeria.

**Blood collection and preparation:** The 24 h after the last treatment, the rats were anaesthetized with diethyl ether and blood collected through the retro-orbital venous plexus into EDTA-coated sample bottles for hematological analysis while the sample for serum lipid profile was collected in plain sample bottles. The blood in plain bottles was kept at room temperature for 1 h and the serum obtained by centrifugation at 3000 rpm for 15 min was used for lipid profile analysis. All samples were stored in a refrigerator at 4°C before analysis.

**Analysis of lipid and hematological parameters:** All hematological and lipid profile parameters were determined by standard methods. From the blood samples, hemoglobin concentration (Hb) by cyanmethaemoglobin method, Red Blood Cell (RBC) and White Blood Cell (WBC) were counted using haemocytometer method as described by Ochei and Kolhatkar (2008). Pack cell volume (WBC) was determined by microhematocrit method as described by Ochei and Kolhatkar (2008). Total cholesterol concentration was determined by the method of Searcy and Bergquist (1960). Triglyceride concentration was determined according to the principle described by Tietz (1990). The HDL-cholesterol concentration was estimated using the precipitation method of Warnick *et al*. (1982) and LDL-cholesterol concentration was calculated using Friedwald's formula (Friedewald *et al*., 1972).

**Statistical analysis:** Data are presented as Mean±SEM and statistical significance was determined for the difference between the control and the experimental groups using the Student's t-test. $p<0.05$ was considered statistically significant.

## RESULTS

**Effect of methanolic extract of *Hibiscus sabdarifa* (MEHS) on hematological parameters in rats:** The effects of $CCl_4$ administration and the modulatory potential of methanolic extract of *Hibiscus sabdariffa* on the hematological parameters of the Wistar rats are shown in Table 1. The $CCl_4$ administration at a dose of 2.5 mL $kg^{-1}$ significantly decreased ($p<0.05$) the RBC, Hb concentration and PCV while WBC count was significantly higher ($p<0.05$) when compared with the control. There were non-significant effects of treatment with MEHS alone on the hematological parameters. Pretreatment of the rats with MEHS at every dose significantly ameliorated the deleterious $CCl_4$-induced alterations in hematological parameters, although the favorable decrease in WBC was not significant at the lowest dose (200 mg $kg^{-1}$). The pretreatment with vitamin E had significant effects on the parameters ($p<0.05$) compared to the $CCl_4$-treated rats.

Table 1 also presents the post-treatment with MEHS which elicited different effects on hematological parameters. The post-treatment with MEHS and vitamin E significantly ($p<0.05$) improved PCV as compared to the $CCl_4$-treated group. Although there were insignificant dose-dependent increases in RBC count and Hb concentration in all treatments, significant increases were particularly observed at 1000 and 9.7 mg $kg^{-1}$ doses of MEHS and vitamin E, respectively for Hb concentration ($p<0.05$). Similarly, treatment with vitamin E alone significantly decreased WBC count (Table 1).

**Effect of methanolic extract of *Hibiscus sabdarifa* (MEHS) on serum lipids of rats:** In Table 2, the results show that administration of MEHS and vitamin E for pre and post-treatment demonstrated no significant changes in all lipid parameters observed in this study ($p>0.05$).

Table 1: Effect of pre and post-treatment with methanolic extract of *Hibiscus sabdariffa* (MEHS) on hematological parameters of rats

| Treatment groups | RBC ($\times10^6$) $L^{-1}$ | WBC $mm^{-3}$ | PCV (%) | Hb (mg $dL^{-1}$) |
|---|---|---|---|---|
| Control | 196.00±5.09 | 9280±320 | 40.60±0.87 | 12.20±0.56 |
| $CCl_4$ | 152.00±6.63* | 10680±307* | 29.80±2.97* | 10.00±0.72* |
| MEHS | 200.00±1.60 | 9180±421 | 41.40±1.63 | 12.28±0.21 |
| MEHS I+$CCl_4$ | 183.00±3.74** | 9960±592 | 39.00±0.93** | 11.28±0.16** |
| MEHS II+$CCl_4$ | 189.00±14.26** | 9580±496** | 39.20±1.32** | 11.50±0.26** |
| MEHS III+$CCl_4$ | 190.00±3.53** | 9400±334** | 39.40±0.93** | 11.30±0.34** |
| Vit E+$CCl_4$ | 194.00±11.57** | 9700±564** | 40.40±0.87** | 12.00±0.17** |
| $CCl_4$+MEHS I | 166.00±11.61 | 10660±188 | 36.00±1.52** | 11.00±0.69 |
| $CCl_4$+MEHS II | 170.00±9.52 | 9900±462 | 36.20±1.39** | 11.30±0.58 |
| $CCl_4$+MEHS III | 177.20±5.69 | 9850±454 | 38.60±1.63** | 11.55±0.58** |
| $CCl_4$+Vit E | 167.00±11.26 | 9280±547** | 37.20±1.11** | 11.78±0.48** |

MEHS I: 200 mg $kg^{-1}$ methanolic extract of *Hibiscus sabdariffa*, MEHS II: 600 mg $kg^{-1}$ methanolic extract of *Hibiscus sabdariffa*, MEHS III : 1000 mg $kg^{-1}$ methanolic extract of *Hibiscus sabdariffa*. The results are the Mean±SD for 5 rats in each group, Significantly different from controls: *$p<0.05$. Significantly different from $CCl_4$-treated group: **$p<0.05$, RBC: Red blood cells, WBC: White blood cells, PCV: Packed cell volume, Hb: Hemoglobin

Table 2: Effect of pre and post-treatment with methanolic extract of *Hibiscus sabdariffa* (MEHS) on serum lipids of rats, expressed in mg $dL^{-1}$

| Treatment groups | HDL-C | LDL-C | TG | TC |
|---|---|---|---|---|
| Control | 1.46±0.04 | 0.98±0.03 | 1.32±0.06 | 3.12±0.18 |
| $CCl_4$ | 1.32±0.03 | 1.16±0.05 | 1.40±0.04 | 3.12±0.10 |
| MEHS | 1.38±0.06 | 0.72±0.06 | 1.52±0.08 | 2.78±0.17 |
| MEHS I+$CCl_4$ | 1.42±0.10 | 1.05±0.07 | 1.48±0.07 | 3.26±0.08 |
| MEHS II+$CCl_4$ | 1.40±0.01 | 0.92±0.04 | 1.20±0.07 | 3.10±0.14 |
| MEHS III+ $CCl_4$ | 1.48±0.09 | 0.90±0.06 | 1.32±0.02 | 2.96±0.19 |
| Vit E+$CCl_4$ | 1.44±0.08 | 0.86±0.03 | 1.32±0.02 | 3.32±0.11 |
| $CCl_4$+MEHS I | 1.44±0.03 | 1.06±0.04 | 1.52±0.08 | 3.40±0.21 |
| $CCl_4$+MEHS II | 1.46±0.02 | 0.96±0.04 | 1.48±0.07 | 3.46±0.15 |
| $CCl_4$+MEHS III | 1.46±0.02 | 0.48±0.06 | 1.40±0.06 | 3.04±0.13 |
| $CCl_4$+Vit E | 1.48±0.02 | 0.48±0.06 | 1.42±0.06 | 2.92±0.02 |

MEHS I:200 mg $kg^{-1}$ methanolic extract of *Hibiscus sabdariffa*, MEHS II: 600 mg $kg^{-1}$ methanolic extract of *Hibiscus sabdariffa*, MEHS III: 1000 mg $kg^{-1}$ methanolic extract of *Hibiscus sabdariffa*, The results are the Mean±SD for 5 rats in each group. Significantly different from controls: *p<0.05. Significantly different from $CCl_4$-treated group: **p<0.05, HDL-C: High density lipoprotein-cholesterol, LDL-C: Low density lipoprotein cholesterol, TG: Triglycerol, Tc: Total cholesterol

## DISCUSSION

The toxicity of $CCl_4$ depends upon its metabolism by cytochrome P-450 in hepatocytes, which generates highly reactive trichloromethyl free radicals, leading to lipid peroxidation and membrane damage (Hamza, 2010). The effect of the free radicals may culminate into deformations in cytoskeletal membrane protein of RBC due to its vulnerability to oxidative damage to development of anemia (Budinsky, 2000; Hale *et al*., 2011). Although hematotoxicity is not prevalent (Budinsky, 2000), it is useful to be aware of the antidote and natural treatment available with a great deal less harm than conventional therapies.

In this study, we investigated the effect of MEHS against hematotoxicity induced by carbon tetrachloride by the estimation of some hematological parameters. Oral administration of $CCl_4$ was found to cause anemia and leucocytosis in the blood as evidenced by marked decrease in RBC, PCV and Hb concentration with a corresponding increase in WBC. The leucocytosis may be suggestive of induced stress by $CCl_4$ in the rats, as it is known that $CCl_4$ is an established hepatotoxicant (Adesanoye and Farombi, 2010) that generates oxidative stress through CYP P450-mediated metabolism. This confirms the earlier reports on $CCl_4$ toxicity associated with microcytic hypochromic anemia, thrombocytopenia, increased erythrocyte fragility and leukocytosis (Saba *et al*., 2010). The non-significant effect of the extract on hematological parameters in MEHS control rats indicates that consumption of *Hibiscus sabdariffa* extract may not cause any significant deleterious effect on red blood cells, hemoglobin concentration and white blood cells. Similar result was obtained in a study by Olatunji *et al*. (2005a) that reported non-significant effect of red and green petal extracts of *Hibiscus sabdariffa* on hematological parameters. Agbai and Nwanegwo (2013) conducted an acute toxicity test study on methanolic calyx extract of *Hibiscus sabdariffa* and found that $LD_{50}$ was above 5000 mg $kg^{-1}$. These findings are consistent with the dose administered in MEHS control group in this study that demonstrated no toxicity in the blood. In contrast however, Olatunji *et al*. (2005b) found that *Hibiscus sabdariffa* extract decreased RBC, Hb concentration, hematocrit and platelet count, although WBC count, percentage neutrophils and lymphocytes were not significantly affected.

Our study demonstrated hematoprotective role of *Hibiscus sabdariffa* in $CCl_4$ challenge, particularly in the pretreatment procedure. The administration of MEHS at various doses ameliorated hematotoxic effects of $CCl_4$ on hematological parameters. Reduction of RBC and/or hemoglobin concentration has been associated with microcytic hypochromic anemia which is similar to our observation in $CCl_4$-treated rats in the current study. Pretreatment with *Hibiscus sabdariffa* extract was observed to significantly increase the PCV, RBC, Hb values in dose dependent manner with beneficial decrease in WBC. Our results corroborated earlier reports that the extract increased PCV, RBC and Hb concentration (Olusola *et al*., 2012; Agbai and Nwanegwo, 2013; Emelike and Dapper, 2013). Studies have highlighted the role of polyphenolic acid, flavonoids and anthocyanins that may act as antioxidants contributing to *Hibiscus sabdariffa* medicinal merits (Da-Costa-Rocha *et al*., 2014). The observed effects of the extract in this study might be due to the high ascorbic acid component (Falade *et al*., 2005) or other antioxidants present in the extract, which possibly reduced the free radicals and enhanced erythropoiesis for blood cell formation. For example, anthocyanins, a group of flavonoid derivatives and natural pigments present in the dried flowers of *Hibiscus sabdariffa* (Da-Costa-Rocha *et al*., 2014), which reduces loss of blood cells to lipid peroxidation (Degenhardt *et al*., 2000; Ologundudu *et al*., 2009) has been implicated to induce renal secretion of erythropoietin, a biological signal for differentiation and multiplication of the pluripotent stem cells involved in blood formation (Kaur and Kapoor, 2005; Agbai and Nwanegwo, 2013). Increase in hemoglobin concentration further support the use of this medicinal plant in folk medicine against anemia. Authors of previous research reported high concentration of iron in the calyx of this plant (Heda and Bhatia, 1986; Ismail *et al*., 2008) and this may be associated with the

observed increase in hemoglobin concentration for this study. The effect of the extract on hematotoxicity was comparable with Vitamin E, a known lipid free radical scavenger. The increase in white blood cells was an immunological response to the foreign agent, $CCl_4$ in the animals. Pretreatment of animals with MEHS extract prior to $CCl_4$ intoxication showed a feedback effect with an observed significant reduction in WBC count. However, we found non-significant effect of the administration of the extract at all doses on all lipid parameters (Table 2), although in the pre and post-treatments, there were some gradual changes in dose-dependent manner. A number of studies, however, it has reported hypolipidemic effect of *Hibiscus sabdariffa* extract in experimental rats at lower doses but not in $CCl_4$-intoxication model (Olatunji *et al*., 2005a; Yang *et al*., 2010; Da-Costa-Rocha *et al*., 2014). Our data might be interpreted to mean that the consumption of methanolic extracts of *Hibiscus sabdariffa* calyx up to 1000 mg $kg^{-1}$ b.wt., may not cause any significant deleterious effect on HDL-cholesterol, LDL-cholesterol and triglycerides (Table 2). There may be a need for further investigation on the role of this important extract on cholesterol metabolism especially in the context of $CCl_4$ toxicity.

## CONCLUSION

The findings from this study suggest a prophylactic hematoprotective role of methanolic extract of *Hibiscus sabdariffa* calyx against carbon tetrachloride-induced anemia and leucocytosis. This beneficial effect may be due to the bioactive antioxidant phytochemicals associated with the extract of *Hibiscus sabdariffa*. Serum lipids traditionally associated with health status may not be adversely affected by the consumption of *Hibiscus sabdariffa* crude extract. However, further studies on the effect of chronic administration of *Hibiscus sabdariffa* extract on cellular blood components and lipid metabolism is suggested.

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