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Hepatoprotective effects of hydroethanol extracts of *Citrus aurantifolia* fruits in CCl₄-induced hepatotoxic rats.**Israel Oghenevwodokohwo Okoro**

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Abstract:

The current study assessed the hepatoprotective effects of hydroethanol (ethanol: water 50:50 v/v) extract of *Citrus aurantifolia* fruit in carbon tetrachloride (CCl₄) intoxicated rats. Male Wistar albinos rats (150 -200 g) were separated into six groups (n =6) and treated for 12 days (orally); Group 1= Rats + distilled water (negative control), Group 2 = Rats + CCl₄ (positive control), Group 3 = Rats + 25 mg/kg p.o of silymarin, while Groups 4, 5 and 6 rats received 150, 300 and 600 mg/kg bw of *C. aurantifolia* extract respectively. The results indicated that exposure of animals to CCl₄ caused significant (p <0.05) increase in the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), malondialdehyde (MDA), triglycerides (TAG), cholesterol (CHOL) and LDL and decreased levels of HDL, protein, albumin and superoxide dismutase (SOD) and catalase (CAT) in the liver of rats relative to the positive control group. However, pre-treatment and continuous treatment of CCl₄ induced hepatotoxic rats with extracts of *C. aurantifolia* fruits caused significant (p <0.05) dose dependent reduction in AST, ALT, ALP, MDA, TAG, CHOL and LDL levels and increase in the levels of HDL, protein and albumin and activities of SOD and CAT in the liver tissues, when compared with the positive control rats, with 600 mg/kg dose of extract exhibiting good hepatoprotective effect comparable to that of silymarin. Thus, the extract of *C. aurantifolia* fruits possess liver protecting effects, which may be partly, connected to its antioxidant properties.

Keywords: Hepatoprotective effects, *Citrus aurantifolia*, carbon tetrachloride (CCl₄), Wistar albino rats, silymarin, antioxidant properties.

Introduction

The liver is a key organ linked with several aspects of physiologic homeostasis and metabolism of xenobiotics in an organism. It is principal in lipid metabolism modulation, has significant role in cholesterol metabolism, gluconeogenesis, lipoproteins and triglycerides synthesis from fatty acids. Liver risk factors which results in cirrhosis, hepatitis and other liver related diseases include free radicals, food additives, alcohol, xenobiotics, environmental and pharmaceutical chemicals and pollutants. Thus, as a main detoxifying and metabolizing organ in the body, the liver is likely to be endangered by resultant toxicities from its role in conversion of xenobiotics and other vast arrays of metabolic activities (Valko *et al.*, 2007; Farrel *et al.*, 2002).

Liver injury which can be caused by numerous features like viruses, organic chemicals, alcohol, genetic and metabolic abnormalities can be life frightening (Robin *et al.*, 2012). Hence, there is a growing need for other natural solutions to the problems of liver damage apart from liver transplantations, which have several limitations. In this regard, plant-based products with potent antioxidant and liver rejuvenation/protective abilities have been suggested as alternatives, since they are affordable and may have lower risk factors and limitations when compared to liver transplantation (Van Wyk and Wink, 2015).



Furthermore, the search for safer and better hepatoprotective agents is necessitated by the growing incidence of substance abuse, alcoholism, cigarette smoking and other unwholesome lifestyle choices such as eating fatty foods, which have added to the indisposition and related mortality rates occasioned by liver disease (Scott Luper, 1998). Topical researches have revealed that plants in Rutaceae family like *Citrus limon*, *Murraya koenigii*, *Tephrosia purpura* and *Citrus aurantium* possess liver protective activity (Bhavsar, 2007; Jain, 2006; Karaca, 2005). Hepatoprotective analysis of *C. maxima* and *C. hystrix* leaves showed that they have the capacity to restore liver function and the markers of oxidative stress affected by paracetamol induced hepatotoxicity to the desirable levels in rats. This is supported by the antioxidant ability of the extracts against paracetamol induced free radical toxicity (Abirami *et al.*, 2015).

Citrus aurantifolia (Key lime), is of South East Asia origin and of the Citrus family. *C. aurantifolia* comes naturally as either sweet or sour, with the sour type having more citric acid and sugar contents than lemons (Enejoh *et al.*, 2015; Bina *et al.*, 2010). The nutritional profile of *C. aurantifolia*, which includes carbohydrates, insoluble and soluble fiber, sugar, sodium, fatty acids, vitamins, minerals and amino acids have been investigated. Key lime is shown to contain rare flavonoid compounds with antioxidant and antibiotic properties (Tomotake, 2006). It is also known to display bioactive activities for sore throats, colds, fevers, asthma, sinusitis and bronchitis (Khan *et al.*, 2012). *C. aurantifolia*, has been shown to reduce oxidative stress through inhibition of cellular lipid peroxidation and by causing an increase in cellular antioxidant systems (Karoui and Marzouk, 2013). Its juice is known to possess antioxidants which have been shown to stop metal-induced toxicities (Obiamme *et al.*, 2010; Sajitha *et al.*, 2010).

Correspondingly, *C. aurantifolia* has been used customarily as dyspepsia, laxative, stomachic, appetizer, digestive, anthelmintic, flatulence and helminthiasis (Liu *et al.*, 2012). Several researches and reports about the medicinal importance of *C. aurantifolia* leaves have been

given, which include, radical scavenging activity, anticancer, antimicrobial and antifungal activity (Oikeh *et al.*, 2016; Pathan *et al.*, 2012; Jayaprakasha *et al.*, 2008; Poulouse *et al.*, 2005). Also, its insecticidal and repellent activity against mosquitoes and *Camponotus nearcticus* has been reported (Mensah *et al.*, 2014; Effiom *et al.*, 2012). The phyto-constituents of *C. aurantifolia* leaf extracts using different solvents (saponins, reducing sugars, flavonoids, alkaloids, tannins, steroids, cardiac glycosides) and also their antimicrobial activities had been reported (Akinnibosun and Edionwe, 2015).

The essential oils from *C. aurantifolia* has been stated to cause reduction in body weight/ obesity and food consumption by promoting anorexia, and has been recommended for weight loss and in the management of drug-induced obesity and allied diseases (Patil *et al.*, 2013). Also, the oil has antidepressant property due to its ability to promote refreshment to the mind. The leaves are also useful against rheumatism arthritis, clearing of oily skin and acne and as remedy against insect bites, herpes and cuts (Srinivasan *et al.*, 2008; Scora, 1975). The cytoprotective effect of *C. aurantifolia* extracts against Aflatoxin B1-induced liver injury has been reported as well (Forastiere *et al.*, 2000). However, little or no information exist in the literature about the liver protective ability of the fruit of *C. aurantifolia*. Hence, this work was designed to investigate the hepatoprotective effects of the Hydroethanol (ethanol: water 50:50 v/v) extract of *C. aurantifolia* fruit in CCl₄ intoxicated rats.

Materials and methods

Collection and extraction of fruits

Citrus aurantifolia fruits were purchased at Abraka market and authenticated at the Plant Science Department, University of Benin, Benin City, Edo State-Nigeria. They were washed thoroughly with tap water, after which their outer skins were carefully removed and extracted using juice extractor. The juice was concentrated at 40°C and extracted (20 g) with 200 mL hydroethanol for 48 h and filtered using Whatman No. 1 filter paper. The filtrate was rotary evaporated at reduced pressure and temperature and the extract was kept at 4°C until use.

Animals and experimental design

The male Wister albino rats (150–200 g) used for the study were obtained from the Anatomy Department of the Delta State University. They were acclimatized for two weeks, and fed with standard feeds (Top Feeds Ltd., Sapele, Delta State) and water *ad libitum* throughout the experiment. The rats were separated into six groups with six animals per group and were treated for 12 days (orally) as follows:

Group 1 – Rats + distilled water (negative control).

Group 2 – Rats + CCl₄ (positive control).

Group 3 – Rats + 25 mg/kg p.o of silymarin.

Group 4 – Rats + 150 mg/kg bw of *C. aurantifolia* extract.

Group 5 – Rats + 300 mg/kg bw of *C. aurantifolia* extract.

Group 6 – Rats + 600 mg/kg bw of *C. aurantifolia* extract.

The CCl₄ (1 mL/kg body weight), prepared in liquid paraffin (30% concentration) was given 72 hours following the first silymarin/extract administration and next every other 72 hours. The rats were fasted after the last administration and sacrificed to get the blood and organs used for the biochemical assessments.

Biochemical Assays

The biochemical markers of liver function like ALP, ALT, AST, total protein and albumin, and lipid profile (TAG, CHOL, HDL and LDL) were done according to specifications of the suppliers (Randox Kits). While superoxide dismutase (SOD) was by Marklund and Marklund, 1974 method, catalase (CAT) by the method of Aebi, 1983, and malondialdehyde (MDA) was done by Ohkawa *et al.*, (1979) method.

Statistical Analysis.

Data are presented as Mean \pm standard deviation by GraphPad Prism (6.0 version) and were analyzed utilizing the one-way analysis of

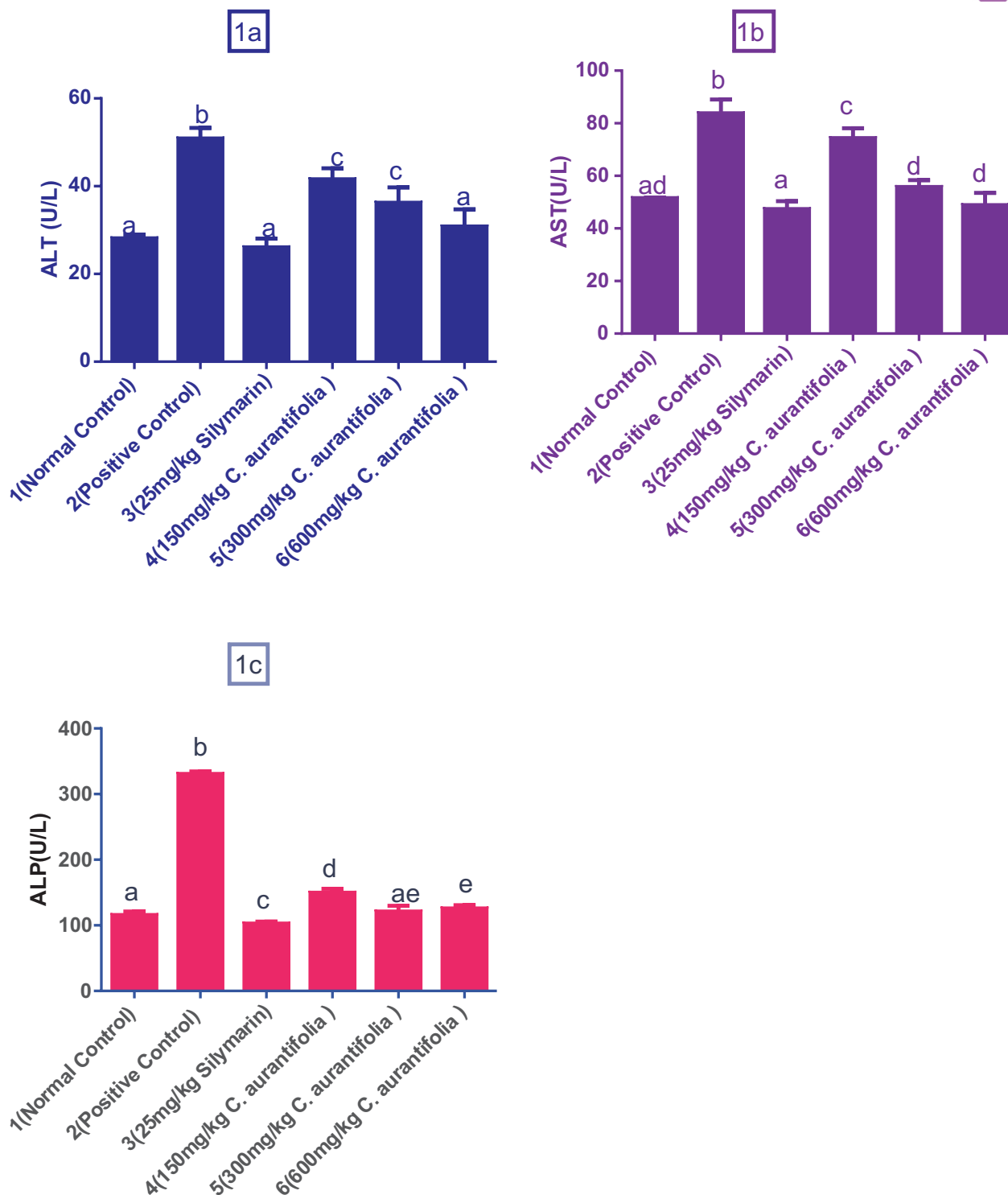
variance (ANOVA), where differences were compared by Tukey's Multiple Comparison Test. A p-value of < 0.05 was considered statistically significant in statistical comparison.

Results

Effects of hydroethanol extracts of *Citrus aurantifolia* fruits on liver functioning of CCl₄ intoxicated rats.

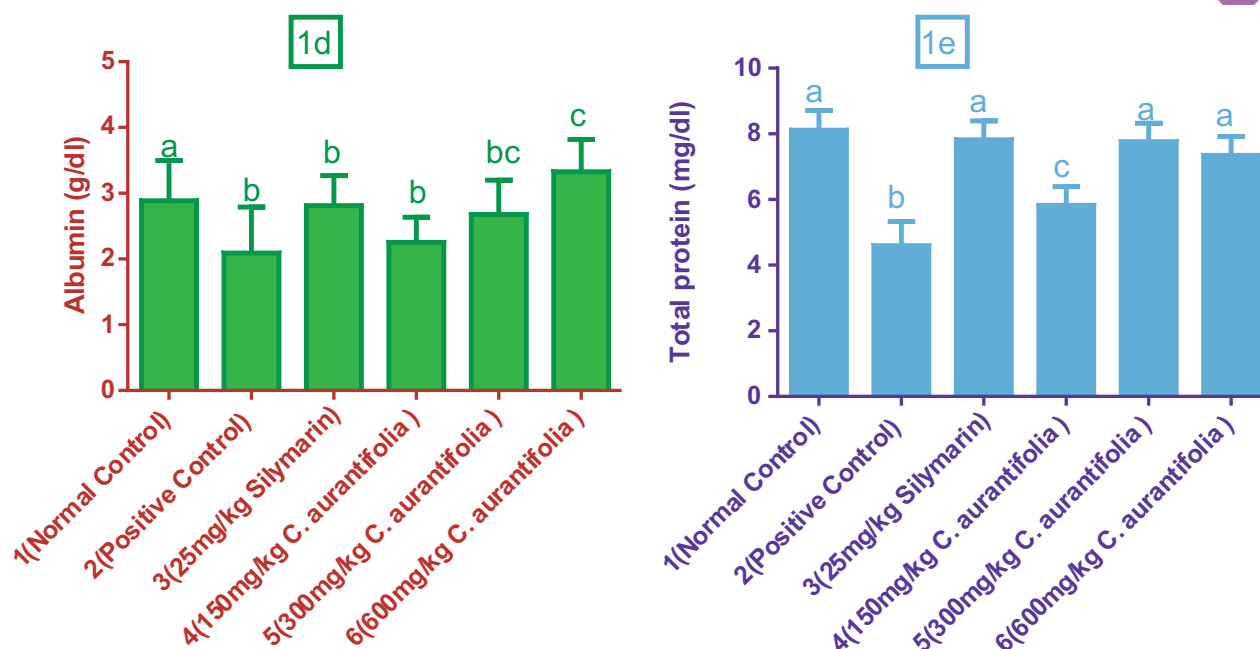
The exposure of animals to CCl₄ displayed severe perturbations in the levels of hepatic indicators (AST, ALT, and ALP) in the rats. The results shown in Figures 1 a, b and c, indicated that CCl₄ caused suggestive rise in the activities of the indicator enzymes. In difference, there was considerable ($p < 0.05$) reduction in these enzymes' activities for the normal control rats. Upon pretreatment and continuous treatment of CCl₄ induced rats with extracts of *C. aurantifolia* fruits, a significant improvement comparable to that of standard drug, in the levels of the main enzymes' activities (AST, ALT, and ALP) was seen both in the medium and highest extract dose. Thus, the extent of improvement was dose dependent. Equally, a distorted pattern was seen for other markers; a significant ($p < 0.05$) decreased level of protein and albumin was noted in the CCl₄ induced (positive control) rats relative to the normal control group (Figures 1 d and e). However, there was no significant difference between the treated groups (150 and 300 mg/kg) when compared with the CCl₄ control rats for albumin except for the highest dose (600 mg/kg). Also, significantly ($p < 0.05$) higher total protein levels were observed when the treated rats were compared with the CCl₄ control ones.

Consequently, the administration of CCl₄ to rats caused considerable hepatocellular damage as proved by the raised serum liver enzymes (ALT, AST and ALP) and decreased protein and albumin levels.



Figures 1a, b and c: Effects of hydroethanolic extracts of *Citrus aurantifolia* fruits on liver marker enzymes of CCl₄ intoxicated rats.

* Values are as Mean \pm standard deviation (n=6) **Bars with unlike letters vary significantly (p<0.05)



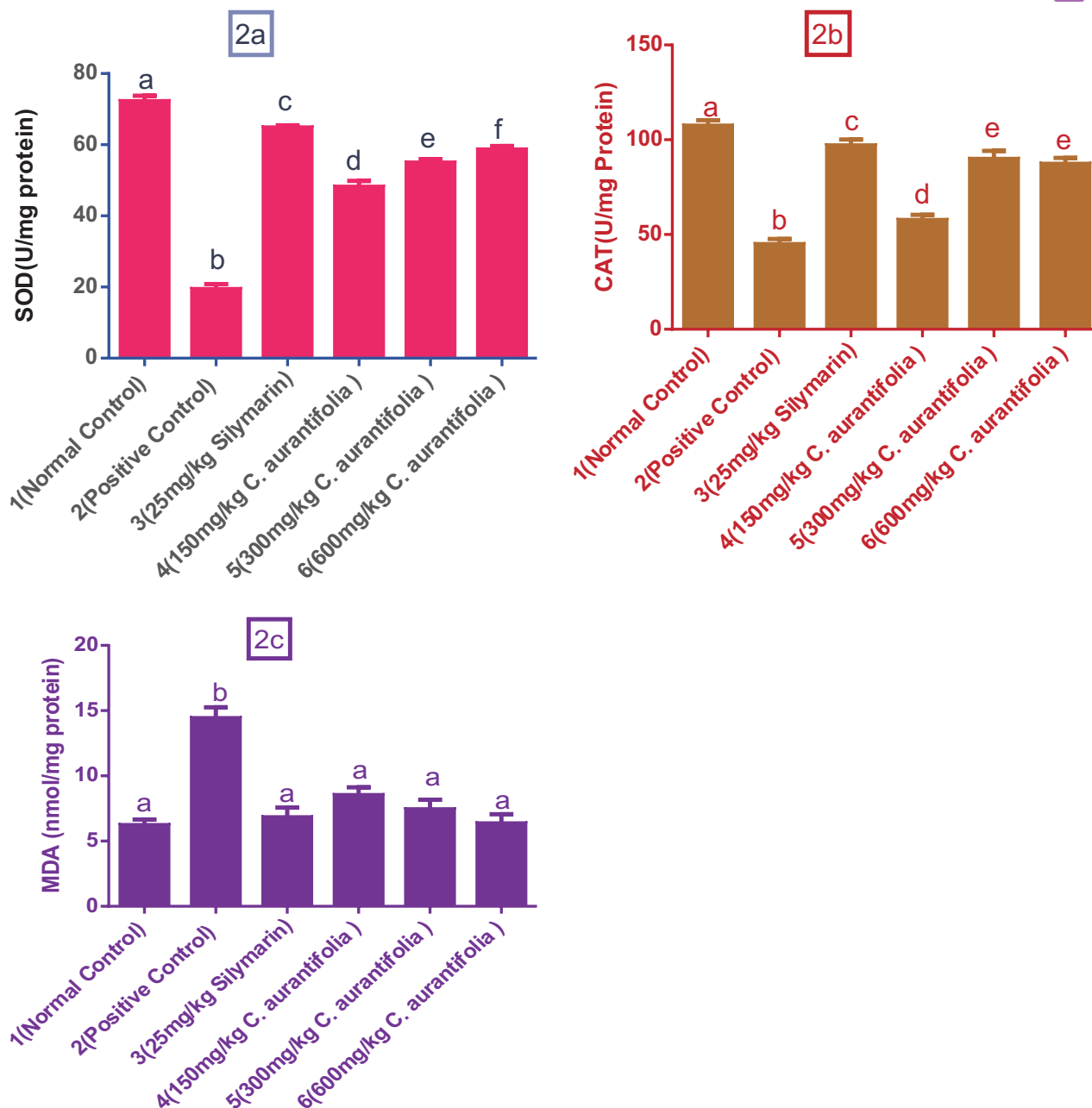
Figures 1d and e: Effects of hydroethanol extracts of *Citrus aurantifolia* fruits on liver functioning of CCl₄ intoxicated rats.

* Values are as Mean ± standard deviation (n=6) **Bars with unlike letters vary significantly (p<0.05)

Effects of hydroethanol extracts of *Citrus aurantifolia* fruits on oxidative stress markers of CCl₄ intoxicated rats.

The administration of CCl₄ was seen to cause significant (p <0.05) increase in the MDA levels in rat liver (Figure 2). However, treatment of animals with extracts of *C. aurantifolia* fruits caused significant decrease in the MDA levels to near normal values. Also, intoxication of rats with CCl₄ produced marked (p <0.05) decline in activities of the antioxidant enzymes (SOD and CAT) in the liver. But, treatment with either silymarin or the extracts of *C. aurantifolia* fruits

brought about significant (p <0.05) reduction in SOD and catalase activities relative to the CCl₄ induced control rats. Thus, the results showed a reduction in liver biochemical parameters (SOD and CAT) and an increase in lipid peroxidation in the CCl₄ induced rats when compared with the normal control ones (Figure 2). However, treatment of the CCl₄ intoxicated rats with extracts of *C. aurantifolia* fruits caused significant recovery in the CCl₄ induced rats as reflected in the results for the oxidative stress markers in this experiment.



Figures 2 a, b and c: Effects of hydroethanol extracts of *Citrus aurantifolia* fruits on oxidative stress markers of CCl4 intoxicated rats.

* Values are as Mean ± standard deviation (n=6) **Bars with unlike letters vary significantly (p<0.05)

Effects of hydroethanol extracts of *Citrus aurantifolia* fruits on lipid profile of CCl4 intoxicated rats.

Shown in Figure 3 are the results for the effects of hydroethanol extracts of *C. aurantifolia* fruits on the lipid profile of CCl4 intoxicated rats. The results showed changes in the total cholesterol, HDL, TAG and LDL levels of CCl4 induced animals. Thus, a significantly (p <0.05) higher

levels of TAG, CHOL and LDL, and decreased level of HDL was seen in the positive control rats relative to the normal control ones. However, administration of *C. aurantifolia* fruits extracts was seen to cause a significant (p <0.05) recovery in the rats as reflected in the lower TAG, CHOL and LDL levels and higher level of HDL in the extract treated groups when compared with the CCl4 control rats.

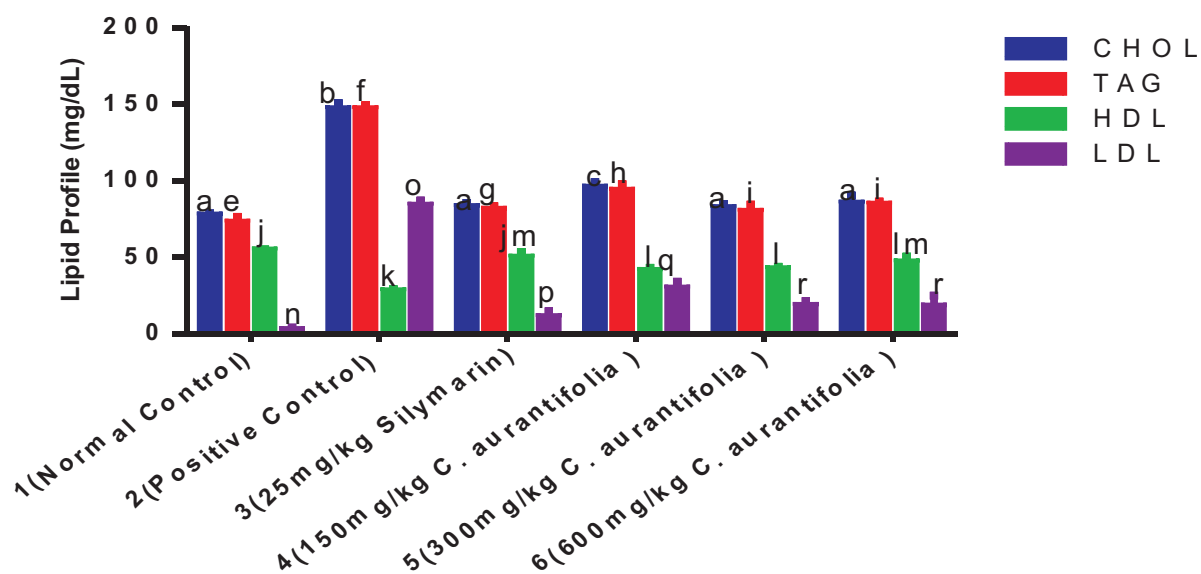


Figure 3: Effects of hydroethanol extracts of *Citrus aurantifolia* fruits on lipid profile of CCl4 intoxicated rats.

* Values are as Mean \pm standard deviation (n=6) **Bars with unlike letters vary significantly (p<0.05)

Discussion

The liver is a main detoxifying and metabolizing organ in the body, and so likely to be endangered by resultant toxicities from its role in conversion of xenobiotics and other vast arrays of metabolic activities (Valko *et al.*, 2007). Injury to the liver can be caused by numerous features like viruses, organic chemicals, alcohol, genetic and metabolic abnormalities, which can be life frightening (Robin *et al.*, 2012). Hence, there is a growing need for other natural solutions to the problems of liver damage apart from liver transplantations. The medicinal value of *Citrus aurantifolia*, particularly the leaves have been widely reported, but little is known about its protective effects on the liver. This work investigated the hepatoprotective effects of the hydroethanol (ethanol: water 50:50 v/v) extract of *C. aurantifolia* fruit juice in CCl4 intoxicated rats.

In this study, exposure of rats to CCl4 caused severe perturbations in the levels of hepatic indicators (AST, ALT, and ALP). Thus, the CCl4 caused a rise in the activities of the indicator enzymes, while, a considerable reduction in these enzymes' activities were seen for the normal control rats. Consequently, the administration of CCl4 to rats caused considerable hepatocellular damage as proved

by the raised serum liver enzymes (ALT, AST and ALP) and decreased protein and albumin levels. This agrees with earlier work where, CCl4 was reported to trigger a substantial surge in the levels of serum ALP, AST, ALT and a decline in serum levels of ALB and TP (Kokhdan *et al.*, 2017). Carbon tetrachloride (CCl4) has been described to generate free radicals, that cause disturbance of hepatocytes cellular permeability thereby leading to raised levels of some serum biochemical parameters such as ALP, ALT and AST (Vinoth *et al.*, 2009). An increase in the activities of serum ALP, AST, ALT seen in this experiment may be due to hepatic damage, resulting in leakage of these enzymes to serum from the liver tissues (Appidi *et al.* 2009).

Upon pretreatment and continuous treatment of CCl4 induced rats with extracts of *C. aurantifolia* fruits, a significant improvement comparable to that of standard drug (silymarin), in the levels of the main enzymes' activities (AST, ALT, and ALP) was seen both in the medium and highest extract dose. Equally, a distorted pattern was seen for other markers; a significant decreased level of protein and albumin was noted in the CCl4 induced (positive control) rats relative to the normal control group. However, treatment with the extract prevented



the CCl₄ induced decrease in the level of protein and albumin. This is in agreement with earlier reports that the xylem of *C. aurantifolia* possesses hepatoprotective activity (Gokulakrishnan *et al.*, 2010). Similar observations were made by Bhavsar *et al.* (2007) about the ethanolic fruit extract of *C. limon* in carbon tetrachloride intoxication in rats. In another study, *Citrus maxima* peels were reported to possess hepatoprotective action against CCl₄ induced hepatotoxicity, an action which has been attributed to the presence of antioxidant compound such as epicatechin and caffeic acid (Sriparna *et al.*, 2011). Also, Casimiro *et al.* (2010) reported that the peel extract of *Citrus microcarpa* displayed hepatoprotective activity against acetaminophen-induced liver injury in rats.

The administration of CCl₄ in this experiment caused significant increase in the MDA levels in rat liver. However, treatment of animals with extracts of *C. aurantifolia* fruits caused a reversal of this trend (decrease in the MDA levels) to near normal values. Also, intoxication of rats with CCl₄ produced marked decline in activities of the antioxidant enzymes (SOD and CAT) in the liver. But, pretreatment and continuous treatment with either silymarin or the extracts of *C. aurantifolia* fruits brought about significant reduction in SOD and catalase activities relative to the CCl₄ induced control rats. Thus, treatment of the CCl₄ intoxicated rats with extracts of *C. aurantifolia* fruits caused significant recovery in the CCl₄ induced rats in this study. In previous studies where *Citrus maxima* leaves were investigated for their hepatoprotective effects against paracetamol-induced liver damage in rats, the methanolic extract exhibited significant effect on TBARS and normalized the levels of glutathione and catalase to a comparable level with that of silymarin (Chowdhury *et al.*, 2015). Thus, the antioxidant effects displayed by the hydroethanol extract of *C. aurantifolia* fruits may be due to its antioxidants contents as earlier report showed that the fruit of *C. aurantifolia* is rich in vitamin C and is a good source of flavonoids with exceptional antioxidant activities (Karoui and Marzouk, 2013; Patil *et al.*, 2009). Jaiswal *et al.* (2015) attributed the

hepatoprotective effect of *C. limon* extract to the presence of vitamin C, flavonoids and other antioxidants in the fruit.

The results of this study showed changes in the total cholesterol, HDL, TAG and LDL levels of CCl₄ induced animals. However, the administration of *C. aurantifolia* fruits extracts was seen to cause a significant recuperation in the rats as reflected in the lower TAG, CHOL and LDL levels and higher level of HDL in the extract treated groups when compared with the CCl₄ control rats. The results are in concord with earlier work by Jaiswal *et al.* (2015) who reported that *Citrus limon* fruit extract administration to carbofuran induced animals caused appreciable recovery in the CHOL and HDL levels. Trovato *et al.* (1996) reported that juices of *C. limon* and *C. sinensis* exhibited hypolipidemic activities in rats. Also, Patil *et al.* (2013), observed that the essential oils from *C. aurantifolia* caused reduction in body weight/obesity and food consumption by promoting anorexia, and therefore recommended it for weight loss and in the management of drug-induced obesity (Patil *et al.*, 2013).

The effectiveness of any hepatoprotective drug has been reported to be basically reliant on its ability of either lessen the destructive effects or sustain the ordinary physiologic roles that are usually distressed by hepatotoxic agents (Tripathi *et al.*, 1999). In this study the extract at the highest dose (600 mg/kg) exhibited good hepatoprotective effect, which was akin to that of silymarin on the liver enzymes and the markers of oxidative stress. Related results had been described from other Citrus extracts. Therefore, the findings of this study agree with previous reports on Citrus extracts. Citrus maxima leaves extract has been reported to have marked hepatoprotective effects (Chowdhury *et al.*, 2015).

Conclusions

The results of the current experiment lucidly proved hepatoprotective effects of *Citrus aurantifolia* fruits extract in CCl₄ induced liver injury in rats. These protecting effects may be partly connected to its antioxidant properties.



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