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Toxicological assessment of ethanol seed extract of *Citrus paradisi* Macfad (grapefruit) on oxidative status, organ function and histoarchitecture in Wistar rats

Godswill J. Udom[1, 2*], Omoniyi K. Yemitan[3], Daniel N. Obot[4], John A. Udobang[4], Nkechi J. Onyeukwu[1], Emmanuel O. Ogbuagu[5], Sunday S. Soyemi[6]

[1]*Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo, PMB 1017 Uyo, Nigeria;*
***Email:** *udomgodswill@gmail.com*
[2]*Gimmex Health Consult, Suites B6 Real Towers Complex, 26 Ekukinam Street, Utako District, Abuja, Nigeria;*
[3]*Department of Pharmacology, Therapeutics and Toxicology, Lagos State University College of Medicine, Lagos, Nigeria;*
[4]*Department of Clinical Pharmacology and Therapeutics, Faculty of Basic Medical Sciences, University of Uyo, PMB 1017 Uyo, Nigeria;*
[5]*Department of Pharmacology and Therapeutics, Faculty of Basic Clinical Medicine, Abia State University, Uturu, Nigeria;*
[6]*Department of Pathology and Forensic Medicine, Lagos State University College of Medicine, P.M.B. 21266 Ikeja, Lagos, Nigeria;*

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ABSTRACT

Background & Aim: *Citrus paradisi* Macfad (Rutaceae) seed extract (CPE) is used in folkloric medicine for the management of diabetes, blood deficiencies and as immune booster, which may require long term usage. This study aimed to evaluate the toxicity profile of ethanol seed extract of grapefruit in adult Wistar rats in order to determine its safety profile in whole organism's systems.

Experimental: Acute toxicity study was conducted using OECD–425 procedure. Subchronic toxicity study (90 days) was conducted using 40 adult male Wistar rats, randomly divided into four groups (10/group) and orally-treated daily, thus: Group I-Control (normal saline, 10 ml/kg), Groups II-IV received CPE (4, 40 and 400) mg/kg body weight, respectively. On the 92nd day, animals (6/group) were euthanized under diethyl ether anaesthesia and sacrificed. Vital organs were eviscerated, blotted, weighed and stored for oxidative stress measurement; some samples of the organs were fixed in formalin for histopathological examination. Other animals (4/group) were retained for reversibility studies.

Results: Results showed significant increase and decrease in weights of the kidneys and spleen, respectively. Significant increase in malondialdehyde level and decreases in superoxide dismutase, glutathione and catalase activities were recorded. Histopathology of the kidney, liver, and lungs showed some degree of pathologies. Reversibility studies showed reversal of test effects on extract discontinuation. Despite the diverse biological usefulness of ethanol seed extract of *C. paradisi* (CPE), it may also induce an array of toxicities especially on long term use.

Recommended applications/industries: Contrary to the tradomedical claims that CPE is absolutely safe, the study revealed that CPE may induce oxidative stress and organ toxicity especially on long term use. It is imperative the plant seed extract and its derivatives be used with utmost caution, and where possible be avoided.

1. Introduction

The occurrence of toxic outcomes or adverse effects following exposure to exogenous or endogenous toxicants could occur at the molecular level, cellular level, specific organs, and/or the whole organism. Chemicals (including those from plant sources) can interact with biomolecules like the proteins, lipids and DNA at the molecular level (Valko and Cronin, 2005; Halliwell and Gutteridge, 2007; Halliwell and Gutteridge, 2015) causing the modification of proteins like fragmentation of peptide chains, alteration of proteins' electrical charges, oxidation of specific amino acid amongst others. Most substances with the propensity to cause harm induce such via lipid peroxidation, thus disrupting the membrane rich phospholipid bilayer arrangement. Such alterations may desensitize or down-regulate surface receptors, inactivate enzymes and increase tissue permeability (Girotti, 1985). Other substances generate free radicals (reactive oxygen and nitrogen species), with ability of attacking biological molecules such as lipids, proteins, and DNA, resulting in oxidative stress, which is well known to be involved in the pathogenesis of lifestyle-related diseases, including the multifactorial metabolic diseases and malignant tumours (Halliwell and Gutteridge, 2015). The interaction of chemicals with biomolecules at the cellular level may interfere with receptor-ligand binding, membrane function, and cellular energy production as well as perturb homeostasis (Valko and Cronin, 2005). As earlier reported by Dudkina *et al.* (2011), chemical interference with cellular physiology disrupts normal functioning and is a hallmark in the pathogenesis of several diseases.

The use of medicinal plants and their derivatives labelled as 'herbal remedies and/or medicines' is chiefly due to the quest for sound health in the human population. Thus, humans have exploited the phytotherapeutic potentials of plants in the treatment and/or management of various disease conditions. At present, especially in developing nations of the world, there is an increased patronage of herbal medicines primarily due to its perceived efficacy, absolute safety and cheapness. However, the concept that herbal medicines are completely safe, devoid of any toxicity whatsoever, has indeed raised genuine public health concerns on its safety due to a lack of proper pharmacological and toxicological data. Thus, the principal aim of this study was to assess the toxic outcomes of repeated exposure of experimental animals to ethanol seed extract of *Citrus paradisi* (CPE) using a 90-day subchronic toxicity test approach, in an attempt to ascertain its safety claims as well as to provide useful information regarding the use of grapefruit seed extract.

2. Materials and Methods

2.1. Collection of plant material and preparation of extract

As earlier described and reported by Udom *et al*. (2018), the plant was collected, identified and authenticated by Prof. (Mrs) Margaret Bassey, a taxonomist in the Department of Botany and Ecological Studies, University of Uyo, Nigeria. The pulverized plant material was gradiently and successively extracted following the method earlier described by Udom *et al*. (2018).

2.2. Experimental animals

Male Wistar rats were obtained from and kept at the Department of Pharmacology and Toxicology Animal House, University of Uyo, Nigeria. The animals were maintained under standard environmental conditions and had access to standard rodent pellets (Livestock Feeds, Nigeria Ltd) as food and water *ad libitum* and were housed at room temperature in well cross-ventilated rooms. All experimental animals were acclimatized to the laboratory condition 14 days before the onset of the experiment. The care and use of animals was conducted in strict adherence to the National Institute of Health Guide for the Care and Use of Laboratory Animals and ethical approval for use of lower animals in research was obtained from the Experimental Ethics Committee on Animal Use of the Faculty of Pharmacy, University of Uyo, Nigeria.

2.3. Acute toxicity test

Determination of the median lethal dose (LD_{50}) was conducted using the limit dose test of the staircase phenomenon, also known as OECD Test Guideline 425 (OECD, 2001) as earlier reported by Udom *et al*. (2018).

2.4. Experimental design

A total of 40 Wistar rats were weighed and randomly divided into four groups of 10 animals each. The control group was administered 10 ml/kg body weight of normal saline, while the three extract-treated groups were treated with 4, 40, and 400 mg/kg body weight of CPE (which represented one-thousandth, one-hundredth and one-tenth of the LD_{50}, respectively). The doses were administered daily using oral gavage for 90-day test duration (Tanira *et al*., 1988; Yemitan and Adeyemi, 2004), during which the experimental animals were closely observed for any behavioural changes, feeding and drinking habits, as well as body weight and general morphological changes. After the specified test period, six animals per group were euthanized under diethyl ether (Sigma, USA) anaesthesia and sacrificed. Internal organs were eviscerated for internal macroscopic, oxidative stress measurement and histopathological investigations. Where significant changes were derived, the retained animals (4 per group) were subjected to re-measurement of the parameters after 14 days reversibility of toxic test effects on extract withdrawal or discontinuation (Yemitan *et al*., 2015).

2.5. Qualitative weight measurement

At the end of the study, the weights of vital organs (heart, lungs, liver, kidneys, spleen and testes) were measured using a sensitive weighing balance (Ohaus, USA). The organs were eviscerated from the euthanized animals, blotted with tissue paper and weighed fresh on a sensitive balance. The qualitative weights of each organ were obtained per 100 g body weight of the control and extract treated rats.

2.6. Biochemical analysis - Oxidative stress biomarkers

Each of the sectioned rat kidney, heart and spleen was homogenized in 10 ml of 100 mMol KH_2PO_4 buffer containing 1 mM EDTA (pH 7.4) and centrifuged at 12,000 rpm for 30 min at 4 $^{\circ}$C. The supernatant was then collected for enzymatic studies. The protein concentration of the tissue was determined using crystalline bovine serum albumin (BSA) as standard, according to the method of Lowry *et al.* (1951).

Reduced glutathione (GSH) was estimated by the method of Jollow *et al.* (1974) by precipitating 1 ml sample of homogenate with 1 ml of 4% sulfosalicylic acid. The samples were kept at 4 $^{\circ}$C for 1 h and centrifuged at 1200 rpm for 20 min at 4 $^{\circ}$C. The total volume of 3 ml assay mixture contained 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M; pH 7.4) and 0.2 ml DTNB (100 mM). The yellow colour developed from the reaction mixture was read immediately at 412 nm on a SmartSpecTM plus Spectrophotometer and GSH was expressed as U/g tissue.

SOD activity was estimated by the method of Kakkar *et al.* (1984). The reaction mixture of this method contained 0.1 mL of Phenazine methosulphate (186 µmol), 1.2 ml of sodium pyrophosphate buffer (0.052 mM; pH 7.0). About 0.3 ml of the homogenate was added to the reaction mixture and enzyme reaction was initiated by adding 0.2 ml of NADH (780 µM) and stopped after 1 min by adding 1 ml of glacial acetic acid. The amount of chromogen formed was measured by recording colour intensity at 560 nm. The results were expressed in U/g protein.

CAT activities were determined by the method of Chance and Maehly (1955). The reaction solution of CAT activities contained 2.5 ml of 50 mM phosphate buffer (pH 5.0), 0.4 ml of 5.9 mM H_2O_2 and 0.1 ml enzyme extract. Changes in absorbance of the reaction solution at 240 nm were determined after 1 min. One unit of CAT activity was defined as an absorbance change of 0.01 as k/mg. The results were expressed in mM/g tissue.

MDA which is a measure of lipid peroxidation was measured spectrophotometrically at 532 nm by the method of Colado *et al.* (1997), using 1, 1, 3, 3-tetraethoxypropane as standard. To 500 µl of tissue homogenate in phosphate buffer (pH 7.4), 300 µl of 300% trichloroacetic acid (TCA), 150 µl of 5N HCl and 300 µl of 2% w/v 2-thiobarbituric acid (TBA) were added and then the mixture was heated for 15 min at 90 $^{\circ}$C. The mixture was centrifuged at 12,000 rpm for 10 min and a pink coloured supernatant was obtained. MDA level was expressed as nmol/g.

2.7. Histopathological assessment

After the collection of blood from the sacrificed/dissected rats, the liver, kidneys, lungs, heart, spleen and testes were immediately excised, freed from adventitia, blotted with tissue paper, weighed, sectioned and fixed in 10% formol saline for histological studies. Fixed sections were passed through xylene, alcohol and water to ensure that the tissue was totally free of wax and alcohol. Each section

was then stained with haematoxylin and eosin for photo-microscopic assessment using light microscope at a magnification of 400. Knowledge of the doses and treatments given to animals in the different groups were withheld from the pathologist, in order to minimize bias (Yemitan *et al.*, 2015).

2.8. Statistical analysis

Data obtained from the study were statistically analysed using SPSS statistical package (version 16.00). Statistical significance between the groups was analysed by means of one-way analysis of variance (ANOVA). Results were presented as Mean ± S.E.M. Values less than (P<0.05) were considered significant.

3. Results and discussion

3.1. Acute toxicity test

The result of the acute toxicity profile of ethanol seed extract of *Citrus paradisi* is as earlier reported by Udom *et al.* (2018) "and the LD_{50} values were estimated to be>2000 mg/kg (p.o.); <4000 mg/kg (i.p.).

3.2. Effect of extract on organs' weight

The effect of CPE on internal organ weights is as presented in Table 1. There was no significant changes in the weights of the heart, lungs, liver and testes at all doses tested. However, a significant (P<0.05) increase in the weight of kidneys of the rats were recorded dose-dependently. Also, a significant decrease in the weight of the spleen at 400 mg/kg body weight was recorded.

Generally, following exposure to a toxic substance, significant increase or decrease in body and internal organ weights are considered observable adverse or toxic outcomes (Farah *et al.*, 2013). Therefore, the recorded increase in weight of the kidneys could indicate hypertrophy, which according to Ping *et al.* (2013) is considered a primary indicator of toxicity after exposure to a chemical and/or biological substance.

Table 1. Qualitative organ weight per 100 g body weight of rats treated with extract.

	Mean organ weight (per 100 g body weight ± S.E.M)					
	Liver	**Lungs**	**Heart**	**Kidneys**	**Spleen**	**Testes**
Control	2.90 ± 0.09	0.66 ± 0.04	0.31 ± 0.01	0.40 ± 0.03	0.36 ± 0.01	1.19 ± 0.09
4 mg/kg	2.90 ± 0.03	0.58 ± 0.04	0.29 ± 0.01	0.95 ± 0.02*	0.40 ± 0.04	1.01 ±0.05
40 mg/kg	2.93 ± 0.05	0.65 ± 0.06	0.31 ± 0.01	1.53 ± 0.01*	0.35 ± 0.03	1.03 ±0.03
400 mg/kg	2.97 ± 0.03	0.75 ± 0.06	0.30 ± 0.02	1.87 ± 0.03*	0.32 ± 0.01*	1.00 ±0.05

Result expressed as mean ± SEM. * Significant (P <0.05) compared with control; n = 6.

3.3. Effect of extract on oxidative statuses of eviscerated organs

Tables 2–4 represent the oxidative statuses of the kidneys, spleen and heart, respectively. For the kidneys, significant (P<0.05) increase in the levels of malondialdehyde (MDA) was recorded dose-dependently at all doses tested. However, the enzymatic activities of superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) were significantly reduced in dose-dependently. The spleen showed significant increase in the level of malondialdehyde (MDA) at 400 mg/kg body weight of extract, reduced activities of superoxide dismutase (SOD) and glutathione (GSH) at 400 mg/kg body weight of extract and reduced catalase (CAT) activity both at 40 and 400 mg/kg dose of extract, respectively. While the heart presented significant decrease in the level of malondialdehyde (MDA) as well as increased activities of superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) at 40 mg/kg dose of extract. However, there were no significant differences in the superoxide dismutase (SOD), glutathione (GSH), catalase (CAT) activities and malondialdehyde (MDA) level of the eviscerated liver and lungs.

Table 2. Effect of extract on the kidneys' oxidative status of treated animals.

	Oxidative stress biomarkers			
	MDA (nmol/g)	**GSH (U/g tissue)**	**SOD (U/g tissue)**	**CAT (U/g tissue)**
Control	0.89 ± 0.02	5.46 ± 0.03	3.06 ± 0.02	0.57 ± 0.004
4 mg/kg	0.96 ± 0.01*	5.09 ± 0.05*	2.98 ± 0.02*	0.56 ± 0.005*
40 mg/kg	1.04 ± 0.01*	4.89 ± 0.03*	2.92 ± 0.02*	0.55 ± 0.008*
400 mg/kg	1.11 ± 0.01*	4.60 ± 0.07*	2.84 ± 0.02*	0.52 ± 0.003*

Result expressed as Mean ± SEM. * Significance (P<0.05) relative to control; n = 6.

Table 3. Effect of extract on the spleen's oxidative status of treated animals.

	Oxidative stress biomarkers			
	MDA (nmol/g)	**GSH (U/g tissue)**	**SOD (U/g tissue)**	**CAT (U/g tissue)**
Control	0.82 ± 0.009	5.60 ± 0.02	3.17 ± 0.01	0.58 ± 0.003
4 mg/kg	0.82 ± 0.008	5.54 ± 0.02	3.12 ± 0.01	0.57 ± 0.002
40 mg/kg	0.84 ± 0.004	5.52 ± 0.03	3.12 ± 0.02	0.56 ± 0.003*
400 mg/kg	0.89 ± 0.010*	5.31 ± 0.03*	2.73 ± 0.06*	0.52 ± 0.002*

Result expressed as Mean ± SEM. * Significance (P<0.05) relative to control; n = 6

Table 4. Effect of extract on the heart's oxidative status of treated animals.

	Oxidative stress biomarkers			
	MDA (nmol/g)	**GSH (U/g tissue)**	**SOD (U/g tissue)**	**CAT (U/g tissue)**
Control	0.86 ± 0.01	5.28 ± 0.05	3.18 ± 0.04	0.59 ± 0.01
4 mg/kg	0.85 ± 0.01	5.37 ± 0.03	3.28 ± 0.02	0.59 ±0.004
40 mg/kg	0.82 ± 0.01*	5.44 ± 0.03*	3.47 ± 0.08*	0.63± 0.01*
400 mg/kg	0.84 ± 0.004	5.22 ± 0.05	3.08 ± 0.12	0.59 ±0.004

Result expressed as Mean ± SEM. *Significance (P<0.05) compared with control; n = 6.

SOD, CAT, GSH and MDA are biomarker of oxidative stress, which is implicated in the pathogenesis and progression of virtually all disease conditions. Free radicals-induced lipid and protein peroxidation is believed to be one of the major causes of cell membrane damage leading to a number of pathological situations (Halliwell and Gutteridge, 2015). In humans, during physiological and pathological processes, reactive oxygen species (ROS) are generated predominantly in the cytosol, peroxisomes, mitochondria, plasma membranes etc. (Hemnani and Parihar, 1998). Once generated, these free radicals due to their unstable state react readily with biomolecules, and perturb homeostasis with consequential pathologies. In order to combat these radicals, living organisms produce free radical scavenging enzymes and coenzymes (e.g. glutathione, catalase, superoxide dismutase and peroxidase). Within their normal range, these enzymes and coenzymes serve as the body's antioxidant defence system. However, if overwhelmed, which is chiefly measured by decrease in the activities of glutathione, superoxide dismutase and catalase, oxidative stress ensues (Wang *et al.*, 2013). With regard to the heart, significant decrease in the activity of MDA and increases in GSH, SOD and CAT compared to control suggests that its antioxidant defence system were not overwhelmed following the repeated exposure to CPE. Generally, the result of this study suggests the occurrence of oxidative stress in some tissues of the vital organs tested except the heart tissue.

Furthermore, lipid peroxidation is one of the frequent and major outcomes of free radical-mediated injury that directly damages cell membranes and generates a number of secondary products including aldehydes, such as malondialdehyde. The latter is the most abundant aldehyde following peroxidation, and is used as an important biomarker for lipid and protein peroxidation (Slater, 1984; Draper *et al.*, 1988; Taib *et al.*, 2013; Goudah *et al.*, 2015). Thus, the kidneys and spleen tissues showed levels of oxidative stress from lipid peroxidation as well as decreases in the enzymatic activities of GSH, CAT and SOD. However, these observed effects were reversed on extract withdrawal and/or discontinuation.

3.4. Effect of extract on histopathology of eviscerated organs

At the end of the subchronic toxicity study, histopathological examinations of the liver, lungs, kidney, heart, and spleen of rats in the control group presented preserved/normal cellular architecture. At a low dose of CPE (4 mg/kg body weight), the spleen and heart tissues presented preserved/normal cellular architecture, whereas the kidneys, liver and lungs presented some forms of pathologies (vascular congestions and edema of lung interstitium respectively). Also, at 40 mg/kg body weight, the liver showed vascular congestion and moderate scars of necrosis, whereas the lungs showed vascular congestion of the interstitium and presence of inflammatory cells in interstitium and alveoli. At high dose of CPE (400 mg/kg body weight), the liver

showed marked vascular congestion at the periportal zone as well as striking vacuolation of the hepatocytes (as diabetic manifestations), the lungs showed congestion and widening of the interstitium as well as pulmonary edema (intra-alveolar fluid). While the kidney showed vascular congestion (Fig. 1–3).

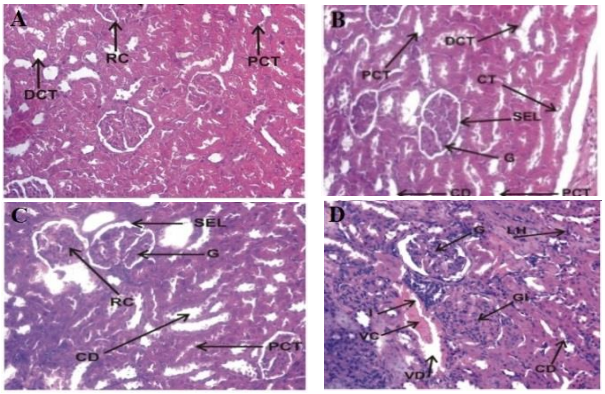

Fig. 1. Typical kidney sections from control (A) and extract-treated rats (B) showed normal architecture, renal corpuscle (RC), distal convoluted tubule (DCT), normal glomeruli (G), inflamed glomeruli (GI), congested tubules (CT) and vascular congestion of the interstitial blood vessels (VC) x 400 magnification.

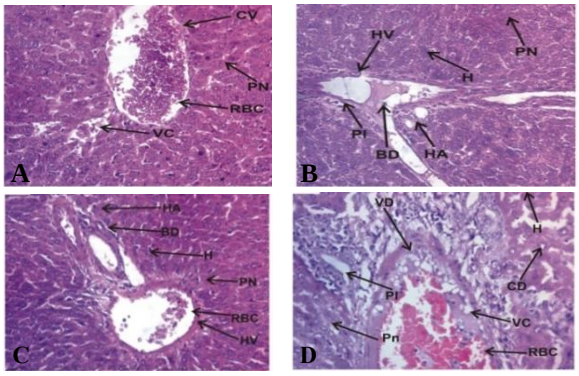

Fig. 2. Typical liver sections from control (A) and extract-treated rats (B, C and D) showed normal hepatocytes (H), central vein (CV), red blood cell (RBC), vascular degeneration (VD), vascular congestion (VC), cellular degeneration (CD), periportal inflammation (PI) and moderate scar of necrosis x 400 magnification.

The histopathology of the kidneys at 400 mg/kg body weight of extract showed vascular congestion. In a pathologic kidney, vascular congestion is associated with renal ischemia characterized chiefly by cellular swelling in its generation. Mason *et al.* (1989) asserted the cause of vascular congestion to be the loss of vascular integrity during ischemia, with a resultant poor perfusion and impaired renal function thereafter. Ischemia-reperfusion produces excessive ROS that overwhelms the tissues normal radical scavenging system. The excessive ROS generated are toxic to tubular epithelial cells and leads to cell damage by lipid peroxidation (Kim *et al.*, 2009; Brede and Labhasetwar, 2013). Congestion within peripheral vascular tissues, in addition to renal and cardiac tissues, triggers local as well as systemic inflammatory responses, which promote additional fluid retention when endogenous anti-oxidative, anti-inflammatory, and vasodilating defences are all overwhelmed (Ganda *et al.*, 2010). Therefore, the kidneys are susceptible organs of concern in this subchronic toxicity test.

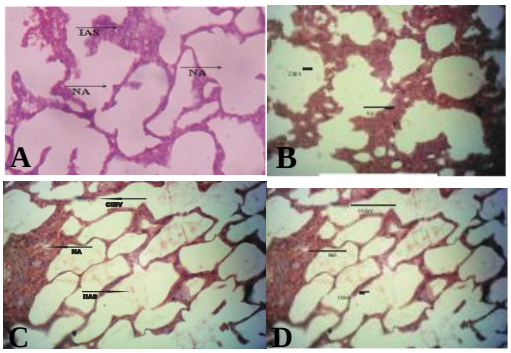

Fig. 3. Typical lung sections from control (A) and extract-treated rats (B, C and D) showed normal architecture, normal alveoli (NS) and inter-alveolar septae (IAS), vascular congested interstitial blood vessels (CIBV), widening of the interstitium and pulmonary oedema, x 400 magnification.

The histopathological assessment of the liver revealed moderate scars of necrosis, marked vascular congestion at the periportal zone and striking vacuolation of the hepatocytes. Vascular congestion within the liver is usually followed by oedema. In pathologic terms, an oedema is the result of an "exudation process" (Riede and Werner, 2004). Chronic congestion at frequent or regular intervals leads to fibrosis of the necrotic parenchyma as well as destruction of the nodular parenchyma, which are prominently seen in congestive cirrhosis. Therefore, the histopathological examination of the liver suggests a possible liver damage at all doses tested.

The histopathology of the lungs revealed some forms of pathologies at all doses of extract tested. As earlier reported by Riede and Werner (2004), some medications, perhaps with herbal preparations inclusive, induces lung damage in one or varied ways.

Most medication causes toxic pulmonary edema with fluid percolating into the interstitial space of the alveoli, with a resultant inflammatory pleuropulmonary fibrosis (Riede and Werner, 2004). Pulmonary congestion is an excessive accumulation of fluid in the lungs, usually associated with either an inflammation or congestive heart failure. Therefore, from this subchronic toxicity study, the lung pathologies recorded could be considered as adverse/toxic effects of grapefruit seed extract seen especially on long term use.

4. Conclusion

Findings of this study suggest that despite the diverse biological usefulness of ethanol seed extract of *C. paradisi*, it may also induce an array of toxicities especially on long term use. Grapefruit seed extract is relatively safe on acute oral exposure as well as at therapeutic doses. However, contrary to the tradomedical claims and/or belief that grapefruit seed extract is devoid of any toxicity whatsoever, this study has revealed varying degrees of toxicities such as oxidative stress, cellular toxicity and organs' toxicity following the subchronic administration of the extract. However, these toxic outcomes were reversed on extract withdrawal and/or discontinuation. Since toxicity is evaluated based on the amount, frequency and duration of exposure to a toxicant, therefore, the chronic (intermittent, continuous) or long-term use of grapefruit seed extract should be done with utmost caution, and wherever possible be avoided. The phenomenal reversal of test outcomes within 14 days of reversibility studies requires further investigation.

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