International Journal of Science and Research Archive
eISSN: 2582-8185
Cross Ref DOI: 10.30574/ijsra
Journal homepage: https://ijsra.net/

(RESEARCH ARTICLE)

Check for updates

Histological based sub-chronic toxicity testing of target heavy metals of crude oil spill; using the Histo-morphometry of lung of Wistar rat

Allison Theodore Athanasius [1, *] and Ogoun Timipa Richard [2]

[1] *Department of Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, University of Port Harcourt, Nigeria.*
[2] *Department of Anatomy, Faculty of Basic Medical Sciences, Bayelsa Medical University, Nigeria.*

International Journal of Science and Research Archive, 2025, 15(02), 1791–1798

Publication history: Received on 11 April 2025; revised on 27 May 2025; accepted on 30 May 2025

Article DOI: https://doi.org/10.30574/ijsra.2025.15.2.1609

Abstract

This study was aimed to evaluate in histological term, the sub-chronic toxicity of some target crude oil contaminants on the histo-morphometry of the lungs of exposed Wistar rats. The following target chemicals (TCs; cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb) and nickel (Ni) were selected based on findings from literature review of chemical analysis done on crude oil spill site. Thirty (30) inbred male Wistar rats of average weight 150-200g (5 for control and 25 for experimental – 5 for each of the five TC treated groups). Rats were later sacrificed and the target organ (lung) excised and used for qualitative histological evaluation. Gross anatomical assessment showed that there was no significant difference (P> 0.05) when correlating weight gain between the treated and control groups. Histological evaluation showed the following major lesions: glomerular congestion, degeneration and necrosis; tubular degeneration and necrosis; Interstitial inflammation, hemorrhage and necrosis. This study gives credence to the fact that histology-based evidence is a veritable tool for assessing sublethal level of environmental stressors in the certification of toxicity

Keywords: Wistar rat; Lung; Histology; Sub-chronic toxicity; Toxicity; Heavy Metals

1. Introduction

Toxicology is a branch of biology, chemistry, and medicine (more specifically pharmacology) concerned with the study of the adverse effects of chemicals on living organism. It also studies the harmful effects of chemical, biological and physical agents in biological system that establishes the extent of damage in living organisms. The relationship between dose and its effects on the exposed organism is of high significance in toxicology. Factors that influence chemical toxicity includes; the dosage (and whether it is acute or chronic), the route of exposure, the species, age, sex and environment.

Toxicity tests can measure lethal and/or sublethal effects. These effects are known as measurement endpoints: that is, they are ecological attributes that may be adversely affected by exposure to site contaminants and that are readily measurable. In addition, each measurement endpoint is closely related to an assessment endpoint. Because of this close relationship, a measurement endpoint can approximate or represent the assessment endpoint if the assessment endpoint is not amenable to direct measurement (USEPA, 1992). Based on the measured end points, toxicity testing can be divided into acute toxicity testing, sub-chronic toxicity testing and chronic toxicity testing. Sub-chronic toxicity testing was applied for this study, which is defined as a prolonged toxicity test for 14 day. This prolonged toxicity test may be used in place of the acute toxicity test if a longer observation period is considered appropriate (OECD, 1984), for example if testing highly lipophilic, poorly water soluble substances, and/or the reporting of additional information is considered necessary. The principle of the test is that threshold levels of lethal and other observed effects and NOEC are determined at intervals during the test period (OECD, 1992a).

* Corresponding author: Allison Theodore Athanasius

Organism exposure to hazardous chemicals causes increase in the levels of stress proteins, which induces specific detoxification system responses, reflecting their compensatory potential. When these systems are overwhelmed, it causes sublethal effect which usually begins as biochemical alterations at the molecular levels of biological organization. This change might not impair cellular function until when it results in the formation of a lesion, which is any structural damage or alterations in an organ, tissue or cell of an organism due to an injurious stimulus. Lesion diagnosis is a report on the qualitative and/or quantitative gross morphological alterations of organs or histo-morphological alteration of tissues, cells and organelles, including the histo-chemical changes that occur at the molecular level of biological organization. Diagnostic statement of a lesion is based on the predominant lesion(s) in the tissue, while lesion description can be based on the severity (mild moderate or severe), distribution (focal, multi-focal or diffuse), location (intracellular, epithelial or interstitial) and pathogenesis (adaptive degenerative, inflammatory, and neoplastic). In so far that sublethal lesions of toxic substances is insidious and usually begins at the subcellular levels of biological organization, even before its manifestation of impairment of the physiology of the affected cell, histology as an assessment tool is therefore a gold standard in certification of toxicity – in a laboratory toxicological setting or pollution – in an ecotoxicological setting. It is noteworthy that physiologic and biochemical changes are predominantly due to structural abnormality of tissue which histopathology investigates. Hence lesion diagnosis is a gold standard in diagnosis of disease pathogenesis and pathognomonic histopathologic features. Some lesions are pathognomonic histopathological features that are specifically distinctive or characteristic of some diseases or pathological conditions.

The respiratory system is divided into three regions:

- **Nasopharyngeal**: It extends from nose to larynx. These passages are lined with ciliated epithelium and mucous glands. They filter out large inhaled particles, increase the relative humidity of inhaled air, and moderate its temperature.
- **Tracheobronchial**: consists of trachea, bronchi, and bronchioles and serves as conducting airway between the nasopharyngeal region and alveoli. These passage ways are lined with ciliated epithelium coated by mucous, which serves as an escalator to move particles from deep in the lungs back up to the oral cavity so they can be swallowed. These ciliated cells can be temporarily paralyzed by smoking or using cough suppressants.
- **Lungs**: This is the basic functional unit of the respiratory system which contains the ***pulmonary acinus***; the primary location of gas exchange. It consists of small bronchioles which connect to the alveoli. The alveoli, of which there are 100 million in humans, contact the pulmonary capillaries.

Many environmental chemicals produce a generalized systemic disease due to their effects upon a number of target sites. Lung toxicity is damage to the gas exchanges portion of the respiratory system upon exposure to xenobiotics. Lungs are not only the target for many harmful agents but the site of entry of toxic substances which pass through the lungs into the bloodstream without any damage to the lungs. However, when distributed by the blood circulation to various organs, the lungs can be damaged. Approximately half of heavy metals absorbed systemically are rapidly accumulated in the liver, which resulted in the reduced availability of heavy metals to other organs like the kidneys, lungs and testes, which are more sensitive to its toxic actions (DelRaso et al., 2003).

The health effects of toxic substances and hazardous wastes are not yet fully understood. Research to better understand how these exposures may impact health is ongoing. Meanwhile, efforts to reduce exposures continue. Reducing exposure to toxic substances and hazardous wastes is fundamental to environmental health.

2. Material and methods

The study was a sub-chronicity testing for 14 days. Thirty (30) inbred male Wistar rats were obtained from the animal house of the Department of Anatomy, Faculty of Basic Medical Sciences, University of Benin for this study. The rats were divided into experimental and control groups:

- Control Group: Five (5) rats were used for this group. They were untreated with target chemical (TC), and were only given only water and food.
- Experimental Group: Twenty five (25) rats were used for the experiment group. They were treated with the study TCs. This group was sub-divided into 5 Wistar rat per TC treated group of Cd, Cr, Cu, Ni and Pb.

In estimation of the rat exposure dose (RED) for the study, OEDC (2001) reference oral LD_{50} dose for Wistar Rat (in mg/Kg of body weight) of the TCs – Cd (63mg/kg); Cr (46 mg/kg), Cu (481 mg/kg); Nickel (300 mg/kg) and Pb (600 mg/kg), was used as a guideline standard for the upper limits of dose administration (OECD, 2001). Ten times (10x) the TC concentration that is above maximum allowable toxicant concentration (MATC) for surface fresh water, but below

the median lethal dose (LD_{50}) reference concentration for Wistar Rats was used as the guideline for estimation of the RED (Hounkpatin et al., 2013; Thinkratok, et al., 2014). Thus analytical grade metallic salts of the TCs concentrations that are 10x >MATC standard per TC, but below the LD50 per TC for Wistar Rat was dissolved in 100litres of distilled water to make the stock solution. 1ml of the stock, for each of TCs, was administered orally/day for 14 days to the test rats (Thinkratok, et al., 2014). Therefore RED in mg/ml for this study was: Cd (0.0001 mg/ml), Cr(0.01 mg/ml), Cu(0.02 mg/ml), Ni(0.25 mg/ml) and Pb(10.0 mg/ml). Oral route was chosen as the route of administration of the test solution because it is the most common mode of exposure of the target toxicants (ATSDR, 2004; ATSDR, 2007; ATSDR, 2012a, 2012b).

All animals used in this study were handled with regards to international, natural and institutional guidelines for care and use of laboratory animals in biomedical research as promulgated by the Canadian Council of Animal Care (CCAC, 1984). Study animals were housed in cages with wire bar lids used to hold water bottle and feeds to prevent contamination with urine or faeces. Bedding was placed directly into the shoe box cage to allow the absorption of urine. Test animals were kept in well ventilated room at ambient temperature of 28.0±2.0 ^{o}C under 12hour light/dark cycle well fed with food and water ad libitum. Generally, the study was conducted in accordance with the recommendations from the declaration of Helsinki on guiding principles in care and use of animals (Obianime and Roberts, 2009).

Histological tissue processing and qualitative analysis of prepared tissue slides was done at the Histology Laboratory of the Department of Anatomy, School of Basic Medical Sciences, University of Benin. Resected target organ of lung was collected in vials filled with preservative (10% neutrally-buffered formalin solution), and transported to the University of Benin Histology laboratory for tissue processing and staining. The prepared tissue slides were used for quality histological evaluations. (Drury and Wallington, 1980; Allison and Paul, 2014; Allison and Paul, 2018)

3. Results

3.1. Gross Anatomical Assessment

The Wistar rat sub-chronic toxicity showed that, rats exposed to the daily rat exposure doses (RED) of Cu1, Ni1 and Pb1 died. Only those exposed to the daily doses of Cd1, Cr1 and control survived the 14day experiment. Table 1 showed that there was no significant difference ($P \geq 0.05$) when correlating weight gain between the treated and control groups.

Table 1 Correlating weight gain between experimental and control groups using t test analysis

Group	No of Male Rats	Daily RED (mg/ml)	Exp. Days	Mean Wo (g)	mean W_1 (g)	Weight Gain	Sig. (2-tailed)	
Control		Nil	14	175.5	208.4	32.9	0.300	Not Significant
Cd1	5	0.0001	14	180.6	212.3	31.7	0.419	Not Significant
Cr1	5	0.01	14	175.3	203.6	28.3	0.215	Not Significant
Cu1	5	0.02	14	198.4	228.4	30.0	0.216	Not Significant
Ni1	5	0.025	14	201.0	230.4	29.4	0.650	Not Significant
Pb1	5	0.01	14	180.7	210.5	29.8	0.188	Not Significant

Key: TC = RED – Rat Exposure Dose; Exp. = Experiment; W0 = Initial Weight; W1 = Final Weight; Cd1, Cr1, Cu1, Pb1 and Ni1 = Heavy metal exposure concentration; Conc = Concentration; Sig: Significance Mean values ($p<0.05$) are significantly different

3.2. Histological Assessment

Plate A-D are observed micrographs of lungs of Wistar rats:

3.2.1. Plate A: A micrograph of the control Wistar rat

- **Diagnostic Lesion**: Normal architecture
- **Diagnostic Note**: Normal histological architecture of the lung consisting of:
    - **Bronchioles (BR)**: Bronchioles are thin-walled airways lined by variably ciliated or non-ciliated columnar (Clara cells) epithelium. The walls are composed of tangentially arranged smooth muscle. Bronchioles lack cartilage and glands which, along with their smaller size, distinguish them histologically from bronchi.

- **Alveoli (A)**: The alveoli are responsible for the spongy nature of the lung. These alveoli are lined by flattened epithelial cells called pneumocytes with a single opening. The alveolar wall or septum is made up of three tissue components: surface epithelium, supporting tissue, and an extensive network of continuous capillaries.
- **Pulmonary Vessels (PV)**: A small branch of the pulmonary vessel accompanies the respiratory bronchiole into the lung
- **Interalveolar Septum:** The adjacent alveoli share a common interalveolar septum (arrows) or alveolar wall. It consists of simple squamous alveolar cells, fine connective tissue fibres and fibroblasts, and numerous capillaries located in the thin interalveolar septa. The thin interalveolar septa bring the capillaries close to the squamous alveolar cells of the adjacent alveoli.

3.2.2. Plate B: A micrograph of Wistar rat exposed to Cd

- **Diagnostic Lesion**: Mild Alveolar Epithelial Degeneration
- **Diagnostic Note**: There is alveolar epithelial cell swelling with nuclear pyknosis indicating necrosis (AEN). It is associated with:
  - **Focal Interstitial Necrosis**: Interstitial cells are also shows focal areas of pyknosis
  - **Intra-bronchiolar Hemorrhage**: with associated areas pf extravasation of blood cells into the bronchioles (IBH)

3.2.3. Plate C: A micrograph of Wistar rat exposed to Cr

- **Diagnostic Lesion**: Mild Bronchiolar/Alveolar Hyperplasia
- **Diagnostic Note**: There is alveolar epithelial hyperplasia associated with:
  - **Interstitial congestive necrosis (ICN):** The cells shows consistent eosinophilic appearance
  - **Congested Pulmonary Vessel (CPV)**: Pulmonary capillaries are enlarged with blood cells
  - **Desquamated mucosa in lumen of bronchiole (DMLB):** The bronchiole vessel epithelial disruption with cells sloughing into the lumen

3.2.4. Plate D: A micrograph of Wistar rat exposed to Pb

- **Diagnostic Lesion**: Moderate Bronchiolar/Alveolar Hyperplasia
- **Diagnostic Note**: There is alveolar epithelial hyperplasia associated with:
  - **Collapsed Alveoli (CA):** Hyperplasia of the alveolar epithelium has resulted in collapse of some alveolar sacs;
  - **Alveoli Capillary Congestion (ACC**): Alveolar capillaries are enlarged with blood cells
  - **Alveoli inter-septal thickening (ST**): Hyperplasia of the alveolar interseptum has resulted in thickening of the wall
  - **Immune Cell Infiltration (ICI):** There is multi-focal areas of immune cell infiltration

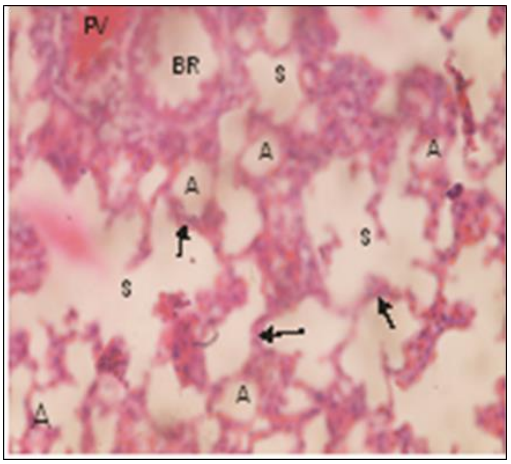

Figure 1 Photomicrograph (H&E:400x magnification) of the normal architecture of the lung of the control wistar rat showing normal alveoli (A) with inter alveolar septa (arrows), alveolar sacs (S), pulmonary blood vessels (PV) and a bronchiole (BR)

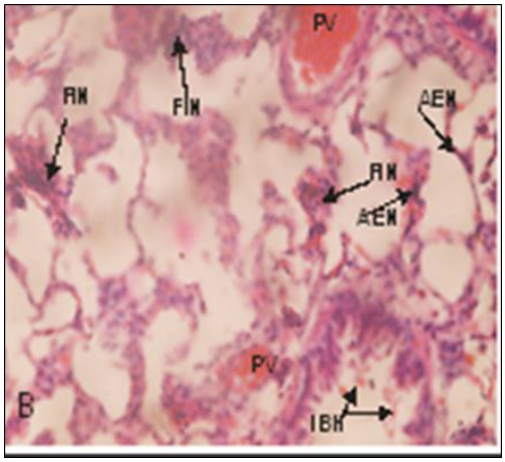

Figure 2 Photomicrograph (H&E:400x magnification) of lung exposed to Cd showing multiple focal interstitial necrosis (FIN), Alveoli epithelial necrosis (AEN), and intrabronchiole hemorrhage (IBH)

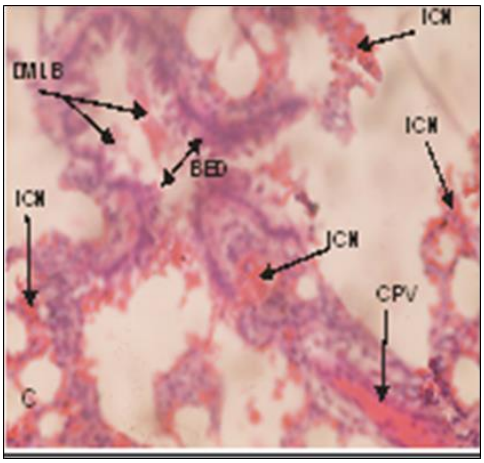

Figure 3 Photomicrograph (H&E:400x magnification) of lung exposed to Cr. showing multiple interstitial congestive necrosis (icn), congested pulmonary vessel (cpv), desquamated mucosa in lumen of bronchiole (dmlb) and bronchiole epithelial disruption

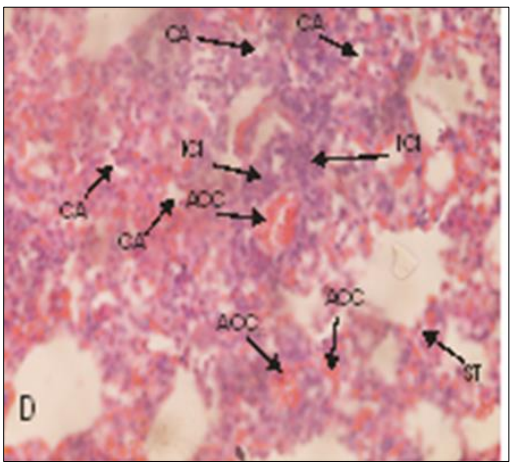

Figure 4 Photomicrograph (H&E:400x magnifications) of lung exposed to Pb showing multiple collapse alveoli inter-septal thickening (ST) and multiple foci of interstitial immune cell infection (ICI)

4. Discussion

4.1. Gross Assessment

This involves the weight relationship between the control and experimental group. There was no significant difference in mean weight gain between experimental Wistar Rats groups exposed to TCs Rat Exposure Doses (REDs) of Cd1, Cr1, Cu1, Ni1 and Pb1, and the control group. This implies that the RED does not have significant effect on the weight of WISTAR RATS, which is consistent with other studies in which Wistar Rats were exposed to heavy metals (Sun et al., 2017).

4.2. Histology

In this study, lung showed the following major alterations:

- **Epithelial degeneration**: It is considered to be parts of a spectrum of lung tissue response to injury consisting of adaptive changes, degeneration and necrosis. The adaptive change and degeneration represent reversible cell response, while necrosis represents irreversible cell response (NTP, 2014; Allison et al., 2022). The light microscopic terminal hallmarks of reversible cell response to injury (degeneration) include cellular swelling, cytoplasmic vacuolation, perinuclear clear spaces, formation of cytoplasmic blebs, loss of normal apical blebs from Clara cells, and loss of cilia. In some cases, detachment of viable cells from the epithelial surface and nuclear condensation (pyknosis) and cellular shrinkage of scattered cells within the epithelium, suggestive of imminent death of individual cells, may be interpreted as epithelial degeneration because it may be consistent with reversible damage to an epithelial surface and evidence of outright necrosis may be lacking. The light microscopic features of necrosis include nuclear pyknosis, karyorrhexis, or karyolysis, cell swelling, loss of cellular detail, cell fragmentation, and cytoplasmic hypereosinophilia (in which the cytoplasm often has a homogeneous appearance). Necrosis of the epithelial cells lining the airways as a result of toxic injury is often characterized by sloughing of necrotic cells or cellular debris into the lumen. Other lesions often accompany necrosis and degeneration, such as inflammation and hemorrhage (NTP, 2014; Allison et al., 2022). The anatomic location of degenerative lesions may vary due to the physicochemical properties of the test or toxicological agent or the susceptibility of a particular cell type to the test agent. The epithelium of the terminal bronchioles and alveolar ducts (i.e., the centriacinar region) and alveoli are particularly susceptible to injury due to the large surface area and fragility of the alveolar type I cells, the metabolic activity of P450 enzymes in Clara cells, and the generally thinner mucous layer (NTP, 2014).
- **Bronchiolar/Alveolar epithelial hyperplasia**: It is hyperplasia of the epithelial cells in the centriacinar region (terminal bronchiole/alveolar duct and adjacent alveoli) of the lung. It is this consistent location that differentiates this lesion from alveolar epithelial hyperplasia, which is randomly located in the alveolar parenchyma. The cells are generally cuboidal with round to oval nuclei. The majority of the cells can be nonciliated, with a variable number of ciliated cells, and some appear to have apical blebs. Given the location of this lesion, it may be a precursor to bronchiolar/alveolar neoplasia (NTP, 2014; Allison et al., 2022). The proliferating cells may originate in the terminal bronchioles or in the alveolar ducts. The morphology of the cells is similar to that of the terminal bronchiole cells. However, in bronchiolar/alveolar neoplasms, in some of the cells immunohistochemical staining is consistent with Clara cells, and in other cells it is consistent with alveolar epithelial cells (NTP, 2014).

Associated lung histological alterations include: collapsed alveoli (atelectasis) alternating with dilated alveoli. There is Necrosis of alveolar epithelial cells and formation of characteristic eosinophilic hyaline membranes lining the respiratory bronchioles, alveolar ducts and the proximal alveoli. The membrane is largely composed of fibrin admixed with cell debris derived from necrotic alveolar cells. There is Interstitial and intra-alveolar oedema, congestion and intra-alveolar hemorrhages. In some cases a compensatory proliferation of pneumocytes into alveolar lumen may be seen as tufts of alveolar epithelium. There is also interstitial fibrosis obliterating alveolar spaces. Findings in this study were consistent with previous studies: Rat treated with heavy showed oedema, air space enlargements, thick interalveolar septa, Interstitial lymphocyte infiltration, shedding of the mucosal lining, dilatation and congestion of pulmonary vein, some cellular debris in the bronchiole, marked thickening of the wall of pulmonary vein and deposited inflammatory cells inside alveolar sacs (El-Refaiy, 2013). Also, findings in this study are in agreement with the findings of Shin et al. (2004) who reported that the lung is a primary target organ of systemic exposure to heavy metals. Mckenna et al. (1997) found that heavy metals exposed lungs showed acute and more chronic pulmonary inflammation in both rats and mice with bronchiolar and alveolar lesions. Heavy metal exposure was deleterious to the lung tissue causing mild to severe inflammation (Bell et al., 2000).

5. Conclusion

This study was ecologically relevant. It was able to demonstrate that known contaminants of oil spill sites, if consumed at rates equal to or higher than their oral reference dose for mammalian species can cause lung disorders, even at sub-chronic toxicity period. The study has once more given credence to the use of histology as a biomarker to assess sublethal level of environmental stressors, and in determination and extrapolation of the ecosystem pollution capabilities of the exposure to the studied target chemicals.

Compliance with ethical standards

Acknowledgments

We wish to thank the Department of Anatomy, University of Benin, Nigeria for the use of their Anatomy House for this study.

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

Ethical approval was obtained from ethical committee.

References

[1] Allison, T.A. and Paul, C.W., (2014). Histological Based Biomonitoring: A Baseline Ecotoxicological Evaluation of New Calabar River Using Chrysichthis Nigrodigitatus. Europe American Journal: International journal of Environment and Pollution Research. Vol. 2. No. 3.

[2] Allison, T.A. and Paul C.W. (2018). Histological based Biomonitoring: A Baseline Ecotoxicological Evaluation of Ekerekana and Okochiri Creeks using Mudskipper. Scholars Bulletin (Anatomy). An Official Publication of ―Scholars Middle East Publishers. Dubai, United Arab Emirates ISSN 2412-897X.

[3] ATSDR (Agency for Toxic Substances and Disease Registry), (2004). Public Health Statement on Copper. Department of Health and Human Services, Public Health Service Agency for Toxic Substances and Disease Registry

[4] ATSDR (Agency for Toxic Substances and Disease Registry), (2007). Public Health Statement on Lead. Department of Health and Human Services, Public Health Service Agency for Toxic Substances and Disease Registry

[5] ATSDR (Agency for Toxic Substances and Disease Registry), (2012a). Public Health Statement on Chromium. Department of Health and Human Services, Public Health Service Agency for Toxic Substances and Disease Registry

[6] ATSDR (Agency for Toxic Substances and Disease Registry), (2012b). Public Health Statement on Cadmium. Department of Health and Human Services, Public Health Service Agency for Toxic Substances and Disease Registry

[7] Bell, R.R., Nonavinakere, V.K., Soliman, M.R., (2000). Intratrachealexposure of the guinea pig lung to cadmium and/or selenium: a histological evaluation. Toxicology Letters 114 (1), 101–109

[8] CCAC (Canadian Council on Animal Care), (1984). Guide to the Care and Use of Experimental Animals: husbandry of experimental animals on a species by species basis. Vol. 2, 1st Edition, www.ccac.ca

[9] Bennicelli, C., A. Camoirano, S. Petruzelli, P. Zanacchi, and S. De Flora, (1983). High sensitivity of salmonella TA102 in detecting hexavalent chromium mutagenicity and its reversal by liver and lung preparations. Mutat. Res. 122:1-5.

[10] Drury, R.A. and Wallington, E.A., (1980). Carleton's Histology Technique. 5th Edition, Oxford University Press, Newyork.

[11] El-Refaiy, A.L. and Eissa, F.L. 2013. Histopathology and cytotoxicity as biomarkers in treated rats with cadmium and some therapeutic agents. Saudi Journal of Biological Sciences, 20: 265–280

[12] Hounkpatin, A. S. Y., Edorh, P. A., Guédénon, P., Alimba, C. G., Ogunkanmi, A., Dougnon, T. V., Boni, G., Aissi, K. A., Montcho, S., Loko, F., Ouazzani, N. and Mandi, L., (2013). Haematological evaluation of Wistar rats exposed to chronic doses of cadmium, mercury and combined cadmium and mercury. African Journal of Biotechnology. Vol. 12(23), pp. 3731-3737. DOI: 10.5897/AJB12.2669. ISSN 1684-

[13] Jemai, H., Lachkar, H.A., Messaoudi, I., Kerkeni, A., 2010. Effect ofzinc pre-treatment on blood glutathione, serum zinc and kidneyhistological organization in male rats exposed to cadmium. Journalof Trace Elements in Medicine and Biology 24 (4), 277–288

[14] Mckenna, I.M., Waalkes, M.P., Chen, L.C., Grdn, T., 1997. Com-parison of inflammatory lung responses in Wister rats and C57 andDBA mice following acute exposure to cadmium oxide fumes.Toxicology and Applied Pharmacology 146 (2), 196–206

[15] Obianime, A. W. and Roberts, I. I., (2009). Antioxidants, Cadmium-Induced Toxicity, Serum Biochemical And The Histological Abnormalities Of The Kidney And Testes Of The Male Wistar Rats. Nigerian Journal of Physiological Sciences 24 (2): 177 -185 ©Physiological Society of Nigeria

[16] OECD. (Organisation for Economic Co-operation and Development), (1984). OECD guidelines for testing of chemicals. 204. Fish prolonged toxicity test: 14 day study. Organisation for Economic Co-operation and Development, Paris, France.

[17] OECD. (Organisation for Economic Co-operation and Development), (1992a). OECD guidelines for testing of chemicals. 203. Fish, acute toxicity test. Organisation for Economic Co-operation and Development, Paris, France.

[18] (OECD) Organisation for Economic Co-operation and Development 210, (2001), OECD Guideline for Testing of Chemicals: Acute Toxicity and Acute Toxicity Class Method. Test 423

[19] Shin, H.J., Lee, B.H., Yeo, M.G., Oh, S.H., Park, J.D., Park, K.K.,Chung, J.H., Moon, C.K., Lee, M.O., 2004. Induction of orphannuclear receptor Nur77 gene expression and its role in cadmium-induced apoptosis in lung. Carcinogenesis 25 (8), 1467–1475

[20] Sun H, Wang N, Nie X, Zhao L, Li Q, Cang Z, et al. (2017) Lead Exposure Induces Weight Gain in Adult Rats, Accompanied by DNA Hypermethylation. PLoS ONE 12(1): e0169958. doi:10.1371/journal.pone.0169958

[21] Thinkratok A, Suwannaprapha P, Srisawat R., (2014). Safety assessment of hydroethanolic rambutan rind extract: acute and sub-chronic toxicity studies. Indian J Exp Biol. 2014 Oct;52(10):989-95. PMID: 25345248.

[22] USEPA (United States Environmental Protection Agency), (1992).