ACUTE AND CHRONIC HISTOPATHOLOGICAL EFFECTS OF SMOKED MARIJUANA ON ALBINO RATS AND ITS OXIDATIVE STRESS INDICES

ABSTRACT

Smoking marijuana (cannabis Savita), a psychoactive substance may result in side effect on living cells of the system and oxidative stress may meditate some of these effects. This study aims to evaluate the acute and chronic histopathological effect on wistar albino rat as well as oxidative stress indices and some of the haematological parameters evaluation. This study was conducted at the animal house of Ebonyi State University, Presco campus Abakaliki. The experimental animals were share into control group, acute and chronic administered group which was housed in the temperature and humidity controlled facility of 22°C+1, standard laboratory mice chow diet with tap water freely available at ad libitum to the animals. The weight of the male wistar albino rat were taken twice daily which range from 150g to 200g after which was exposed to smoke of burnt wrapped 2mg of marijuana morning and evening for 21 days for experimental animals in group B and 42 days for group C animals respectively. The acute histopathological effect and that of chronic of the lungs of smoked marijuana wistar albino rat displayed histomorphological changes with Haematoxylin and Eosin, methanamine silver staining and phosphotungstic acid staining method when compared with the control lung sections that shows normal histomorphological pattern with the present of alveolar pneumocytes and alveolar space. Evaluation of the lungs of smoked marijuana rat shows increased MDA and decreased level of CAT, SOD, GSH and GPx but examining some of the haematological parameters show elevated Twbc and platelet while packed cell volume decreased the level as compared with control animals. I hereby concluded that marijuana smoked is a potent source of cellular oxidative stress that contributes significantly to cellular injury, dysfunction, and pulmonary edema as well as histomorphological changes from the lungs of marijuana smokers and disorder in some of the haematological parameters.

***Keywords**: acute, chronic, histopathological effects, marijuana, albino rats, oxidative stress indices*

INTRODUCTION

Cannabis also known as Marijuana is the most frequently consumed illicit drug in most developed and developing countries. In recent years, there has been noticeable increase in cannabis and its products consumption among teenagers and young adults (Appendino *et al.*, 2011).They are derived from the flowering tops, leaves, and resin from the female plant of Cannabis sativa L. (family Cannabidaceae). Cannabis is abused for its mood altering properties. These psychotropic effects of cannabis are mediated by its main psychoactive constituent Δ9-tetrahydrocannabinol (Δ9-THC). Cannabis also contains over 60 other cannabinoids, a C21 terpenophenolic compound that is uniquely produced by the cannabis plant, such as cannabinol, cannabidiol, cannabigerol, and cannabichromene. Africa is the second largest producer of herbal cannabis in the world.

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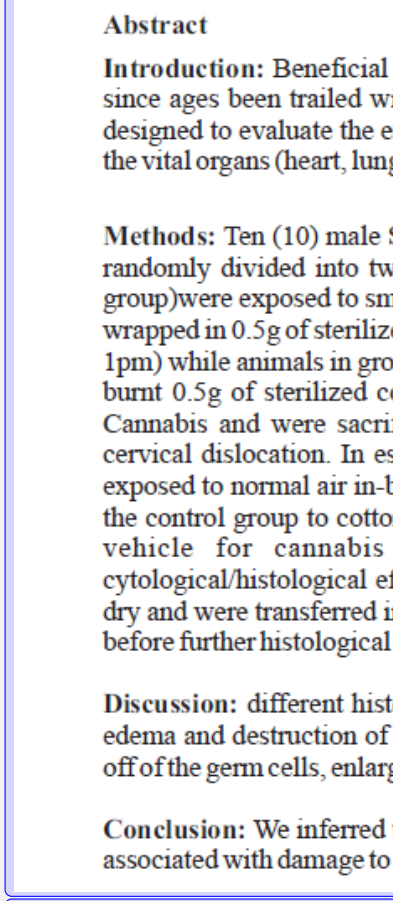

Abstract

Introduction: Beneficial
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MATERIALS AND METHODS

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Study Area

This study was conducted at the Animal house of Ebonyi State University, Presco Campus, Abakaliki.

Sample size estimation/ sample selection

Resource Equation method (E) was used for the sample size estimation; the formular: E = (Total number of animal in a group multiply by number of groups) –(Number of groups) as recommended by Jaykara and Kantharia (2013) on how to calculate sample size in animal studies

= (7×3) - 3, **= 21-3**

= 18

Sample size (E) =

Corrected sample size= sample size **E÷ (1-% attrition)**

In this study 10% attrition is expected hence,

Corrected sample size= 18/1-10/100

= 18/0.9

= 20

Inclusion criteria: Eligibility of the subjects includes; healthy albino rat, body weight between 150g to 200 g and must be male albino rat.

Exclusive criteria: Other species of rats except albino rat, non-healthy albino rats, body weight below 150g or above 200 g are excluded in this study.

Ethical Consideration

Animal procedures were performed in accordance to the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals were dully observed (Publication No. 85-23, revised 1985).

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Experimental animals

In this study, 21 Albino rats were housed in a temperature- and humidity-controlled facility of (22^0C+1^0C) and were maintained on a 12 h light/dark cycle. Standard laboratory mice chow diet and tap water were freely available ad libitum to the animals. The albino rats were randomly divided into 3 groups with 7 animals in each group. The rats were divided into three (3) groups made up of seven in each group. Each group was housed in clean capacious macro lane cage.

Laboratory Investigations

Sacrifice and Collection of Samples

The animals would be anaesthetized using chloroform vapour in an enclosed transparent plastic jar**,** blood samples collected through cardiac puncture in to EDTA and plain containers and thereafter dissected to remove the lungs and wash with normal saline. Part of the lungs was fixed in 10% Buffered formal saline for histopathological evaluation and the remaining part was preserved using cool ice block for oxidative stress indices determination. The EDTA blood sample was send to the laboratory for haematological analysis (packed cell volume, total white cell and platelet counts).

Histopathological Procedure

The fixed specimens of the kidney and lung were processed overnight for dehydration, clearing, and impregnation using an automatic tissue processor (Sakura, Japan). The specimens were embedded in paraffin was using an embedding station (Sakura, Japan) and serial sections of 4um thickness were cut using a microtome (ModelRM2245, Leica Biosystems, Wetzlar, Germany). Conventional stain, Histochemical stain and special stain were used. The mounted specimens were observed and were scored under light microscopy. For a semi-quantitative comparison of the structural changes, the abnormalities in the tissue sections were graded from 0 (normal structure) to 3 (severe pathological changes).

The following staining methods were employed:

Haematoxylin and Eosin Stain (conventional method)

SpecialStain**:** Phosphotungstic Acid Haematoxylin (PTAH) Stain

(A) Conventional Stain (Haematoxylin and Eosin Stain)

Procedure: Tissue Sections were dewaxed in three changes of xylene for 5-minutes. Hydrated in descending grade of alcohol (Absoluteand90%) for 5-minutes each. Sections were brought to water. Sections were dipped in hematoxylin to stain for 15-minutes it and rinsed with water. 1% Acid alcohol was used to differentiate the sections briefly and rinsed with water. Sections was blued with Scot's water for10-minutes and rinsed with water. The sections counter stained with 1% eosin for4-minutes and rinsed with water. Dehydrated with ascending graded alcohol (90%, absolute1, 2 & 3) for15-seconds each. Sections were cleared with xylene 1&2 for 3-minutes each. Stained sections were mounted DPX mountant. Sections were examined with microscope and photographed.

(B) Special Stain (Phosphotungstic Acid Haematoxylin Stain)

Procedure: Sections were deparafinized using three changes of xylene for 5-minutes each. There were taking to graded alcohol (Absolute and 90%) for5-minutes each. Sections were brought to water.

There were Oxidized in potassium permanganate for 10-minutes and rinsed with water. Sections were bleached in oxalic acid for5-minutes and washed thoroughly with distilled water.

Sections were stained with PTAHfor15-hours.Stainedsections were transferred directly to 95% alcohol, followed by absolute for 15-secondseach for dehydration. There were cleared in two changes of xylene for 3-minutes each and mounted with DPX mountant. Mounted Sections were examined with microscope

DETERMINATION OF OXIDATIVE STRESS PARAMETERS

Determination of Catalase (CAT) Activity

Procedure

Exactly 2.5 ml of phosphate buffer and 2 ml of hydrogen peroxide (H_2O_2) were added to the test tube. After that, 0.5 ml of the sample homogenate was also added to the test

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tube. To 1 ml portion of the reaction mixture, 2 ml of dichromate acetic acid reagent was added. Absorbance was read at 240 nm against the bank at a minute interval.

Calculation

$$\text{Catalase concentration (U/L)} = \frac{0.23 \times \log \text{Absorbance 1/Absorbance 2}}{0.00693}$$

Estimation of Superoxide Dismutase (SOD) Activity

This was estimated according to the method described by Fridovich and Mc-Cord (1969).

Procedure

Exactly 0.2 ml of the sample homogenate was introduced into 2.5 ml of 0.05 phosphate buffer. At pH of 7.8, 0.3 ml of newly prepared adrenaline solution was added to the reaction mixture followed by quick mixing by inversion in the cuvette. The increase in absorbance was taken every 30 seconds for 3 minutes at 480 nm against blank. Blank contained 0.3 ml of adrenaline and 2.5 ml buffer.

Super Oxide Dismatase (SOD) activity was measured by determining the inhibition of auto oxidant of adrenalins.

Determination of Malondialdehyde (MDA) Level

Procedure

Exactly 0.1 ml of sample homogenate, 0.9 ml of distilled H_2O, 0.5 ml of 25% TCA reagent and 0.5 ml of 1% TBA reagent in 0.3% NaOH were added to a test tube. The test tube was incubated at 95^0C for 40 minutes. After that, the test tube was allowed to cool in water and exactly 0.1 ml of 20% SDS (sodium dodecyl sulphate) was added to the test tube. The absorbance of the sample was read against the blank reagent at 532 and 600 nm.

Calculation

$$\%\text{TBARS} = \frac{\text{A532} - \text{A600}}{0.5208 \times 0.1} \times 100$$

Reduced Glutathione Determination (GSH)

Glutathione (GSH) concentration was measured according to the method of Ellman (1959).

Procedure

One mililitre of the sample was added 4.0 % sulfo-salicyclic acid and the mixture centrifuged at 3,000 rpm for 15 minutes at 2 ^{o}C. The sampleshomogenate was introduced to 4.5 ml of Ellman reagent and absorbance was measured at 412 nm. The blank were prepared by addition of 0.5 ml of 4 % sulfo-salicyclic acid to 4.5 ml of Ellman reagent while absorbance was measured at 412 nm.

$$\text{Plasma GSH concentration} = \frac{\text{Asample - Ablank}}{\text{Astandard - Ablank}} \text{X Cstandard}$$

Determination of Glutathione Peroxidase Activity

Procedure

The reaction mixtures were prepared by putting in a testtube 14.0 ml of distilled water, 2.0 ml 5 % pyrogallol solution, 1.0 ml of 0.147 MH_2O_2 solution and 2.0 ml of 0.1 M phosphate buffer (pH 6.0). The mixture was then equilibrated at 20 ^oC for about 5 minutes, afterwhich there was the addition of 1.0 ml of the sample homogenate solution, with mixing of the resulting solution.This was followed by the addition of 1ml of 2.0 N H_2SO_4 to stop the reaction after exactly 20 seconds. The optical density of the resulting solution was measured at 420 nm against a blank (prepared like the test except that no sample is added to it and 15 ml of distilled water is used where 14 is used in the test solution).

Calculation

The activity of peroxide can be calculated for using the formula:

$$\text{Volume of activity}(\mu/\text{ml}) = \frac{\Delta\text{OD x df}}{0.117 \text{ x Vs}}$$

where: $\Delta OD = (OD_{Test} - OD_{Blank})$

= ΔOD x 8.547 x df -Weight activity (µ/mg) = (µ/ml) x $\frac{1}{c}$

$$\frac{\Delta OD \ 8.547 \times df}{c}$$

Where: Vs = sample volume (1.0ml); 0.117 = Ooptical density at 420 nm corresponding to 1 mg % purpurogallin in ether; df = dilution factor (if used during the study); c = enzyme/sample concentration in dissolution.

HEMATOLOGICAL ESTIMATION OF PARAMETERS

Automated Hematological Analyzer (Sysmex KX-21N).

Statistical analysis

The data generated from this research were analysed using graphpad instat 3 version 3.02, comparison of groups using analysis of variance (ANOVA) with post hoc Bonferroni Multiple comparison test to identify differences in means where appropriate and $p < 0.05$ was taken as statistically significant value

RESULTS

The control group of the lung section of wister albino rats showed normal histomorphological patterns as shown in plate 1 bellowed as compared to that of the test groups (B&C) of the experimental animals.

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Plate1Mag.X40

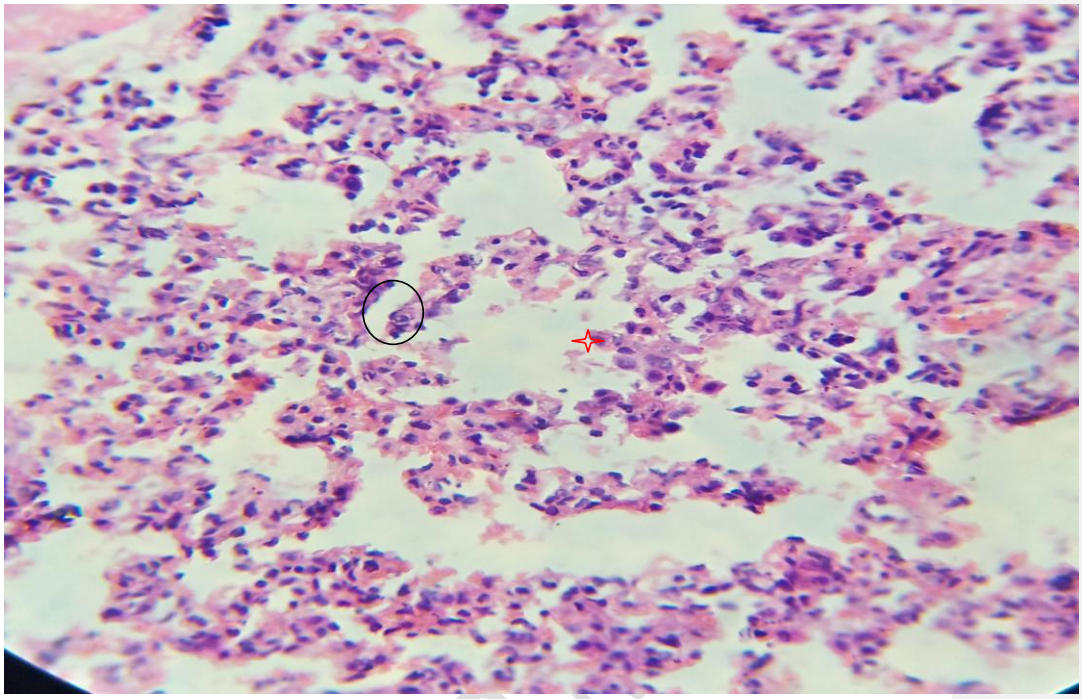

Plate 1 is a lung section of Wistar on smoked Marijuana control section stained with Haematoxylin and Eosin technique; the alveolar pneumocytes (oval) and alveolar spaces (star) are well demonstrated and shown normal lung section.

Plate 2.Mag.X40

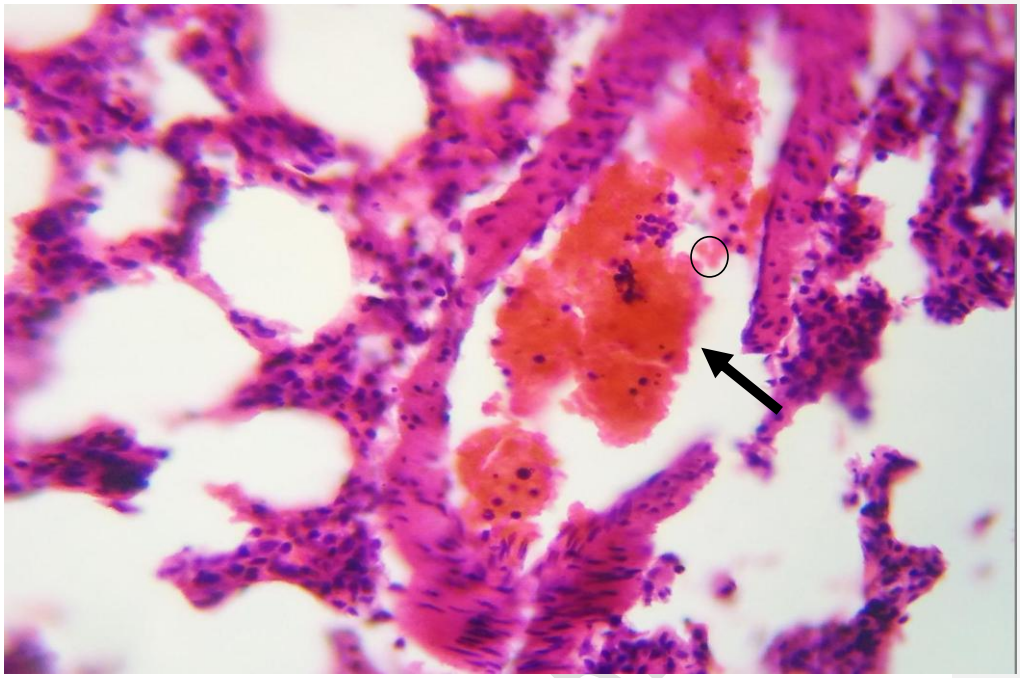

Plate 2 is a lung section of Wistar rat Acute Smoked Marijuana stained with Haematoxylin and Eosin technique; section shown acute alveolar hemorrhage occluding the alveolar space consequence of acute injury, with lymphocytic granules at the background. Type II pneumocytes are remarkably present.

Plate 3.Mag.X40

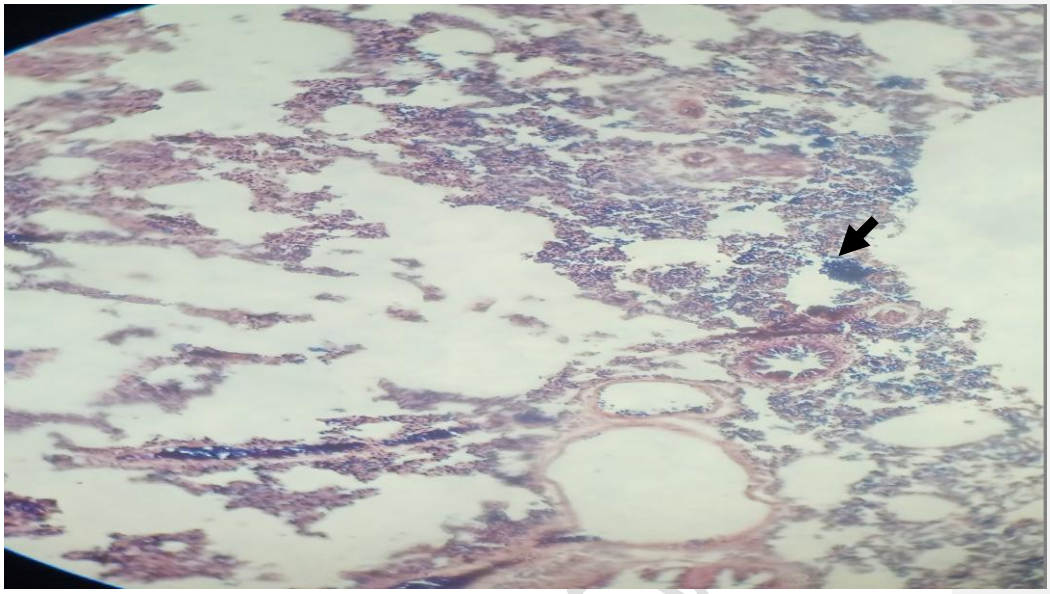

Plate 3 is a lung section of Wistar rat Acute Smoked Marijuana stained with Methanamine silver staining technique; section shown hypertrophy of the smooth muscles with remarkable hyperplasia of the mucus glands. Smoker's macrophages were seen around the haemorragic alveolar sacs.

Plate 4Mag.X40

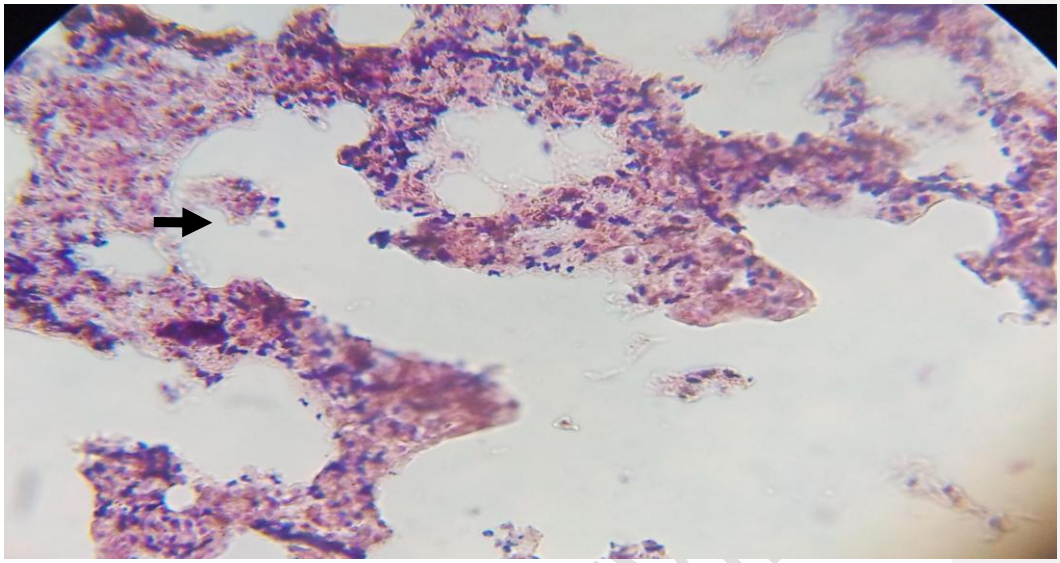

Plate 4 is a lung section of Wistar rat Chronic Smoked Marijuana stained with phosphotungstic acid staining technique; section shown streaming of fluid into the alveolar space (arrow) and an already pulmonary edeoma seen as consequence of the alveolar congestion **(E**).

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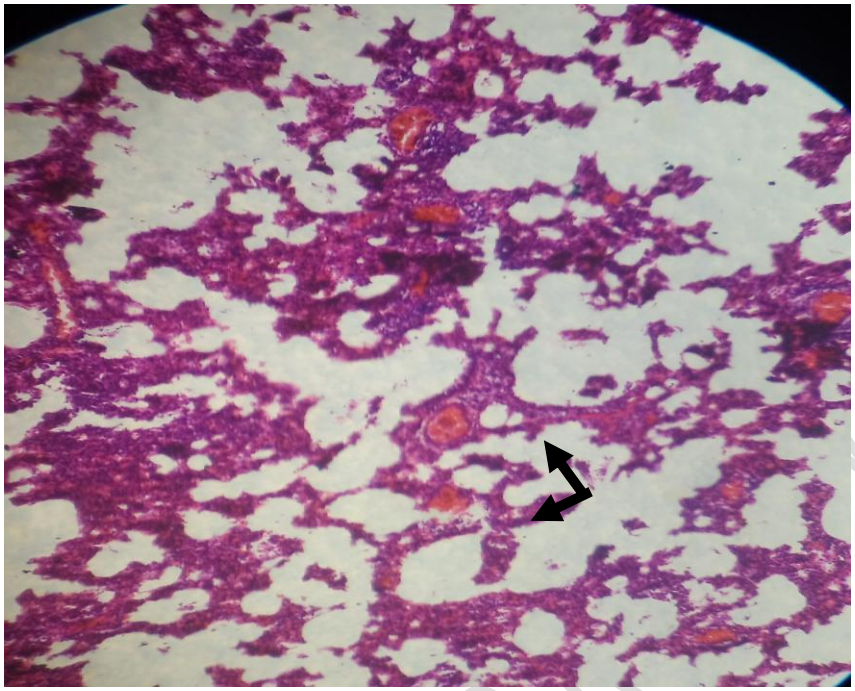

Plate 5 is a lung section of Wistar rat Chronic Smoked Marijuana stained with haematoxylin and Eosin staining technique; section shown smooth muscle hyperplasia **(arrows**) with glandular hyperchromasia, seen also include smokers macrophages(**M**)

Plate 6Mag.X40

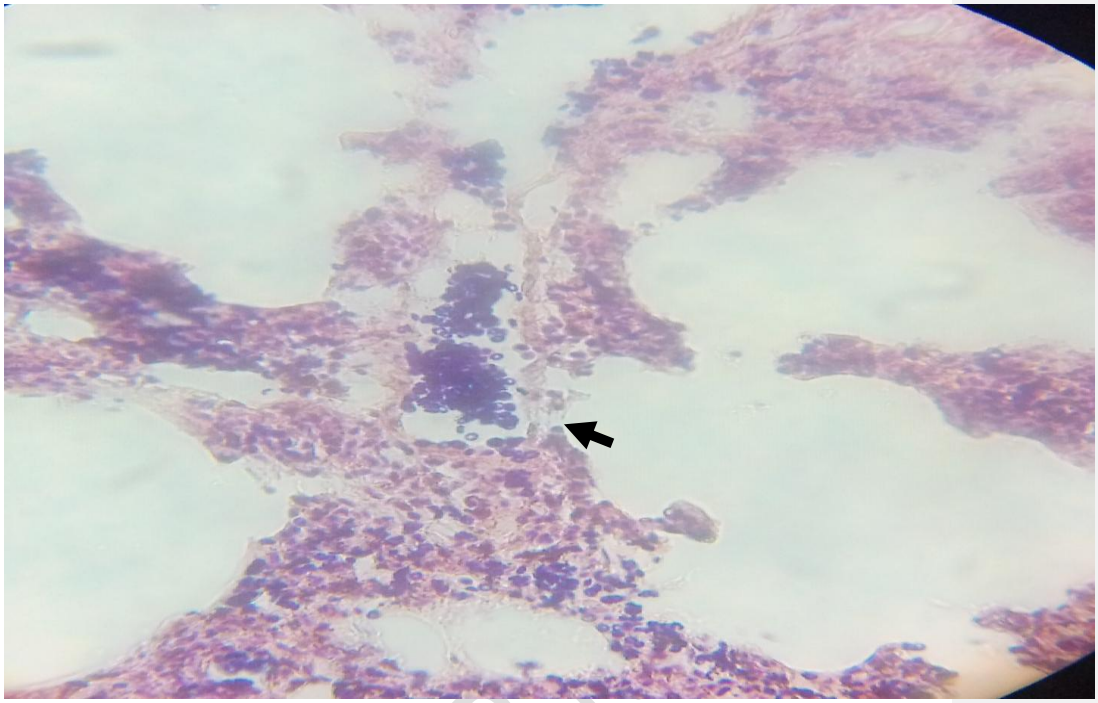

Plate 6 is a lung section of Wistar rat Chronic Smoked Marijuana, stained with phosphotungstic acid haematoxylin staining technique; section shown alveolar congestion (arrow) and remarkable type II pneumocytes.

BODY WEIGHT OF WISTER ALBINO RATS

Administration of smoked marijuana in rats significantly ($p<0.05$) reduced the body weight of the experimental rats (Figure 1).

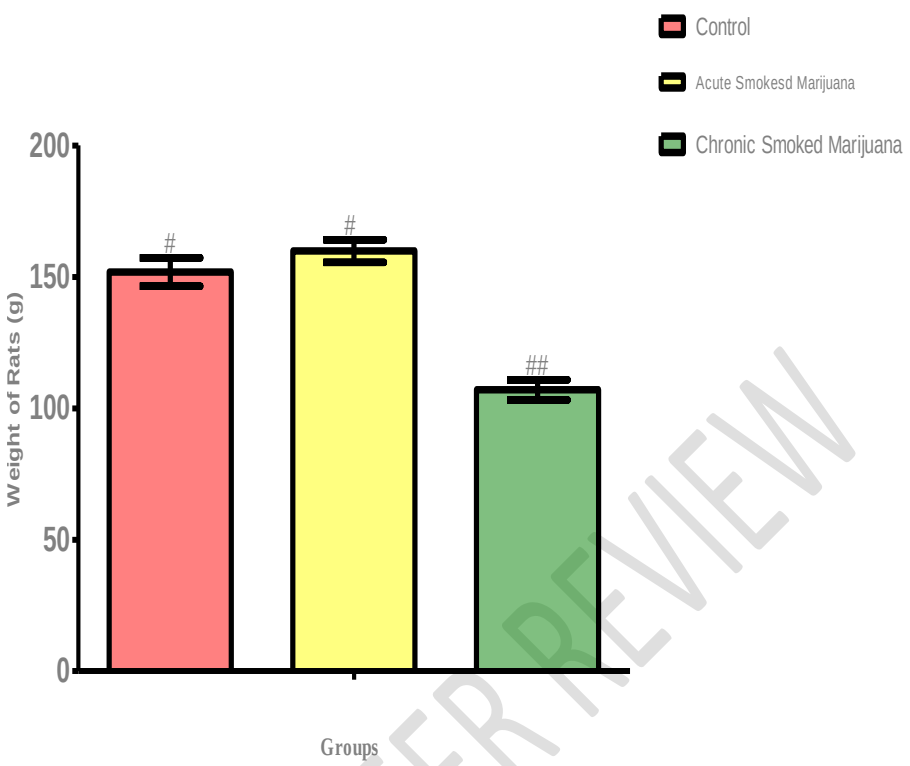

Figure 1: Effects of Smoked Marijuana on Body Weight of Rats. Data are shown as mean ± S.D (n=6). Mean values with different sign are significantly different at $P<0.05$.

The animals appreciated in weight during acclimatization, but during the exposure, their weight dropped gradually as shown in figure 1. Apparently, the animals looked healthy throughout the duration of the experiment. However, some were very excitable while some were dull and sluggish in movement. Both the test and the control animals showed marked differences in their behavioral and physical features during the acclimatization.

The initial increase in body weight of the albino rats observed in this experiment could be attributed to increase in feeding. However the weight loss at the later stages of the experiment could be due to the inability of the albino rats to gets to food as a result, depression and consequent inability of the muscles of the body caused by cannabis smoked administration. Also, there was general anorexia. It is possible therefore, that a significant loss of body weight could be an index of cannabis toxicity in albino rats. The test animals showed much resistance to cannabis smoked administration within the first three days. More so, after the first two days of smoked exposure they become receptive. Most of the experimental animals were seen to calm

immediately after the smoke administration, they were also aggressive during the administration. This is attributed to the effect of cannabis smoked administration. The control group (A) of the albino rats showed normal strength and appetite more than the test group (B&C).

Oxidative Stress indices of the Lungs of Albino Rats

Exposure of smoked Marijuana in albino rats significantly (p**Rat Lung**<0.05) elevated the level of MDA (Melondialdehyde) and significantly($p<0.05$) reduced the activities of Catalase (CAT), Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and level of Reduced Glutathione(GSH) in albino rat lung in time dependent manner as compared to their control group shown in figure 2,3,4,5&6 respectively.

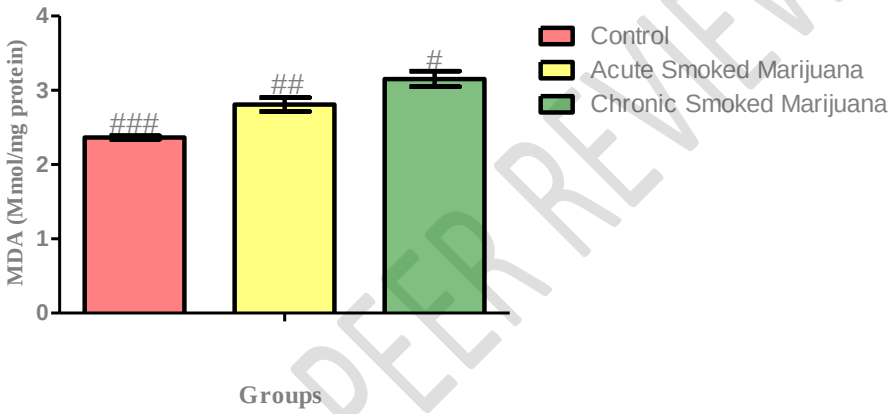

Figure 2: Effects of Smoked Marijuana on Lung MDA Level in Rats. Data are shown as mean ± S.D (n=6). Mean values with different sign are significantly different at $P<0.05$.

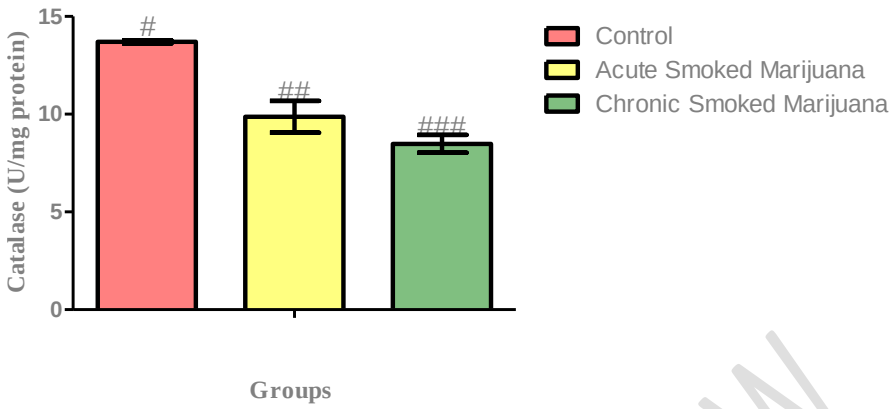

Figure 3: Effects of Smoked Marijuana on Lung Catalase Activity in Rats. Data are shown as mean ± S.D (n=6). Mean values with different sign are significantly different at P<0.05.

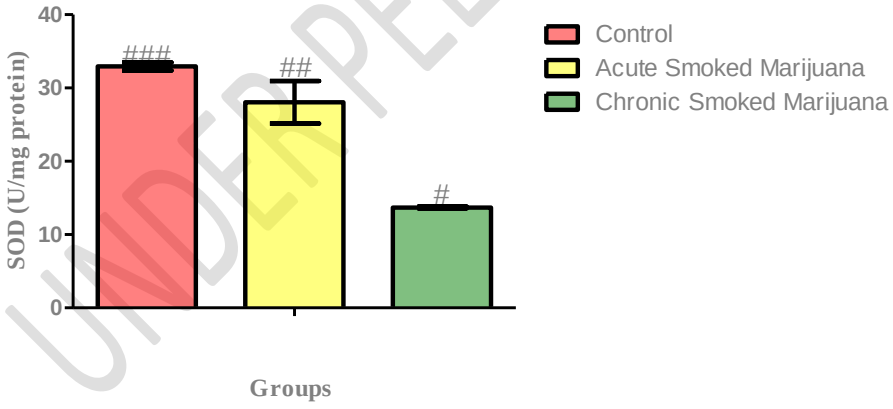

Figure 4: Effects of Smoked Marijuana on Lung SOD Activity in Rats. Data are shown as mean ± S.D (n=6). Mean values with different sign are significantly different at P<0.05.

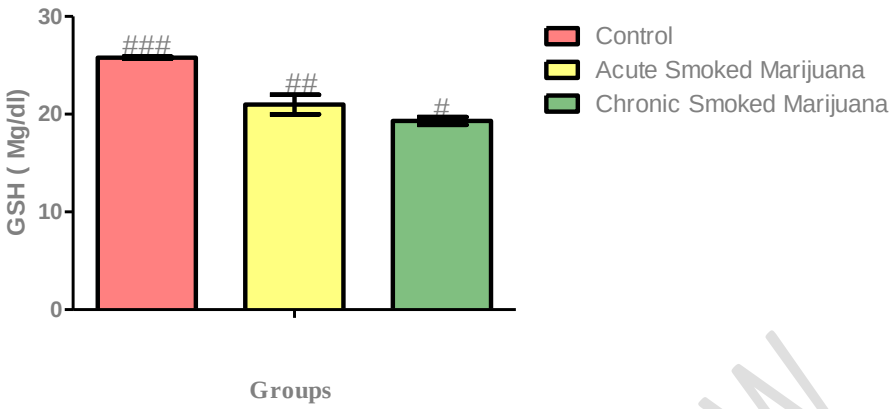

Figure 5: Effects of Smoked Marijuana on Lung GSH Activity in Rats. Data are shown as mean ± S.D (n=6). Mean values with different sign are significantly different at P<0.05.

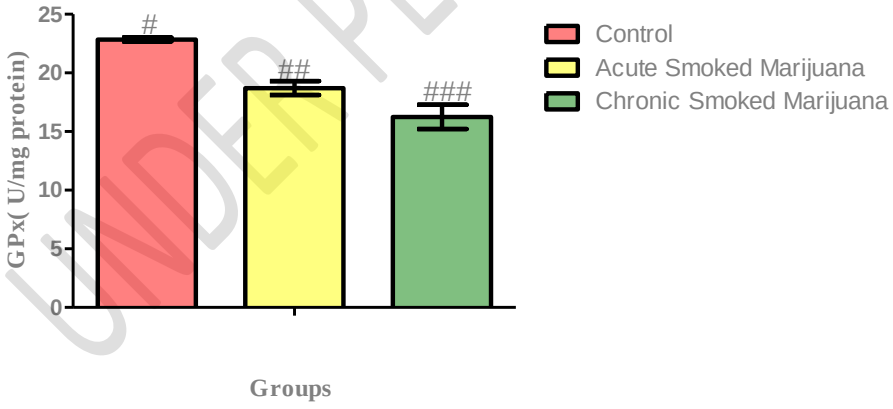

Figure 6: Effects of Smoked Marijuana on Lung GPx Activity in Rats. Data are shown as mean ± S.D (n=6). Mean values with different sign are significantly different at P<0.05.

Acute and Chronic Effects of Smoked Marijuana on Haematological Parameters of Albino Rat

Exposure of smoked Marijuana in rats significantly ($p<0.05$) elevated the levels of Total white blood cells and platelet. A significant ($p<0.05$) reduction was observed in the levels of Packed cell volume (Haematocrit [HCT]) in time dependent manner in smoked marijuana exposed rats (Figures 7-9).

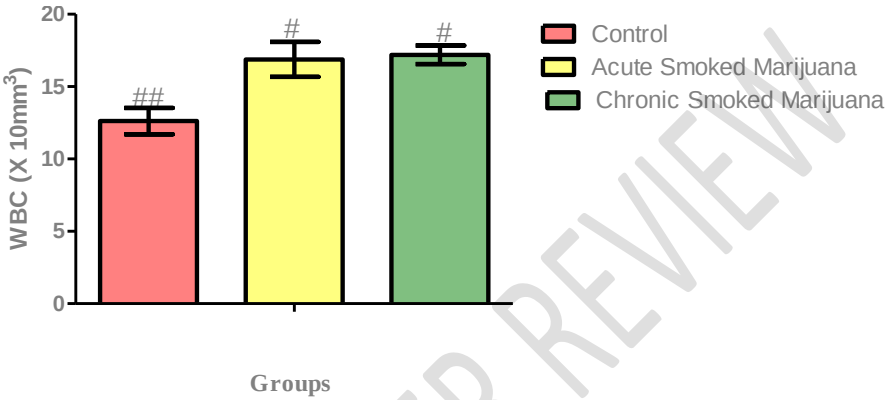

Figure 7: Effects of Smoked Marijuana on WBC Level in Rats. Data are shown as mean ± S.D (n=6). Mean values with different sign are significantly different at $P<0.05$.

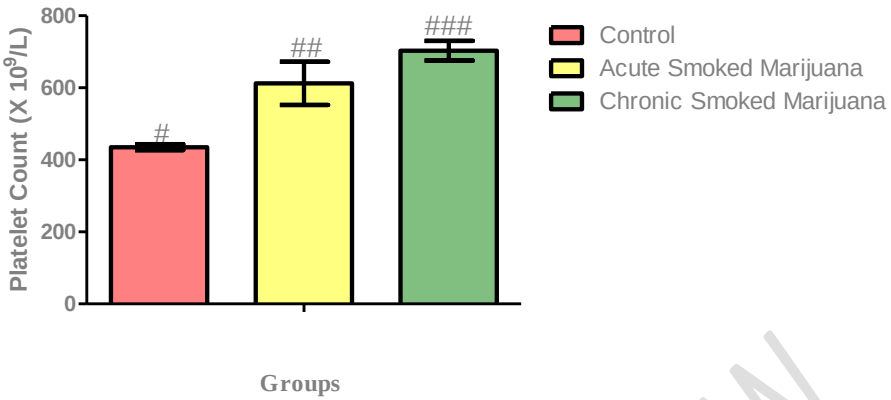

Figure 8: Effects of Smoked Marijuana on Platelet Level in Rats. Data are shown as mean ± S.D (n=6). Mean values with different sign are significantly different at P<0.05.

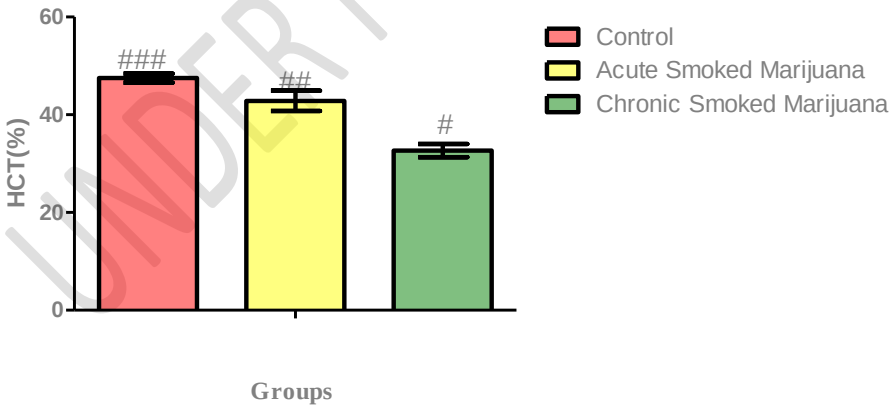

Figure 9: Effects of Smoked Marijuana on HCT Level in Rats. Data are shown as mean ± S.D (n=6). Mean values with different sign are significantly different at P<0.05

DISCUSSION

The current uncontrolled use of marijuana (cannabis savita) in Nigeria will in time lead to such a scale that would dexterously affect the health of human being and animal inclusive. The present experiment set out to investigate the acute and chronic histopathological effects of smoked marijuana on albino rats and its oxidative stress indices which were conducted on the lungs of albino rats and evaluation of some hematological parameters (haematocrit, total white cells count and platelets). The idea was to stimulate a situation whereby the albino rats were exposed to cannabis smoked for the period of 21days (acute administration for group B) and 42 days (chronic administration for group C) for morning and evening respectively.

In my present study, there were pathological changes ranging from mild variation in group B (acute) of the albino rats treated with 2mg of cannabis smoked twice daily to more severe variation seen in group C (chronic) body weight, there were histological changes in the lungs such as acute alveolar hemorrhage occluding the alveolar space consequence of acute injury, with lymphocytic granules at the background. Type II pneumocytes are remarkably present in Haematoxylin and Eosin staining method shown in plate 2 and Methanamine silver staining technique shown hypertrophy of the smooth muscles with remarkable hyperplasia of the mucus glands. Smoker's macrophages were seen around the hemorrhagic alveolar sacs of plate 3 in acute administration of smoke marijuana as shown in photomicrograph when compared to the control group of albino rat photomicrograph of plate 1. That shown alveolar pneumocytes and alveolar spaces which shown normal lung section with Haematoxylin and Eosin staining method.

The photomicrograph of the lungs of wistar rat chronic marijuana smoked stained with phosphotungstic acid staining technique shown streaming of fluid into the alveolar space and an already pulmonary edema seen as consequence of the alveolar congestion in plate 4, as well as smooth muscle hyperplasia with glandular hyperchromasia and smokers macrophages in Haematoxylin and Eosin staining method shown in plate 5, but plate 6 also indicate alveolar congestion and remarkable type II pneumocytes with phosphotungstic acid staining technique.

In this study as well cannabis was found to have toxic effect that grew worse as the days of exposure to cannabis smoked were increases. It might be the reasons for it insignificant decreased in the body weight of the albino rats as in test group ($P<0.05$) of figure 1, with increased dullness and aggression

Furthermore, the evaluation of oxidative stress indices in the lungs of wistar albino rat exposed to marijuana smoked shown increased level of Melondiaidehyde (MDA) and decreased level of Catalase (CAT), Superoxide dimutase (SOD),Reduced Glutathione (GSH), and Glutathions peroxidase (GPx) as shown in figure 2, 3, 4, 5 & 6 ($P<0.05$) when compared test group with that of the control. This is attributed to the increase level of reactive oxygen species due to marijuana smoked. The alteration of these parameters is attributed to the cellular injury, information and pulmonary edema due

to marijuana smoked and risk factors for lung cancer (Sarafian, et al., 1999). Habitual marijuana smoking has also been shown to alter alveolar macrophage morphology (Davies, et al., 1979; Beals, et al., 1989), phagocytic functions (Baldwin, et al., 1998), fungicidal and bactericidal activity, and oxidative burst superoxide production (Baldwin, et al., 1998).

Additionally, this study was further demonstrated the effects of marijuana smoking on some of the haematological parameter of smokers and observed a significant difference in some of the parameters between wistar albino rats exposed to marijuana smoked and non-exposed albino rat (control group). The results obtained in the study showed that total white blood cell count increased significantly ($P<0.05$) of figure 7 against the control group. This increased level attributed to disturbances in immune system functions and immune response to the smoked inhalation (Amaechi, et al., 2020). This finding also showed a significant increase in platelet count ($P<0.05$) of figure 8. This elevation of platelet count in marijuana smokers is in agreement with the work of Erikssen, et al., (1994) who observed a small but statistically highly significant increase in platelet count in marijuana smokers. His study was further revealed a slightly decreased of prothrombin time and activated partial thromboplastin time amoung marijuana smokers when compared with non-smokers counterpart. The decrease, though not satisfactory significant but was in concordant with the work of Coetzee, et al., (2007), that cannabis savita and the Cannabinoids, display anticoagulant activity and may be useful in the treatment of disease such as type 2 diabetes in which a hypercoagulabe state exist. On the order hand, Haematocrit (Packed Cell Volume) evaluation shows remarkable decreased ($P<0.05$) of figure 9 in Marijuana Smoked wister albino rats as compared to the control group. This was explained by the increase in carbon monoxide level in the blood of smokers which induced erythrocytosis (Tashkin, 1997) which has been suggested to result in the intrathoracic airway obstruction or pulmonary insufficiency leading to ventilation/perfusion imbalance that results in functional hypoxia or hypoxaemia and arterial oxygen unsaturation, increasing the demand of bone marrow for red blood cell production observed as increase haemoglobin concentration to increase oxygen carrying capacity of the blood (Rubin, et al., 1975).

Finally the study was carried out within six weeks with the rate of progression, if the procedure was not interrupted, it would have progressed to more severe toxic, histological and pathological changes due to cannabis smoked on albino rats.

CONCLUSION

In summary, marijuana (cannabis savita) within short time of exposure could cause serious histological changes in the lungs of the albino rats and alteration of oxidative stress and haematological indices. It is observed that there was a dose response relationship, where the more the exposure of the albino rats to cannabis smoked the higher the chances of toxicity, and the longer the length of exposure, the chances of damages. It is believe that smoking cannabis clearly shows that it is unhealthy and dangerous.

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Conflict of Interest
The authors declare there is no conflict of interest.

Consent for Publication
None

Funding
None

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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