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Research Article
Investigation of Acute and Sub-chronic Toxicity of Aqueous Extract of Nigerian Nasal Snuff in Wistar Albino Rats

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Abstract

Background and Objective: The use of dry nasal snuff is growing rapidly and is alarmingly becoming prevalent among different age groups in both rural and urban populations of Nigeria based on certain ethnocultural believes that were not verified scientifically. This study was carried out to evaluate the toxicity of aqueous extract of the local nasal snuff in wistar albino rats. **Materials and Methods:** Twenty rats of both sexes used in the study were randomly divided into five groups of four animals each. Group A served as control, receiving only distilled water and food, while experimental groups (B-E) were orally administered graded doses of the extract at 7.60, 14.80, 22.00 and 29.05 mg kg^{-1} b.wt., representing 20, 40, 60 and 80% of the lethal dose (LD_{50}), respectively, once daily using intubation cannula, for 28 days. The animals were sacrificed on the 29th day, after an overnight fast, blood samples collected for biochemical analysis, livers and kidneys collected for histopathological examination. **Results:** Acute toxicity study revealed that the aqueous extract of the dry snuff has LD_{50} >50 mg kg^{-1} b.wt., of the rats. There was progressive non-significant ($p<0.05$) increase in the body weights of the animals in all the groups. In the liver function tests, there was a significant ($p<0.05$) increase in the activities of enzymes, Alanine Transaminase (ALT), Aspartate Transaminase (AST) and Alkaline Phosphatase (ALP) in the treated groups. The serum total protein, albumin and globulin levels were significantly ($p<0.05$) increased in groups D and E compared with the control. For renal function indices, significant ($p<0.05$) decrease and increase were observed in urea and creatinine levels, respectively in the experimental group E. There was however, no significant ($p<0.05$)difference in the levels of electrolytes, sodium, potassium and chloride compared to the control. Significant($p<0.05$) decrease in the levels of anti-oxidant enzymes, catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD) was equally observed. Histopathological analysis showed degenerative changes in the liver but not the kidney tissues. **Conclusion:** These findings suggest that chronic consumption of high sub-chronic doses of the nasal snuff may cause hepato- and renotoxicity as well as induces an oxidative stress in the test animals.

Key words: Dry snuff, hepatotoxicity, renotoxicity, histopathology, oxidative stress, rats

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Snuff (smokeless tobacco) is a tobacco or tobacco product that is not burned or smoked but used by other means, such as nasally or orally. Nasal snuff is a dry snuff that is inhaled nasally and includes a variety of commercially available products and mixtures that differ according to the nature of its chemicals constituents and the geographical locations[1]. In Nigeria, dry nasal snuff comes in different names including 'Shaqe' in Hausa 'Taba' in Yoruba and'Utaba' in Igbo languages. These products are produced locally from materials such as tobacco wastes, ayoro, potash, clove, alligator pepper, black pepper, salt, flavours and some fragrances. The production and consumption of snuff is growing rapidly and is alarmingly becoming prevalent in most developing countries including Nigeria[2]. This may be related to its widespread marketing, availability, afford ability, addiction potential, lack of legislation against its usage and perhaps the belief that it has some medicinal effects[3]. Consumption of smokeless tobacco products is also popular in other parts of the world including United States of America, Asia, Africa and various European countries[4].

The nasal snuff is utilized for cultural and traditional purposes. It is believed by its users to cure ailments, such as catarrh, toothache and abdominal pain. Some users consider it as an energy booster and a sexual enhancer. However, all these are ethno-cultural believes that are not scientifically verified. On the other hand, smokeless tobacco exposes its users to numerous health risks, including narcotic activity, dizziness, weakness, loss of consciousness, insomnia and coma due to its nicotine contents and other harmful substances[5]. The unprecedented use of dry nasal snuff is growing rapidly among different age groups in both rural and urban populations of Nigeria based on some ethno-cultural believes without scientific verification of its safety. The present study was therefore, designed to investigate the acute and sub-chronic toxicity of aqueous extract of the local nasal snuff in albino wistar rats.

MATERIALS AND METHODS

Study period: This research study was conducted in the Department of Biochemistry, Usmanu Danfodiyo University, Sokoto, Nigeria between 15th August-26th September, 2018 (6 weeks).

Chemicals and reagents: Chemicals and reagents of analytical grade were used in this study, assay kits were used for the assay of some biochemical parameters.

Sample preparation: Locally produced nasal snuff powder was obtained from a factory at 'Takoshayi' Area, Bida, Niger State, Nigeria. Exactly 500.00 g of the sample were soaked in 5 L of distilled water. The mixture was filtered after 24 h using muslin cloth. The filtrate was then evaporated in a drying cabinet at 45°C to obtain a concentrated extract. The concentrated extract was reconstituted in distilled water to obtain the stock concentration of the extract.

Experimental animals: Wistar albino rats, weighing 140-180g of both sexes, purchased from animal house of the Faculty of Animal Husbandry and Veterinary Medicine, University of Ibadan, Ibadan, Nigeria. The animals were housed in hygienic and well-ventilated environment in polycarbonate cages with saw dust bedding at a temperature of 27±3°C in the animal house of Usmanu Danfodiyo University, Sokoto. The animals were fed with standard Finisher's Marsh (Rico Gado Feeds) and clean water and they were allowed to acclimatize to the experimental conditions for a week prior to the commencement of the experiment.

Acute toxicity study: The acute toxicity study was carried out based on OECD guidelines[6]. Five rats were randomly selected and marked to permit individual identification. The animals were weighed prior to the administration of the extract. The lethal dose $(LD_{50})_{oral}$ of aqueous extract of the dry snuff used was 50 mg kg^{-1} b.wt. as the LD_{50} for nicotine in rats[7]. One animal served as the control while the others were administered the extract at 50 mg kg^{-1} b.wt., orally in a single dose using intubation cannula. Each animal was observed at intervals of 1 h, for the first 4 h after administration, for any sign of toxicity and subsequently, at least twice daily for the next 48 h. Observations include mortality, dermal sensitization, eye effects, fur and gait feature, food consumption and neurotoxic behavioral changes in the animals. The animals were re-weighed to determine change in weight 48 h following administration.

Sub-chronic toxicity test: Repeated toxicity study was carried out using twenty rats, randomly divided into five groups of four animals each (n = 4). Group A served as control, receiving 1 mL of distilled water and food only. Experimental groups (B-E) were given the aqueous extract of the snuff at 7.60, 14.80, 22.00 and 29.05 mg kg^{-1} b.wt., as 20, 40, 60 and 80% of the LD_{50}, respectively. The extract was administered orally, once daily for 28 days. The body weights of the animals were measured a day before the start of administration and then weekly. On the 29th day, the animals were sacrificed after

overnight fast. The blood samples were collected into labelled centrifuge tubes for biochemical analysis while liver and kidneys were collected for histopathological examination.

Liver function tests: Total protein was determined by Biuret reaction method[8]. Albumin was determined by Bromocresol Green (BCG) method[8]. Globulin was calculated as the difference between total proteins and albumin. Total and direct bilirubin were determined by Jendrassik and Groff method[9]. Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) activities were determined using Reitman and Frankel method[10]. Alkaline Phosphatase (ALP) was determined using ρ-nitrophenyl phosphate reaction method[11].

Renal function tests: Urea was determined by Urease-Berthelot method[12]. Creatinine was determined by Jaffe's reaction method[8]. Sodium, potassium and chloride were determined by Flame-emission Spectrophotometry[8].

Oxidative status assessment: The oxidative status of the test animals was analyzed by assaying the levels of anti-oxidant enzymes, Superoxide dismutase (SOD) was assayed according to method described by Zuo *et al.*[13]. Catalase (CAT) activity was assayed using the method described by Beers and Sizer[14]. The activity of Glutathione Peroxidase (GPx) was determined by method described by Rotruck *et al.*[15].

Histological analysis

Tissue collection: The liver and kidneys were carefully excised from the bodies of the rats following dissection. The organs were rinsed in normal saline and fixed into separate specimen bottles containing 10% neutral buffered Formalin for preservation and prevention of tissue decay. The samples were taken to Usmanu Danfodiyo University Teaching Hospital, Sokoto, Nigeria for histological analysis.

Tissue preparation: The tissues were processed into thin microscopic sections and fixed onto paraffin support using 10% buffered formalin. The paraffin block containing the tissue sample was sliced or sectioned using as microtome into sections as thin as 5 µm. The paraffin sections produced were floated on a water bath, from which they were mounted on glass slides. The sections were then stained with a combination of hematoxylin and eosin (H and E) dye to reveal the cellular components of the examined tissues and to produce the contrast needed to visualize the tissues through optical microscope. Hematoxylin stains nuclei blue, while Eosin stains cytoplasm and the extracellular cellular connective tissue matrix pink. The histological slides were then analyzed under light microscope by a histopathologist.

Statistical analysis: Statistical analysis was carried out using Graph pad INstat 3 software (San Diego, USA). The results were expressed as mean (±SEM) using one-way analysis of variance (ANOVA), followed by Dunnett's test for multiple comparison. Differences were considered significant at $p<0.05$.

RESULTS

The acute oral dose of aqueous extract of the dry snuff showed neither mortality nor serious toxic effects at 50 mg kg^{-1} b.wt. However, some behavioral changes, including restlessness and mouth scratching (immediately after administration) as well as reduced feed and water intake were observed during the study period.

The effect of repeated sub-chronic oral doses of aqueous extract of the nasal snuff on weight changes in the animals as presented in Table 1, which showed a non-significant ($p<0.05$) increase in body weights in all the groups, which was slower in groups D and E. This suggests that higher sub-chronic doses of the snuff extract may have interfered with feed utilization ratio in the test animals.

The results of the liver function parameters of the rats administered with sub-chronic doses of aqueous extract of the snuff is presented in Table 2 and 3.

The decrease in the levels of total proteins, albumin and globulin was significant ($p<0.05$) in groups D and E. There was also a non-significant decrease and increase in the levels of total and direct bilirubin, respectively in the treated groups compared to the control.

The results showed that there was a significant ($p<0.05$) increase in the activities of the three enzymes, ALT, AST and ALP in groups D and E compared to the control.

Table 4 presents the results of the renal function indices in the rats administered with sub-chronic oral doses of the aqueous extract of the nasal snuff.

The results showed that there were significant ($p<0.05$) increase and decrease in the levels of creatinine in group E. There were also non-significant differences ($p<0.05$) in the levels of electrolytes, sodium, potassium and chloride compared to the control group.

The effect of oral administration of sub-chronic doses of aqueous extract of the nasal snuff on oxidative status of the test animals is presented in Table 5.

Table 1: Effect of sub-chronic oral doses of aqueous extract of the nasal snuff on the weekly weight changes in albino rats

Groups	Week 1	Week 2	Week 3	Week 4
A	150.2±1.94	158.1±3.08	170.2±3.20	177.1±3.22
B	147.2±1.77	155.5±2.69	165.2±3.89	169.2±3.09
C	148.7±1.49	154.2±2.01	165.2±3.17	164.2±3.44
D	147.1±1.17	156.2±4.55	159.7±1.32	162.2±1.94
E	149.5±2.79	152.6±1.32	158.2±2.41	160.7±2.36

Values are expressed as Mean±SEM (n = 4). *Significantly different from control (p<0.05) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test. A: Control, B: 7.60, C:14.80, D: 22.0 and E: 29.05 mg kg^{-1} b.wt.

Table 2: Effect of sub-chronic oral doses of aqueous extract of the nasal snuff on serum proteins and bilirubin in albino rats

Groups	TP (g dL^{-1})	ALB (g dL^{-1})	Globulin	TB (mg dL^{-1})	DB (mg dL^{-1})
A	8.7±0.24	3.3±0.08	6.12±1.59	1.16±0.01	0.07±0.01
B	7.4±0.34	3.4±0.14	4.02±0.49	0.91±0.01	0.07±0.01
C	7.3±0.06	3.4±0.06	3.95±0.12*	0.92±0.10	0.09±0.01
D	6.2±0.28*	2.5±0.15*	3.77±0.26*	0.93±0.08	0.09±0.01
E	6.0±0.50*	2.4±0.14*	3.47±0.69*	0.93±0.15	0.10±0.01

Values are expressed as Mean±SEM (n = 4). *Significantly different from control (p<0.05) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test. A: Control, B: 7.60, C:14.80, D: 22.0 and E: 29.05 mg kg^{-1} b.wt. TP: Total protein, ALB: Albumin, TB: Total bilirubin and DB: Direct bilirubin

Table 3: Effect of sub-chronic oral doses of aqueous extract of the nasal snuff on liver enzymes in albino rats

Groups	AST (U L^{-1})	ALT (U L^{-1})	ALP (U L^{-1})
A	243.0±3.53	92.3±1.03	470.8±2.96
B	270.8±3.63	92.5±0.95	471.8±4.44
C	272.0±0.95*	99.0±1.87	479.5±1.94#
D	277.0±3.24*	105.8±2.10*	518.6±3.25*
E	289.3±3.71*	111.3±3.44*	521.8±3.12*

Values are expressed as Mean±SEM (n = 4). *Significantly different from control (p<0.05) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison test. A: Control, B: 7.60, C:14.80, D: 22.0 and E: 29.05 mg kg^{-1} b.wt. AST: Aspartate amino transferase, ALT: Alanine amino transferase, ALP: Alkaline phosphatase

Table 4: Effect of sub-chronic oral doses of aqueous extract of nasal snuff on kidney function indices in albino rats

Groups	Urea (mMol L^{-1})	Crtn (mg dL^{-1})	Na^+ (mMol L^{-1})	K^+ (mMol L^{-1})	Cl^- (mMol L^{-1})
A	7.4±0.19	0.77±0.09	138.0±0.41	5.98±0.30	102.3±0.25
B	6.9±0.43	1.03±0.08	134.5±0.96	5.42±0.24	99.0±0.40
C	6.7±0.43	1.03±0.07	135.8±1.11	5.65±0.11	98.5±0.64
D	6.5±0.46	1.10±0.13	137.5±0.96	5.45±0.09	99.8±0.62
E	5.4±0.23*	1.25±0.09*	137.5±1.32	5.58±0.03	100.0±0.40

Values are expressed as Mean±SEM (n = 4). *Significantly different from control (p<0.05) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison test. A: Control, B: 7.60, C:14.80, D: 22.0 and E: 29.05 mg kg^{-1} b.wt. Crtn: Creatinine, Na^+: Sodium ion, K^+: Potassium ion and Cl^-: Chloride ion

Table 5: Effect of oral administration of sub-chronic doses of aqueous extract of nasal snuff on anti-oxidant enzymes in albino rats

Groups	CAT (U mL^{-1})	GPx (U mL^{-1})	SOD (U mL^{-1})
A	5.98±0.74	24.99±1.56	3.08±0.27
B	4.25±0.15	18.79±0.89*	2.89±0.53
C	4.14±0.50*	17.33±0.15*	2.66±021
D	3.90±1.01*	17.18±0.55*	2.70±0.93
E	3.70±0.21*	16.17±1.63*	2.63±0.67*

Values are expressed as Mean±SEM (n = 4). *Significantly different from control (p<0.05) using analysis of variance (ANOVA) followed by Dunnett's Multiple comparison test. A: Control, B: 7.60, C:14.80, D: 22.0 and E: 29.05 mg kg^{-1} b.wt. CAT: Catalase, GPx: Glutathione peroxidase, SOD: Superoxide dismutase

There was a significant (p<0.05) decrease in the levels of antioxidant enzymes, catalase, glutathione peroxidase (GPx) and superoxide dismutase compared to the control group. The results of histological analysis of liver and kidney tissues of the rats, orally administered with sub-chronic doses of aqueous nasal snuff extract were presented in Fig. 1 and 2, respectively.

Normal portal triad, central vein and normal hepatocytes arranged in cords are seen in Fig. 1a and b. However, in Fig. 1c-e, hepatocytes exhibited a ballooning degeneration, which is an earlier histological sign of hepatocytes injury or inflammation.

DISCUSSION

The present study investigated the acute and sub-chronic toxicity of aqueous extract of the Nigerian local nasal snuff in albino Wistar rats. The results evaluated some biochemical indicators of toxicity and histological indices of tissue damage. Toxicological studies are carried out in experimental

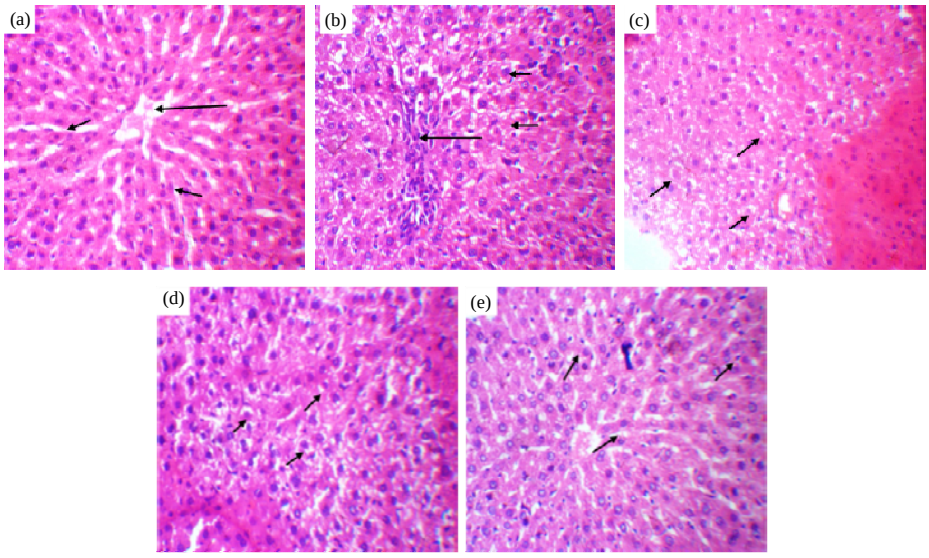

Fig. 1(a-e): Effect of oral administration of sub-chronic doses of aqueous extract of nasal snuff on liver histology of albino rats. Normal portal triad, central vein (long arrows) and normal hepatocytes arranged in cords (short arrows) are seen in (a, b). However, in groups, (c-e) Hepatocytes exhibited a ballooning degeneration (short arrows), which is an earlier histological sign of injury seen on light microscopy. Images are representative of four animals (n = 4) in each experimental group. (H and E, X100)
A: Control, B: 7.60, C:14.80, D: 22.0 and E: 29.05 mg kg^{-1} b.wt.

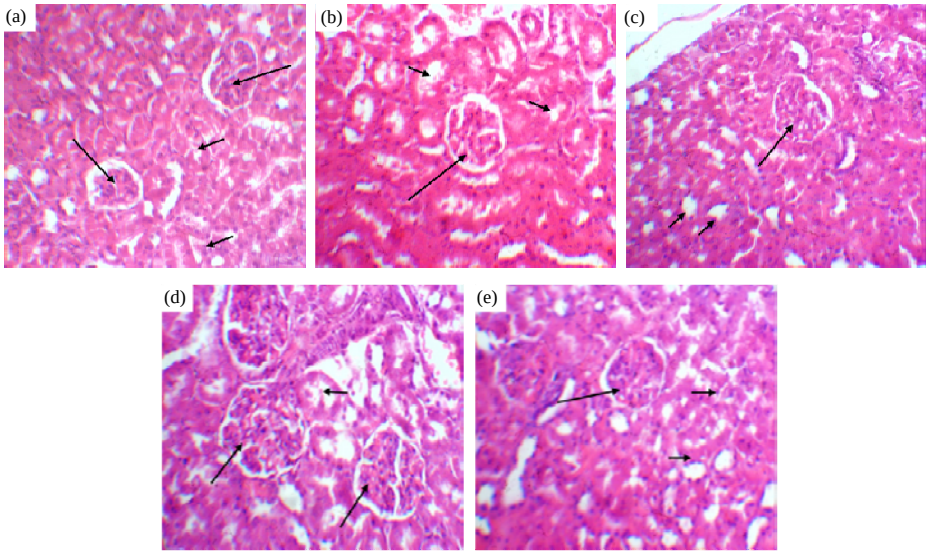

Fig. 2(a-e): Effect of oral administration of sub-chronic doses of aqueous nasal snuff extract on kidney histology of albino rats. Sections shows regular glomerulus (long arrows), renal tubules and interstitium (short arrows). No obvious histological anatomical changes observed. Images are representative of four animals (n = 4) in each experimental group. (H and E, X100)
A: Control, B: 7.60, C:14.80, D: 22.0 and E: 29.05 mg kg^{-1} b.wt.

animal models to evaluate toxicity or safety of various substances and to provide the bases for establishment of safe levels in humans. The estimation of plasma proteins helps in assessing the functional integrity of the liver as most of these proteins, including albumin and globulin are synthesized in the liver cells[16]. Serum total protein alone may not portray the true picture of the metabolic state of the cells, since the levels of the individual proteins do not rise or fall in parallel to each other, but with the levels of albumin and globulins calculated, the consistency in the decrease or increase in the level of total proteins can be established. This synthesis of plasma proteins in the liver diminishes if the hepatocytes are injured or damaged[17]. The significant ($p<0.05$) decrease in the plasma total proteins and albumin levels observed in this study may indicate a degeneration in the synthetic capacity of the liver because of exposure of the animals to the snuff extract. There were non-significant ($p<0.05$) difference in the levels of both total and direct bilirubin in all the experimental groups when compared with the control group. Consequently, it can be deduced that the excretory function of the liver in the test animals is not adversely affected as bilirubin level is a useful index of the excretory function of the liver[18].

A significant ($p<0.05$) increase in the enzymes, AST, ALT and ALP observed in the study is a common indicator of liver cells damage[19]. These enzymes are mainly localized in the cytoplasm and any damage in hepatic cells may result in their release into the blood[20]. Though ALT is more specific to the liver than the AST and its activity is usually greater than AST activity at the earlier stages of acute hepatocellular diseases, AST tends to be released more than the ALT in chronic liver diseases, such as cirrhosis[17]. ALP is a useful diagnostic marker of cholestatic hepatobiliary lesions and osteoblastic bone diseases its activity is increased in liver and bone diseases[21]. Significant increase in ALP activity in groups D and E therefore, suggested a damage to the structural integrity of the liver.

One of the primary functions of the kidneys is to remove creatinine, a waste product of muscle breakdown from the bloodstream which is commonly measured as an index of glomerular function rate[22]. High-levels of creatinine indicated kidney impairment, which can be temporary or permanent[23]. Decreased level of urea is associated with hepatic failure and nephritis. Though, plasma urea concentration is less reliable than the creatinine as an index of glomerular filtration rate (GFR), estimation of the two complement each other in evaluating the functional integrity of the kidneys[17]. The increased serum creatinine and decreased serum urea levels observed in group E indicate likelihood of kidney malfunction at extreme doses. The levels of Electrolytes in the blood is controlled by the regulatory mechanisms of ionic charges and osmotic balance, a homeostasis that is maintained through the functional interplay between the endocrine system, the kidneys and the lungs[24]. The non-significant ($p<0.05$) differences in the serum concentrations of electrolytes, sodium, potassium and chlorides suggested that the aqueous extract of the nasal snuff may not have deleterious effect on the water, electrolytes and acid-base balance in the test animals. These findings correlated with hemostatic parameters reported by Ukoha *et al.*[21].

Antioxidant enzymes form the body's main endogenous defence mechanism against free radicals-induced cell damage. Normally, a state of equilibrium exists between tissue oxidants and anti-oxidants and the imbalance between the level of pro- and anti-oxidant species results in oxidative stress (failure to repair oxidative injury induced by reactive oxygen species)[25]. The ability of many cells to resist oxidative stress is associated with high intracellular levels of anti-oxidants enzymes[25,26]. These enzymes metabolize the oxidative toxic intermediates and control the levels of lipid hydroperoxides to prevent cell damage by the free-radicals[27,28]. The significant ($p<0.05$) decrease in the serum levels of these enzymes in the present study indicates that the nasal snuff extract may have induced an oxidative stress in the test animals and this result agrees with the report by Avti *et al.*[29] that smokeless tobacco impairs the antioxidant defence mechanism in some vital organs in rats.

The histopathological examination revealed a ballooning degeneration in the hepatocytes, which is an earlier histological sign of hepatocytes inflammation/injury seen on the light microscope. The high doses of the snuff extract may have induced an inflammation of the portal tract of the liver. This observation also correlates with that reported by Avti *et al.*[29]. There were however, no obvious histological anatomical changes in the kidney tissues observed as regular glomerulus, renal tubules and interstitium were evident. These results lend credence to those of biochemical parameters in suggesting that the snuff extract may have caused structural damage to the liver but not the kidney tissues at the sub-chronic doses.

CONCLUSION AND RECOMMENDATION

From the results of the current study, it can be concluded that higher sub-chronic doses of the aqueous extract of the nasal snuff may have adverse effects on the liver and kidneys and induces an oxidative stress in the test animals. The extract may however, be safe to the kidney at low doses.

In view of the biochemical and histopathological indices of the rats administered with acute and sub-chronic oral

doses of the aqueous nasal snuff extract, it is apparent to recommend that the snuff may be toxic to health, especially at higher sub-chronic doses. Further research to isolate the bioactive principles of the snuff and uncover their mechanism of actions is also recommended.

SIGNIFICANCE STATEMENT

This study discovered that at higher sub-chronic doses the nasal snuff can be toxic. The study will therefore, help researchers to uncover the deleterious effects of nasal snuff consumption on the health of its users.

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