Enzyme Engineering
ISSN: 2329-6674
OPEN ACCESS Freely available online
Research Article

Effect of Sudan II Adulteration of Palm Oil on the Serum Enzyme, Bilirubin Concentration and Renal Function Biomarkers of Albino Wistar Rats

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ABSTRACT

Aim: This study investigated the impact of Sudan II adulteration of palm oil on serum enzymes (ALT, AST, ALP), bilirubin and renal function biomarkers (creatinine, urea) of albino wistar rats.

Methods: A total of sixty (60) 750 mL bottles of red palm oil were purchased from random markets in Nigeria. Sixty (60) male albino rats weighing 150-180 g were divided into 5 groups of 12 rats each. Group 1 served as normal control. Groups 2 to 5 were fed 90% rat chow supplemented with 10% red palm oil. The Sudan II dyes were co-administered with the red palm oil with the diet (rat chow) to provide levels of 0.025% (PO/0.025) (group 3), 0.03% (PO/0.03) (group 4) and 0.04% (PO/0.04) (group 5) for a period of 30 days (short term) and 90 days (long term). Animals were sacrificed and blood was collected *via* cardiac puncture for biochemical analysis. Calorimetric methods were used to determine the bilirubin, urea and creatinine concentrations while standard methods were used for the kinetic determination of ALT, AST, ALP. Data analysis was carried out with SPSS using one-way analysis of variance (ANOVA).

Key findings: Result showed that the serum enzymes activities and functional biomarkers increased significantly ($P<0.05$) in both short-term and long-term feeding conditions. Intentional addition of Sudan II dye to palm oil had adverse effects.

Significance: The significant increase of the parameters in this study is indicative of an adverse effect of the dye on health and therefore a major public health concern. It is pertinent to create awareness and the need for enforcement of regulatory acts and food safety procedures.

Keywords: Colour; Palm oil; Serum enzymes; Renal biomarkers; Adulteration; Health

INTRODUCTION

Adulteration of food is a global phenomenon that has serious consequences on health and safety. It is an unacceptable practice that is designated as illegal in food safety regulations. The spectra of food products being adulterated varies from nation to nation and may include fruit juices, palm oil, flour and meat products. Food is adulterated mainly to make it attractive and mask the effect of the use of unwholesome ingredients. Thus, food adulteration brings economic benefits to those who engage in it. In Nigeria, palm oils are being adulterated through the use of prohibited food dyes or colourants by food fraudsters.

The oil palm (*Elaeis guineensis*) is believed to originate from West Africa. The commercial value of this crop lies mainly in its oils. The crop is unique in that it produces two types of oil. The fleshy mesocarp produces red palm oil and the kernel produces palm kernel oil. Both of which are edible but with different chemical composition, physical properties and applications. The composition of red palm oil, together with its natural consistency, appearance and pleasant aroma make it an ideal ingredient in the development and production of a variety of edible oils, in particular, margarines and fats and also ideal when making products like biscuits, cakes, sauces [1]. The term "quality" denotes the degree of excellence of a product. The quality of red palm oil is virtually determined by its colour, which is a brilliant red colour. Hence, any palm oil without the bright red colour, would not be acceptable to consumers. Thus consequently, Sudan II dye is added to enhance the colour

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Received: 10-Jan-2022, Manuscript No. EEG-22-47513; **Editor assigned:** 12-Jan-2022, PreQC No. EEG-22-47513 (PQ); **Reviewed:** 26-Jan-2022, QC No. EEG-22-47513; **Revised:** 31-Jan-2022, Manuscript No. EEG-22-47513 (R); **Published:** 07-Feb-2022, DOI: 10.35841/2329-6674.22.11.100017622

Citation: Peters HE, Henshaw AS, Ikpeme CA, Williams I (2022) Effect of Sudan II Adulteration of Palm Oil on the Serum Enzyme, Bilirubin Concentration and Renal Function Biomarkers of Albino Wistar Rats. Enz Eng. 11: 176.

appeal. The palm fruit has carotenoids which are responsible for the bright/orange colour of the red palm oil. According to, any factor which affects the carotene content of the fruit invariably influences the colour of the palm oil. During lipid oxidation, hydro peroxides generated accelerate carotene oxidation which results in bleaching and discoloration of the red palm which then breaks down the carotenoids and consequently deteriorate the bright orange red colour of the red palm oil. Hence, the drive and demand by individuals for brightly coloured red palm oil has exacerbated the increasing use of this dye.

Sudan dyes (I, II, III and IV) are synthetic chemical dyes of similar chemical structure. They are oil-soluble, aromatic compounds containing azo group (-N=N-). Sudan dyes are used in colouring hydrocarbon solvents, plastics and floor polishes, but they are unauthorized food colours. Sudan dyes are fraudulently used in order to maintain the colour of the product. The International Agency Research on Cancer (IARC) classified Sudan II as a suspected carcinogen (group 3) in the Technical Rules for Dangerous Substances (TRGS 905, 201). The amines that may be formed during the azo splitting of the dye after oral intake in the body may be carcinogenic. The inclusion of this adulterating colourant in red palm oil may attain toxic pathological concentrations in humans which may further induce histopathological responses. With the demand for edible oils increasing due to growing population, and changing diets, it is essential that policies and advocacy to stop this trend of adulteration is required. The impact of the addition of this dye *via* red palm oil on human health has not been evaluated.

This study therefore aims to assess the toxicological effects of the Sudan II dye inclusion in red palm oil on the serum enzymes and renal function biomarkers for short (30 days) and long (90 days) term administration in male albino rats.

MATERIALS AND METHODS

Sample collection

A total of sixty (60) 750 ml bottle of red palm oil samples were purchased from four randomly selected markets in different Nigerian cities namely; Aba, Calabar, Kano and Lagos, located in four regions of the country. Five (5) samples of red palm oil were each purchased from three (3) different markets. The samples were then stored in a transparent plastic container on ice and taken to the laboratory for analysis. The samples for qualitative analysis were coded A1 (samples from Aba), C1 (samples from Calabar), K1 (samples from Kano) and L1 (samples from Lagos). The sample that was used as control was purchased from ibiaye oil palm mill in Biase local government area of Cross River state, Nigeria.

Animal experimental protocols

Sixty (60) mature albino rats, weighing between 150 g-180 g were obtained from the animal house of the Physiology Department, University of Calabar and used for the study. The animals were allowed two weeks for acclimatization, after which they were reweighed and housed in plastic cages with plastic bottom and wire-mesh top, under controlled environmental conditions of temperature (28 ± 2°C), relative humidity (50 ± 5%) and a 12 hour light/dark cycle.

The animal facility was adequately ventilated and the animals maintained regularly on the commercial rat chow. Water was provided ad libitum throughout the experimental period. At the end of the acclimatization period of two weeks (14 days), the experimental animals were divided into five groups of twelve animals each. The groups were given 90% commercial rat chow supplemented with 10% red palm oil. Sudan II dye was co-administered in the diet to provide levels of 0% (normal control), 0% (PO) (group 2), 0.025%, (PO/0.025) (group 3), 0.03% (PO/0.03) (group 4) and 0.04% (PO/0.04) (group 5). The experimental animals were given these diets for thirty (30) and ninety (90) days along with water ad libitum. The levels of the Sudan II dye (0.025%, 0.03% and 0.04%) was based on the result of the Sudan II dye content (250-350 ppm or 0.025-0.035%) in the red palm oil (from a previous study) to provide low (0.025%), medium (0.03%) and high (0.04%). The LD50 of Sudan II dye is 1000 ppm (0.1%).

Collection of blood samples

At the end of the treatment period i.e. 30 days (short term) and 90 days (long term), the animals were sacrificed and the blood was collected *via* cardiac puncture. Part of the blood (1 ml) was collected into heparinized or EDTA tubes, and used for haematological studies, while the remaining part was put into non-heparinized (plain) tubes. The blood in the plain tubes was allowed to stand for about two hours (2 hrs.) for proper clotting. Thereafter, the tubes were centrifuged at 3000 rpm for ten minutes and the supernatant (serum) collected using a 5 ml syringe and needle and then was used for biochemical and toxicological assays. Pressure using evaporator, and then kept in a glass flask. The semi solid extract (residue) obtained was stored in a refrigerator for further use.

Estimation of biochemical parameters

Urea and creatinine concentrations were determined based on calorimetric method of Zaanen et al. [2]. Bilirubin concentration was determined based on the method of Gerhard et al., as described by Tietz. Liver enzymes; Alanine Transferase (ALT) and Aspartate Aminotransferase (AST) were determined by the methods described by Reitman et al. and Alkaline Phosphatase (ALP) by standard method described by King et al. [3,4].

Statistical analysis

All data obtained were collated using standard statistical methods. They were expressed as mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) at 5% level of significance. Statistical analysis was performed using SPSS statistical package.

RESULTS

The results of the biochemical and toxicological parameters assessed in the experimental animals that were fed on the different proportion of palm oil adulterated with Sudan II dye on short term and long term are as follows;

Serum enzymes

The serum activities of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP) is presented in Table 1 (short term effect and long term effect). In the short term study, the mean AST activities in the animals showed that there was a significant ($P<0.05$) increase in group

3 (12.09 ± 0.41 iu/L), group 4(12.97 ± 0.41 iu/L), and group 5 (16.82 ± 1.26 iu/L) when compared to group 1 (9.86 ± 0.23 iu/L) (normal control). Group 4 and 5 were significantly (P<0.05) higher than group 1 (normal control) and group 2 (11.00 ± 0.22 iu/L). A non-significant (P>0.05) increase was observed in group 3 (12.09±0.41 iu/L) when compared to group 2 (11.00 ± 0.22 iu/L).

The mean ALT activities in the animals showed that group 5 (53.66 ± 1.86 iu/L) was significantly (P<0.05) higher than the group 1 (45.09 ± 1.51 iu/L) (normal control) and group 2 (47.97 ± 2.05 iu/L). A non-significant increase (P<0.05) was observed in group 3 (49.13 ± 1.58 iu/L) and group 4 (49.36 ± 1.43 iu/L) when compared to group 1 (45.09 ± 1.51 iu/L) (normal control) and group 2 (47.97 ± 2.05 iu/L). The mean ALP activities in the animals showed that groups 4 (17.64 ± 0.32 iu/L) and 5 (18.58 ± 0.41 iu/L) were significantly (P<0.05) higher than group 1 (14.58 ± 0.62 iu/L) (normal control), group 2 (15.61 ± 0.34 iu/L) and group 3 (15.78 ± 0.46) respectively. However, Group 3 showered a non-significant (P>0.05) increase when compared to group 1 (normal control).

In the long term study Table 1, the mean AST activities in the animals that showed that group 3 (13.14 ± 0.54 iu/L), group 4 (14.02 ± 0.83 iu/L) and group 5 (18.98 ± 0.55 iu/L) respectively were significantly (P<0.05) higher than group 1 (9.89 ± 0.29 iu/L) (normal control) and group 2 (10.61 ± 0.30 iu/L). Equally, Group 5 (18.98 ± 0.55 iu/L) was significantly (P<0.05) higher than group 1 (normal control), groups 2, 3, 4. The mean ALT activities in the animals showed that group 5 (53.95 ± 1.85 iu/L) was significantly (P<0.05) higher than group 1 (46.80 ± 1.19 iu/L) (normal control) and group 2 (46.44 ± 2.20 iu/L). A non-significant (P>0.05) increase was observed in group 3 (49.63 ± 1.20 iu/L) and group 4 (50.31 ± 0.89 iu/L) when compared to group 1 (normal control) and group 2. The mean ALP activities in the animals showed that group 5 (18.92 ± 0.39 iu/L) was significantly (P<0.05) higher than group 1 (14.09 ± 0.48 iu/L) (normal control) and group 2 (15.91 ± 0.37 iu/L). There was a non-significant (P>0.05) decrease in group 3 (14.50 ± 0.51 iu/L) when compared to group 2 (15.91 ± 0.37) and group 4 (17.48 ± 0.44 iu/L). Group 4 showed a non-significant (P>0.05) increase when compared to group 1 (normal control), group 2 and group 3.

Serum total bilirubin and direct bilirubin

The serum total and direct bilirubin of experimental animals fed on the different proportion of palm oil adulterated with Sudan II dye on short term (30 days) and long term is presented in Table 2.

Serum creatinine and urea

Table 3 shows the results of short term and long term effect of feeding experimental animals with Sudan II adulterated red palm oil on serum creatinine and urea.

Table 1: Effect of short and long term feeding of experimental animals with Sudan II adulterated red palm oil on serum enzymes.

Groups	Short term			Long term		
	AST (iu/L)	ALT (iu/L)	ALP (iu/L)	AST (iu/L)	ALT (iu/L)	ALP (iu/L)
Group 1 (normal control)	9.86 ± 0.23	45.09 ± 1.51	14.58 ± 0.62	9.89 ± 2.29	46.80 ± 1.19	14.09 ± 0.48
Group 2 (RPO+Feed)	11.00 ± 0.22	47.09 ± 2.05	15.61 ± 0.34	10.61 ± 0.30	46.44 ± 2.20	15.91 ± 0.37
Group 3 (RPO+0.025% dye)	12.09 ± 0.41*	49.13 ± 1.58	15.78 ± 0.46	13.14 ± 0.54*, a	49.63 ± 1.20	14.50 ± 0.51
Group 4 (RPO+0.003% dye)	12.97 ± 0.41*, a	49.36 ± 1.43	17.64 ± 0.32*, a, b	14.02 ± 0.83*, a	50.31 ± 0.89	17.48 ± 0.44
Group 5 (RPO+0.004% dye)	16.82 ± 1.26*, a, b, c	53.66 ± 1.86*, a	18.58 ± 0.41*, a, b	18.98 ± 0.55*a, b, c	53.95 ± 1.85*, a	18.92 ± 0.39*, a

Note: Values are expressed as mean ± SEM; n=6; *significantly different from group 1 (normal control) at p<0.05; a=significantly different from group 2 (palm oil) at p<0.05; b=significantly different from group 3 (0.025% dye) at p<0.05; c=significantly different from group 4 (0.03% dye) at p<0.05; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

Table 2: Effect of short and long term feeding of experimental animals with Sudan II adulterated red palm oil on serum total and direct bilirubin concentration.

Groups	Short term		Long term	
	Total bil. (mg/dl)	Direct bil. (mg/dl)	Total bil. (mg/dl)	Direct bil. (mg/dl)
Group 1 (normal control)	9.3 6 ± 0.79	3.43 ± 0.46	9.33 ± 0.86	3.86 ± 0.41
Group 2 (RPO+Feed)	10.04 ± 0.61	3.96 ± 0.35	9.87 ± 0.68	4.14 ± 0.29
Group 3 (RPO+0.025% dye)	10.93 ± 1.17	4.17 ± 0.37	12.79 ± 0.77*, a	5.54 ± 0.48*, a
Group 4 (RPO+0.003% dye)	10.98 ± 0.61	4.99 ± 0.34*	13.21 ± 1.31*, a	5.28 ± 0.24*, a
Group 5 (RPO+0.004% dye)	13.07 ± 0.44*, a	5.20 ± 0.43*, a	15.59 ± 0.91*, a, b	6.05 ± 0.33*, a

Note: Values are expressed as mean ± SEM; n=6; *significantly different from group 1(normal control) at p<0.05; a=significantly different from group 2 (palm oil) at p<0.05; b=significantly different from group 3 (0.025% dye) at p<0.05; Total bil=Total bilirubin; Direct bil=Direct bilirubin.

Table 3: Effect of short and long term feeding of experimental animals with Sudan II adulterated red palm oil on serum creatinine and urea concentration.

Groups	Short term		Long term	
	Urea (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)
Group 1 (normal control)	63.31 ± 1.01	1.20 ± 0.05	62.47 ± 0.61	1.27 ± 0.03
Group 2 (RPO+Feed)	65.09 ± 0.83	1.49 ± 0.03*	64.27 ± 0.87	1.49 ± 0.03
Group 3 (RPO+0.025% dye)	66.80 ± 2.35	1.96 ± 0.41*	65.11 ± 1.32	1.94 ± 0.23*, a
Group 4 (RPO+0.003% dye)	67.18 ± 3.44	2.45 ± 0.19*, a	68.41 ± 3.80*	2.63 ± 0.17*, a
Group 5 (RPO+0.004% dye)	68.25 ± 1.14	2.30 ± 0.17*, a	69.09 ± 0.90*	2.31 ± 0.14*, a

Note: Values are expressed as mean ± SEM; n=6; *significantly different from group 1 (normal control) at p<0.05; a= significantly different from group 2 (palm oil) at p<0.05.

DISCUSSION

Effect on serum enzymes

The serum enzymes of toxicological importance assessed in this study were Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Alkaline Phosphatase (ALP). Table 1 shows findings for AST activities in animals fed on short and long term basis on different proportion of Sudan II dye adulterated red palm oil. The result showed a significance (P<0.05) within the test groups. The increase in AST activities may be attributed to hepatocellular damage caused by the toxic effect of the dye. Under pathological conditions, the parenchyma cells of the hepatic lobules fail to carry out vital functions, which ultimately results in imbalanced intermediary metabolism. The result of this study is in harmony with findings of Shadia, et al. who reported a significant increase in ALT levels on rats fed on amaranth dye at dose 50 mg/kgb.w/day for two periods (7 and 21 days) [5]. AST is both cytoplasmic and mitochondrial enzyme involved in wide spectrum of protein metabolism and also at the level of amino group transfer between glutamate and pyruvate. The result in this study showed that there may be injury to the cell due to the effect of the Sudan II dye.

The ALT activities in animals that received different proportion of red palm oil adulterated Sudan II dye diet increased significantly (P<0.05) in test group 5 when compared to the control and group 2. Also, an increase was observed within the groups (P<0.05) even though the increase was not statistically significant. The increase in ALT activities in the test groups may be attributed to hepatocellular impairment which subsequently may have caused the release of greater than normal levels of intracellular enzymes into the blood. ALT is the protein metabolizing enzyme responsible for the transfer of amino group between alanine and keto acid to form pyruvate and a new amino acid. It is the primary enzyme responsible for the loading and off-loading of ammonia in the alanine cycle (a major pathway responsible for the transport of ammonia from the muscle to the liver for urea synthesis). The result of ALT activity in this study showed that there was injury to the cells as a result of exposure to red palm oil adulterated with Sudan II dye diet. The result in this study is in agreement with the findings of Imadifon et al., who reported that Sudan IV dye induced a significant increase in serum ALT levels in test rats administered different levels of the dye [6]. Also, Shadia et al. reported a significant increase in ALT levels on rats fed on amaranth dye at dose 50 mg/kgb.w/day for two periods (7 and 21 days) [5].

Furthermore, Ashour et al. reported that tartrazine colouring agent induced a significant elevation in ALT level after 60 days of treatment with 7.5 mg/kgb.w/day [7]. ALT is a mitochondrial enzyme that is specific to the liver, making it a more specific enzyme for detecting liver abnormalities. The ALT activity recorded in this study showed that there was injury to the hepatic cells as a result of exposure to palm oil adulterated with Sudan II dye diet for a period of 30 days and 90 days.

A rise in the ALP levels occurs with all forms of cholestasis, particularly with obstructive jaundice. It is also elevated in diseases of the skeletal system such as hyperparathyroidism as well as fracture and malignant tumor. In this study, a significant (P<0.05) increase was observed in Test groups 4 and 5 respectively when compared to the control, group 2 and test group 3 respectively (Table 1). In the long term study, significant increase was also observed in Test group 5 when compared to the control and group 2 (Table 1). The increase in ALP levels in the Test groups may be suggestive of early biliary tract obstruction due to the toxic effect of this dye. ALPs are a family of zinc metallo-enzymes, with a serine at the active center. They release inorganic phosphate from various organic orthophosphate and are present in nearly all tissues [8]. The findings from this study is in harmony with the findings of Imadifon, et al. who reported elevated serum ALP level in test animals administered 0.01 and 0.015% dye [6]. El-Shamy et al. also observed a significant increase in serum ALP levels in rats treated with a green-colouring dye [9].

Effect on serum total and direct bilirubin

Total serum bilirubin (indirect or unconjugated) is created from red blood cell breakdown in the recticuloendothelial system. It travels into the blood to the liver while the direct (unconjugated) bilirubin reaches the liver, undergoes a chemical change and is moved to the intestines before being removed through the stool.

The serum total bilirubin levels in this study showed a significant (P<.0.05) increase in group 5 when compared to the control (Table 2). In the long term study (90 days), a significant (P<0.05) increase was also observed within the groups 3, 4 and 5 respectively when compared to the control. Additionally, within the test groups, group 5 was significantly (P<0.05) higher when compared to group 3 (Table 2). The observed increase in total bilirubin in the test groups may suggest that the Sudan II dye in the red palm oil diet caused liver damage leading to the leakage of bilirubin into circulation. The result is consistent with the findings of Imadifon et al., who reported elevated levels in total bilirubin in test rats administered with different levels of the Sudan IV dye [6]. In addition, it is also consistent with the findings of Ikechukwu et al.,

who reported elevated level of total bilirubin in rats administered with high dose of amaranth dye [10].

A significant (P<0.05) increase was observed in serum direct or conjugated bilirubin in groups 4 and 5 when compared to the control. A further increase (P<0.05) was observed on the long term study (90 days) in groups 3, 4 and 5 respectively when compared to the control. This marked increase in direct or conjugated bilirubin level in both the short (30 days) and long term (90 days) is consistent with the findings of Imadifon, et al., who reported elevated level in direct bilirubin in test rats administered with different levels of Sudan IVs dye [6].

Effect on serum creatinine and urea

The results of serum creatinine and urea are presented in Table 3. The serum urea level increased insignificantly (P<0.05) in the test groups when compared to the control. However, in the long term study (90 days), a significant (P<0.05) increase was observed for only test groups 4 and 5 when compared to the control (Table 3) in a dose-response manner. The increase in serum urea level might suggest renal function impairment caused by the toxicity of the Sudan II dye in the diet. The changes in serum urea suggest that there was serious protein breakdown in the animals' tissue and the liver's inability to adequately synthesize and secrete the urea shows poor liver's secretory ability. The results of the serum urea are in agreement with the findings of Amin, et al., who observed a significant elevation in serum urea level when rats were administered with high dose (500 mg/kgb.w) of tartrazine dye [11]. The finding is also in agreement with the findings of Ashour, et al., who reported a significant increase in serum urea level of rats dosed with organic azo dye orally for 35 days [7]. Decreased Blood Urea Nitrogen (BUN) is associated with renal failure, negative nitrogen balance, impaired absorption, nephritic syndrome and over hydration. Increased BUN is associated with reduced blood flow to kidney, increased protein catabolism, acute renal failure, chronic renal diseases and urethral destruction by stones. Consequently, intentional addition of Sudan II dye to palm oil would have adverse health concerns.

The serum creatinine levels increased significantly (P<0.05) in Test groups 3, 4 and 5 when compared to the control and group 2 (Table 3). Significant increases (P<0.05) were also observed in the long term study (90 days) in groups 3, 4 and 5 respectively when compared to the control and group 2 (Table 3). Creatinine, being a normal metabolic product in the dephosphorylation of creatinine phosphate by creatine kinase, is a metabolite present in the serum in normal condition and it is cleared normally by the liver. Creatinine serves as a good marker for kidney's filtration and clearance ability, and thus a better analyte than urea. The increased serum creatinine level observed in this study could have resulted from kidney function impairment. The result is in agreement with the findings of El-Shamy, et al. and Helal, et al., who reported an increase in creatinine levels in rats treated with tatrazine and brilliant blue mixture [9,12]. Similarly, Amin, et al. reported a significant increase in serum creatinine level when rats were administered with high dose (500 mg/kgb.w) and low (15 mg/kgb.w) of tartrazine dye. The result of the creatinine level indicated that red palm oil adulterated with Sudan II dye diet may cause harm to kidney function. These findings of significant elevation in both urea and creatinine levels may indicate that the dye could impair kidney function due to the effect of the dye metabolite on kidney tissues.

CONCLUSION

Adulteration of red palm oil samples with Sudan II dye was observed in all the samples in varying levels. This possibly suggests the endemic nature of this unwholesome practice. It could therefore be inferred that Sudan II dye was added deliberately to improve the color of the red palm oil samples. The detection of Sudan II dye in the red palm oil samples is an indication of inadequate surveillance and testing protocols by the Nigerian Regulatory Institutions. The toxicological effects indicates that the red palm oil adulterated with Sudan II dye could impair vital organs (hepatic and renal functions) especially at 0.04% of the dye administration in both short term and long term exposure. Hence, the use of this dye to enhance the red palm oil color is deleterious and should be discontinued. Therefore, it is pertinent to create awareness and the need for enforcement of regulatory acts and food safety procedures. In addition, the Standard Organization of Nigeria (SON) and the CODEX alimentarius commission specifications for edible red palm oil do not permit the use of this dye on any food products.

DECLARATIONS

Author's contribution

Peter Henry contributed to the methodology, laboratory analyses and data collection. Aniekan Henshaw contributed to the writing of the paper. Christene Ikpeme and Ima-obong Williams contributed to the conceptualization of the research, validation and supervision of the research. All authors read and approved the final manuscript.

Funding information

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Conflict of interest

The authors declare no conflict of interest.

Data availability

All data generated or analyzed during this study are included in this published article.

Ethical approval

All protocols were conducted in conformity to the standards for laboratory animal use and care as found in the European Community guidelines (EEC Directives of 1986; 86/609/EEC). Approval was obtained from the Faculty of Basic Medical Sciences Animal Research Ethics Committee.

Acknowledgements

The authors wish to express their profound gratitude to the department of Biochemistry, University of Calabar for granting permission to use the laboratory.

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