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Clastogenic and toxicological assessment of cashew (*Anacardium occidentale*) nut bark extracts in Wistar rats

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Occupational exposures to environmental toxicants have been associated with the onset of skin lesions-including cancers. Identification and reduction of exposure to such compounds is an important public health goal. We examined the effect of cashew shell oil (CSO), used in skin tattooing for its potential to induce skin transformation in rats. Corn oil and CSO (25, 50, and 100%) were topically applied to depilated sections of Wistar' rat skin (groups: I-IV) for six weeks. Effect of treatments on serum transaminases activity, histological changes in hepatocytes and induction of micronuclei in the bone marrow were examined. In addition, CSO-induced hepatocyte proliferation was also quantified. All animals survived the course of the study. Reduced percentage change in body weight and physical trauma were observed in CSO-treated rat. The effects were more prominent in Group IV (100% CSO). Relative liver weights and number of hepatocytes (cells/mm²) increased significantly in groups II–IV relative to control ($p<0.05$). Serum transaminases activities were not significantly ($p>0.05$) affected in treated groups. Hepatic histopathology revealed moderate sinusoidal congestion (group II), in addition to portal congestion in (group III), with mononuclear cellular infiltration (group IV) animals. In addition, CSO induced significant micronuclei formation of polychromatic erythrocyte (mPCEs) in the rat bone marrow ($p<0.05$) when compared with control. Topical application of CSO disrupted skin cells integrity resulting in physical trauma. In addition, CSO appears to be clastogenic and induces hepatocyte proliferation. Occupational exposure to CSO especially for engraving tattoos in humans should be discouraged and further studies need to be conducted.

Key words: Cashew bark oil, tattooing, toxicity, micronuclei, cell proliferation, skin cancer

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INTRODUCTION

Cashew shell oil (CSO) is the main by-product of the cashew industry and makes up to 20–25% of the nut by weight (Ojeh, 1981). CSO has a variety of uses including friction modifying material, floor tiles, laminate resins, baking enamels, adhesives, paints, plastic industries, wood preservation, molluscicide and for medical purpose as a rubefacient in treating various skin diseases in tropical medicine (Morton 1961; Sullivan *et al.,* 1982; Ojeh, 1981). Chemical components consist of anacardic acid (90%) and cardol (10%) (Trosko, 2003). The former is an orthohydroxy benzoic acid with an unsaturated side chain and the latter is a phenol. Commercially available CSO has been reported to be mutagenic (Polasa & Rukmini, 1987). Epidemiological studies have shown that oral submucous fibrosis, an insidious chronic disease that has malignant potential, is high among cashew workers and the CSO may have a positive role in the etiology of oral submucous fibrosis (Varghese *et al.,* 1986). CSO is reported to produce contact dermatitis and eczematous dermatitis (Downing 1940; Aber, 1983; Marks *et al.,* 1984).

Poor occupational safety practice during CSO production, shelling of kernels exposes workers to raw or processed shell oil through dermal, nasal and oral routes. Allergic contact dermatitis has been reported in some people following occupational exposure to the shell oil or the nut (Ratner *et al*., 1974; Downing, 1940). It has been reported that children exhibited contact dermatitis having come in contact with toy burros made from cashew nuts (Orris, 1958). The processed kernels sold in stores as cashew nut in some cases may be contaminated with CSO during production. As such, this may be a means of chronic exposure when consumed regularly and should not be overlooked. Raw cashew nut oil is used as a direct tool for inscribing bodily tattoo amongst other methods in Nigeria, without consideration for its toxic consequences. However, this practice has been going on for hundreds of years and as thus, it is considered as safe. To our knowledge there are no studies in the literature that have investigated this practice with the standpoint of long-term health consequences. Earlier report (Hecker *et al*., 1984) indicated that CSO has inflammatory potential when applied on rat skin. Hazards to human health due to acute exposure to CSO as a tattooing tool need to be studied in order to know the appropriate precautions that should be taken, and the screening of these phytochemicals are essential to understand their functionality(George & Kuttan, 1997).

The present study was therefore carried out to investigate the toxicological and clastogenic potency of CSO in male Wistar rats exposed to CSO through dermal route.

MATERIALS AND METHODS

Chemicals. Alanine amino transferase (ALT), aspartate amino transferase (AST) and gamma glutamyl trans-

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Abbreviations: ANOVA, analysis of variance; ALT, alanine amino transferase; AST, aspartate amino transferase and γ-GT, gamma glutamyl transferase cells/mm²; CSO, cashew shell oil; i.p., intraperitoneal; mPCEs, micronucleated polychromatic erythrocytes; SPSS, Statistical Package for Social Sciences.

ferase (γ-GT) kits were obtained from RANDOX Laboratory, (London, UK). All other reagents and chemicals are of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO., USA).

Test material (CSO). CSO was prepared from fresh cashew purchased from a cashew processing industry in Iseyin, Oyo State, Nigeria. The nuts were cleaned, dried and the shells were removed and cut into small bits: 20 g of the shell was then suspended in 250 ml petroleum ether for 24 h under constant shaking. It was then filtered and concentrated under a water bath. The final preparation appears as a dark brown oily liquid.

Experimental animals. In an *in vivo* experimental study to examine the effect of CSO, twenty (20) male albino rats of the Wistar strain weighing between 100–150 g were purchased from Covenant Farms (Ibadan, Oyo State Nigeria). The rats showed no visible signs of diseases or injuries. The rats were housed in the experimental animal house of the Department of Biochemistry, University of Ibadan and were fed with commercial rat pellets from Vita Feeds (Ibadan, Oyo State, Nigeria) and given water *ad libitum*. The rats were allowed to acclimatize for one week after they were randomized into four experimental treatment groups. All treatment groups contained five rats each, to accommodate for mortality during the study. Rats showing signs of lethargy or less than 100–150 g were excluded from the study. Same sections of the dorsal portion (2 cm square) close to the hind limbs were depilated of hair from each rat using a Wahl™ (Sterling, IL, USA) electric clipper. The animals were restrained in an animal holder and were not anesthetized during depilation. Macroscopically, there were no abrasions after depilation, and to avoid excessive CSO penetration from microscopic abrasion the animals were allowed to heal for a few days. To the best of our knowledge there were no macroscopic and microscopic abrasion on the skin after depilation and before the commencement of treatment with CSO and corn oil.

Animal grouping and treatment. Cashew nut oil (CSO) or Corn oil were applied equally to each animal by volume in the different groups irrespective of their concentrations as shown below:

Group I: Corn oil (control) 100 µL;
Group II: CSO (25%) 100 µL;
Group III: CSO (50%) 100 µL;
Group IV: CSO (100%)100 µL.

CSO or corn oil were topically applied to each group thrice weekly for 6 weeks. The depilated sections were visually observed daily and changes on the skin surface were noted during the treatment period. After six weeks the terminal body weight of all animals was obtained. Rats were sacrificed by cervical dislocation and blood samples collected *via* retro orbital puncture. The liver and femurs were then harvested.

Micronuclei assay. Clastogenic effects of CSO were evaluated in rat bone marrow cells using the method described (Heddle & Salmone, 1981). Animals were injected (i.p.) with 0.04 % colchicine (1 mL/100g body weight 2 h prior to sacrifice). Bone marrow was extruded onto clean slides, the slides were fixed, air-dried and pretreated with May-Grunewald solution and subsequently stained with Giemsa solution. The slides were scored under a light microscope for the presence of micronucleated polychromatic erythrocytes (mPCEs) according to standard procedure with the aid of a tally counter.

Enzyme assays. Blood was collected from each of the sacrificed rats into plain tubes and serum was prepared after the blood has been allowed to clot at room temperature for about two hours. The clotted blood was centrifuged (3 000 × *g*, 4°C for 10 minutes) and the supernatant was transferred into clean tubes stored at –20°C until required. Serum level of hepatic transaminases: γ-GT was assayed in the serum by using the reconstituted reagent kit following standard method (Szasz, 1969). ALT and AST activities were also assayed in the serum using the reagent kits according to the method of Reitman and Frankel (Reitman & Frankel, 1957).

Histopathology. Harvested livers were rinsed in phosphate buffered saline, blotted dry on filter paper and weighed. Sections of the liver were fixed in 4% p-formaldehyde processed and embedded in paraffin. The formalin fixed, paraffin embedded tissue was further sectioned and layered onto glass slide which was stained with haematoxylin–eosin dye and finally read under a microscope by a Veterinary pathologist.

Hepatic cells/mm² count. Slide preparations from liver histology above were used to determine the number of cells/mm² using a Nikon light microscope as previously described (Hochegger *et al*., 2005).

Data analysis. The results were expressed as mean ± Standard Deviation. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) with the aid of Statistical Package for Social Sciences (SPSS) software, SPSS Inc., Chicago, Standard version 10.0.1. *P*-values<0.05 were considered statistically significant for differences in means.

RESULTS

Figure 1 shows local tattoo inscribed on the skins of individual using CSO oil. This is a well-known and common practice dating back to several hundred years, as such it is considered safe without recourse to the short and long term health effects that may occur. Sections of rat skin were depilated and CSO applied topically as shown in Fig. 2.

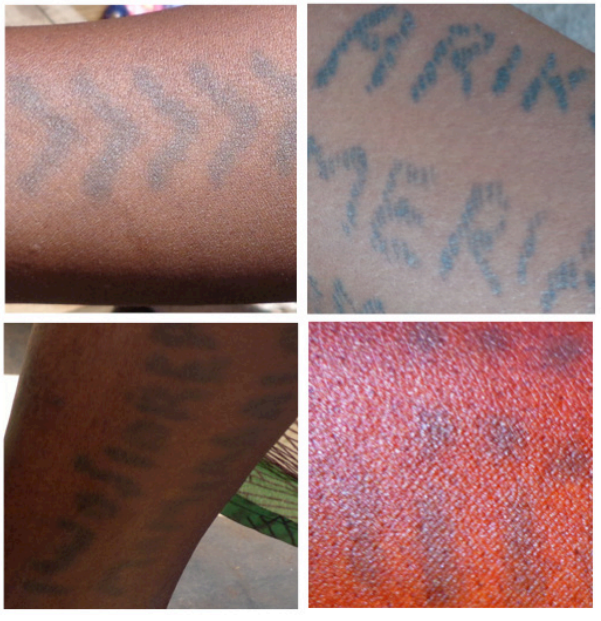

Figure 1. CSO induced tattoo:
CSO marking on the skin in the form of tattoo locally as a means of identification and inscribing the names on skin surfaces.

Table 1. Body and Organ weight and weight change in rat stopically exposed to CSO for 6 weeks.

Group	Initial body weight (g)	Final body weight (g)	Weight change (%)	Liver weight (g)	Relative liverweight (%)
Control (I)	107.0±24.65	130.0±24.50	21.49	2.98±0.79	2.40±0.80
25%CSO (II)	120.0±20.92	142.0±13.03	18.33	4.28±0.41[a]	3.02±0.28
50%CSO (III)	118.0±15.62	126.0±16.73	6.77	3.73±0.16[a]	3.0±0.35
100%CSO (IV)	110.0±13.69	76.0±5.44	–30.90	3.48±0.46[b]	4.44±0.93[abc]

a=mean difference is statistically significant when compared with group I (Control) ($p<0.05$); b=mean difference is statistically significant when compared with group II ($p<0.05$); c=mean difference is statistically significant when comparedwith group III ($p<0.05$). Results are expressed as means ±S.D, n=5.

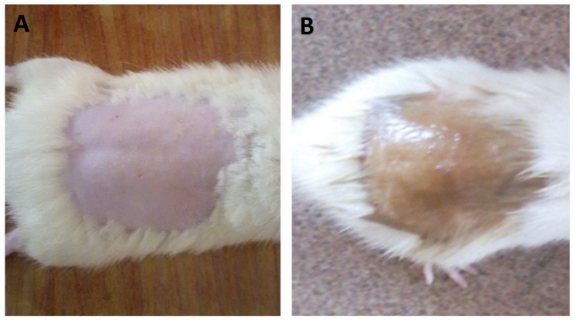

Figure 2. Representative model of rats showing depilated sections of rat skin before application of CSO (A) and after exposure of CSO (B) for 6 weeks.

Table 2. CSO-induced micronucleated polychromatic erythrocytes (mPCEs) in rat bone marrow cells, after topical exposure for 6 weeks.

Experimental group	mPCEs/1000 PCEs
Control (I)	1.00±0.71
25% CSO (II)	1.60±0.82
50% CSO (III)	1.80±0.27
100% CSO (IV)	2.20±0.76[a]

[a]mean difference is statistically significant when compared with group I ($p<0.05$). Results are expressed as mean ± S.D., n=5.

Effect of topical administration of cashew shell oil on the body and liver weight of rats

Table 1, shows the initial, terminal body and organ weights of rats treated with CSO and the control group. Body weight increased in the negative control by about 21.5%. Treatment with CSO (groups I–III) caused a progressive reduction in body weight increase. In fact, there was a dramatic loss of weight in the group of rats treated with 100% CSO (group IV). Also, there was an increase in the liver and relative liver weights of animals in groups (II–IV) when compared with the negative control group. The observed increase ($p<0.05$) in both liver and relative liver weights was CSO dose dependent. There was a significant difference in means between the liver weight of animals in groups II and III compared with the control. The mean difference in the liver weight between groups III and IV is also statistically significant ($p<0.05$).

Prolonged topical administration of cashew shell oil induced micronucleated polychromatic erythrocytes (mPCEs) formation in rats

The relative number of mPCEs scored in the rat bone marrow cells as a result of topical CSO exposure was significantly ($p<0.05$) higher in all treated animals (group II–IV) compared to the negative control (group I). The observed increases (Table 2) in mPCEs scored are dose dependent and with the highest level recorded in the group treated with 100% CSO (group IV). This group recorded about 100% increases in mPCEs formed compared to the negative control. Exposure to CSO at 25 and 50% respectively induced mPCEs values at 60 and 80% fold relative to the negative control group.

Hepatotoxicity following repeated topical administration of cashew shell oil in Wistar rats

Result from the assessment of hepatic transaminases in serum of rat exposed to cashew shell oil is presented in Table 3. Repeated topical administration of CSO resulted in elevated serum transaminases (γ-GT, ALT and AST), markers of hepatotoxicity in the treated animals. Treatment with 100% CSO resulted in significant ($p<0.05$) elevated serum levels of γ-GT (greater than fivefold increase was observed) compared to control animals (group I). Similarly at 25 and 50% CSO serum γ-GT also increased but to a lesser extent than 100% CSO. Serum AST was elevated at 25, 50 and 100% CSO administration (group II–IV). Serum levels of ALT in-

Table 3. Effect of CSO treatment on hepatic transaminases activities in serum of rats treated with CSO for 6 weeks.

Experimental group	GGT (IU/L)	AST (IU/L)	ALT (IU/L)
Control (I)	1.16±0.00	31.67±8.08	5.33 ± 2.31
25% CSO (II)	2.32±0.00	50.33±2.89	6.67± 2.31[a]
50% CSO (III)	2.70±1.34	56.67±4.06	13.60 ±2. 87[ab]
100% CSO (IV)	5.40±2.41[ab]	66.67±19.66[bc]	38.67±4.51[ac]

[a]mean difference is statistically significant when compared with group I ($p<0.05$); [b]mean difference is statistically significant when compared with group II ($p<0.05$); [c]mean difference is statistically significant when compared with group III ($p<0.05$). Results are expressed as means ± S.D., n=5. GGT=gamma glutamyl transferase, AST=aspartate amino transferase and ALT=alanine amino transferase.

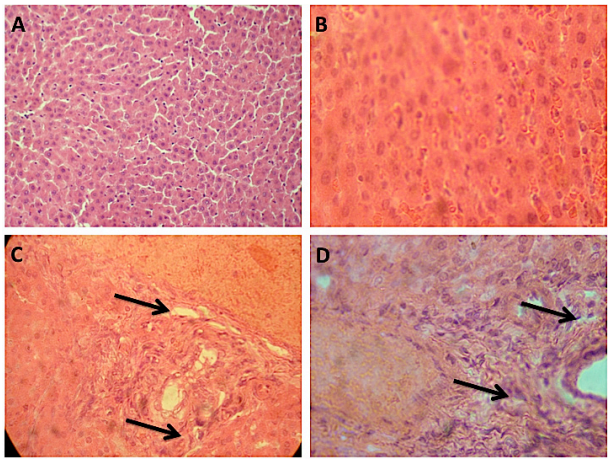

Figure 3. Photomicrograph of rat liver treated with CSO. Histologic examination of animals: control rats (**A**) indicated no visible lesions with normal hepatocyte architecture. Liver of rats (25% CSO) show moderate sinusoidal congestion and mononuclear cellular infiltration (**B**). In contrast, sections of rats liver treated with (50% CSO) (**C**) where characterized by moderate portal congestion, with fibroplasia of the periportal region (black arrows) and (**D**) liver sections of rats treated with (100% CSO) presented with diffused portal congestion and periportal cellular infiltration by mononuclear cell (black arrows). H&E stained; 400X magnification.

creased ($p<0.05$) with CSO treatment in a dose dependent manner (group IV>III>II) compared with the negative control group.

Histological changes in rat's liver cells following topical administration of CSO

Histological assessment of treated rat (group II–IV) liver relative to untreated rats (group I) corroborated the findings of serum transaminases (Fig. 3A–D). There was a disruption of liver architecture and an infiltration of mononuclear cells in the treated groups. The control liver appears to be cellular and structurally normal (A). Treatments with 25% CSO resulted in moderate sinusoidal congestion and mononuclear cellular infiltration (B). In contrast, acute coagulation necrosis of the centrilobular zones, cellular infiltration and moderate portal congestion is observed in rats liver treated with (50% CSO) (C) with fibroplasia of the periportal region and (D) liver sections of rats treated with (100% CSO) exhibited diffused portal congestion and periportal cellular infiltration by mononuclear cell.

Treatment with CSO increases hepatic cell counts

The results presented in Table 4 show the cells/mm^2 in rat liver as a measure of hepatocyte proliferation when exposed to CSO. There was a dose dependent increase in hepatocyte number in treated groups (II-IV). The observed increases were statistically significant ($p<0.05$) compared with the control group. Relative to control, treatment with CSO at 25% (group II) resulted in the highest number of cells/mm^2 in the rat liver.

Table 4. Hepatocytes proliferation assessed by (cells/mm^2) in rat liver after exposure to different concentration of CSO for 6 weeks.

Experimental group	Number of cell/mm^2
Control (I)	62.00±5.66
25% CSO (II)	82.00±8.66[a]
50 % CSO (III)	83.33±3.06[a]
100% CSO (IV)	85.67±12.86[a]

[a]mean difference is statistically significant when compared with group I (control) ($p<0.05$). Results are expressed as means ±S.D, n=5.

DISCUSSION

Skin tattooing is a worldwide phenomenon that is manifested from the ordinary to outrageous designs adorning all body types, skin colour etc.

Local tattooing in Nigeria which has existed for hundreds of years serves as a mark of identification, charms against wild animals and medicinal implants rather than a fashionable addition to enhance skin, project beauty or making of a socio political statement. Local tattooing in Nigeria can be inscribed on the skin using different kinds of tools and means, including the use of raw CSO, which may be toxic and have long-term health consequence on topical exposure. Here, we report findings on the toxicological and clastogenic assessment of CSO in rats exposed to CSO for six weeks. However, we used a conservative approach (increasing concentration of CSO (25–100%) in corn oil) to avoid excessive exposure to CSO that may result in undesirable outcome, all animals survived during the course of the study. In addition, relative to size, the extent of tattoos inscribed on individuals and long term occupational exposure, the amount of CSO used in humans is much higher than those studied here. Making our findings even more so relevant for CSO toxicological implications and the need for this health concern to be addressed. The choice of corn oil is a standard excipient that serves as a carrier vehicle in toxicological biomedical studies. Its application for this purpose is common in the scientific literature (Yuan *et al.*, 1992). In addition, corn oil is used in cooking and consumed as well in food. In case corn oil was supposed to introduce any deleterious effect, a control group (I) was also included in the study exposed to corn oil alone for comparison with the CSO treated groups (II–IV).

Topical exposure to CSO for six weeks did not produce considerable loss in body weight at 25 and 50% CSO doses compared to the negative control. However, the percentage increases in mean body weights of treated animals were lower than observation made with the control group (Table 1). On the other hand, at 100% CSO there was an appreciable loss in body weight (>30%) in rats exposed. It appears that CSO at less than 50% have less overall systemic effect on the animal, whereas at 100% CSO exposure this appears not to be the case. However, there was an overall, dose dependent increase in liver weight and relative liver weight of animals treated with CSO (Group II–IV) (Table 1). This may have implication in the increased number of hepatocytes assessed in the liver. We recorded the highest mean liver weight in group II (given 25% CSO) and the highest number of cells/mm^2.

Clastogenic damage to chromosomes and subsequent recombination plays a role in the onset of various cancers. A predictive index for evaluating the carcinogenic potential of environmental and occupational chemical exposure is the bone marrow micronucleus assay (Celik 2005; Bonassi, 2006). Exposure to CSO induced the formation of mPCEs in the bone marrow cells of rats in a dose dependent manner (Table 2). Group IV animals (treated with 100% CSO) showed a significant mean difference ($p<0.05$) in mPCEs formation, compared with the control (group I). This finding suggests that CSO is

clastogenic in rats with the likelihood of similar implication on human exposure to CSO.

The liver is the major target organ for chemically induced injuries. Several important factors are known to contribute to liver susceptibility. Traditional marker of hepatic injury, diseases and toxicity is the raised level hepatic transaminases (γ-GT, AST and ALT) in serum. Such elevations are an indication of liver dysfunction (Ideo *et al*., 1972; Lum & Gambino, 1972). Our findings indicated that repeated topical exposure to CSO resulted in an increase in mean γ-GT, AST and ALT in the serum of treated animals significantly ($p<0.05$) (Table 3). These increases were more pronounced in Group IV animals treated with 100% CSO, and compared to control animals (group I) suggest that exposure to CSO can be hepatotoxic, damaged hepatocytes and result in the release of hepatic enzymes into the blood. At 25 and 50% CSO concentration there was also an appreciable increase in serum levels of hepatic transaminases.

There was an increase in the number of cell/mm^2 in the rat liver in animal's topically exposed to different concentration of CSO. The observed increase occurred dose dependently (II<III<IV) and was statistically significant ($p<0.05$) compared with the control group. It can be inferred from this observation that topical exposure to CSO may induce hepatocytes proliferation. We, however, did not examine CSO effect on skin cell proliferation but rather gross morphological changes that appear in the forms of increased skin trauma, disruption of membrane integrity (wound) and scars on the skin. This observation correlated with previous findings (Argyris, 1989), who also observed wound and physical trauma in rats treated with CSO (Argyris, 1989). Overall, this finding suggested that topical exposure to CSO is disruptive to the skin and can promote hepatocyte proliferation, with implication in carcinogenesis. In addition, Group IV animals exposed to 100% CSO (without any dilution with corn oil) presented with the highest number of cell proliferation (Table 4). Taken together it can be concluded that exposure to CSO could be toxic to hepatocytes, hepatocyte proliferation increases, and may as well act as a potent clastogenic agent. In addition, exposure to CSO compromises skin integrity and causes trauma in exposed region of the skin. It is therefore suggested that the use of CSO as a tattooing agent should be highly discouraged and the adequate protective measures need to be put in place for occupational health safety, especially for those directly involved in cashew nut processing industries. Further long-term studies from 6–12 months are needed to be conducted in order to fully understand the effect of long-term chronic CSO exposure, in particular on workers in CSO and Cashew nuts processing industries.

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