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## Pathological research on acute hepatic and renal tissue damage in Wistar rats induced by cocoa

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## ABSTRACT

**Objective:** To ascertain the functional integrity of renal and hepatic tissues of Wistar rats fed with processed cocoa bean-based beverages and raw cocoa bean products-containing diets by using biochemical and histological methods.

**Methods:** Thirty Wistar rats were designated on the basis of experimental diets which were given for 28 days. At the end of the feeding period, blood samples were drawn, and renal and hepatic tissues were excised from the experimental rat groups for functional tests and histological examinations, respectively.

**Results:** Serum alanine aminotransferase activities of the experimental rat groups showed no significant difference ( $P > 0.05$ ) and were within a relatively narrow range of  $(32.17 \pm 4.98)$  IU/L to  $(41.00 \pm 10.85)$  IU/L, whereas serum aspartate aminotransferase activities gave wide variation within the range of  $(15.67 \pm 2.13)$  IU/L to  $(34.83 \pm 8.31)$  IU/L with  $P < 0.05$ . Serum bilirubin concentrations of experimental rat groups were less than 1 mg/dL. Serum total protein and albumin concentrations varied within a relatively narrow range. Serum creatinine concentration was significantly lower ( $P < 0.05$ ) than serum urea concentration. Histology showed evidence of moderate disarrangement of hepatic tissue architecture and degenerated tubules as well as glomerular turfs.

**Conclusions:** The pattern of alanine aminotransferase activity being more active than aspartate aminotransferase one in serum appeared to correlate with the extent of disarrangement of hepatic tissue architecture. The experimental rat groups exhibited no hyperbilirubinemia. Also, diets containing processed cocoa bean and raw cocoa bean products did not substantially interfere with the capacity of the hepatocytes to biosynthesize plasma proteins and the functionality of renal tissues.

## 1. Introduction

The cocoa bean tree, *Theobroma cacao* (Linnaeus) belonging to family Sterculiaceae, is originated from Latin America about 500 years ago, from where it was domesticated in other parts of the world<sup>[1]</sup>. Harvested cocoa beans are usually fermented and dried prior to their being processed into finished products<sup>[2,3]</sup>. Cocoa bean-beverages are processed products of the cocoa bean, sold under several brand names in Nigeria and worldwide<sup>[4-6]</sup>. The nutraceutical values of raw cocoa bean products (RCBP) as well as the high acceptability of processed cocoa bean-based beverages (PCB-BB)<sup>[2,7-11]</sup>, and their attractive

flavor and appearance, designate the cocoa tree as a highly prized international cash crop.

The quality parameters of PCB-BB in Nigeria markets have previously been reported elsewhere<sup>[5,6,12-14]</sup>. Previous studies have raised safety concerns about the consumption of RCBP and industrial PCB-BB-containing diets. For the most part, the presence of anti-nutritional factors in RCBP is associated with the toxic outcomes and poor nutritional score when used as feed substitutes for farm animals<sup>[15,16]</sup>. Likewise, the presence of Maillard reaction end-products/chemically modified by-products<sup>[2,17-21]</sup>, heavy metal<sup>[22]</sup>, and microbial contaminations<sup>[12,14,21-25]</sup> of diets containing PCB-BB and RCBP may provoke tissue lesions and organ damage.

The liver and kidney are organs of homeostasis. The hepatic tissues play a central role in the biotransformation of xenobiotics and endogenous molecules prior to their elimination from the body<sup>[26-28]</sup>. The biotransformation of xenobiotics in the

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hepatocytes may elicit the formation of noxious and highly reactive compounds or potentially toxic metabolites, which in the process of their metabolism predisposes the hepatocytes to injuries and dysfunction<sup>[29]</sup>. The renal tissues are highly specialized in ensuring delicate balance in selective excretion or retention of body biomolecules, according to their physiologic renal threshold indices<sup>[30]</sup>. The renal tissues are predisposed to chemical-induced injuries because of their action to concentrate tubular fluid by removal of H<sub>2</sub>O, organic compounds and inorganic salts from the vascular system. Liver (hepatic) function test (LFT) and renal function test are diagnostic parameters for ascertaining organ integrity as well as functionality and level of recovery from pathologic injuries. Histopathological studies are precise methods for the identification and characterization of pathological changes associated with tissue lesions.

Chemical modifications of organic matters in cocoa bean occur through the processes of dextrinization, caramelization, pyrolysis, cyclization, oxidation and esterification reactions<sup>[19,20,31]</sup>, which upon ingestion of the resultant organic derivatives may prompt tissue lesions in biologic systems. Additionally, studies have shown that the physicochemical characteristics of farm and industrial PCB-BBs differ from that of raw cocoa bean<sup>[2]</sup>. There is no available precise empirical information about the effect of PCB-BB-containing diets on integrity and functionality of internal organs. Moreover, study about the effect of RCBP-containing diets on animal physiology is comparatively scanty and has been largely ignored and taken for granted. Accordingly, the present study ascertained the functional integrity of renal and hepatic tissues of Wistar rats fed with diets containing PCB-BB and RCBP by using biochemical and histological methods.

## 2. Materials and methods

### 2.1. Collection and processing of raw cocoa bean seeds

The cocoa bean pods were randomly handpicked from cocoa smallholder in Owerri, Imo State, Nigeria. The pods were harvested on 24th September, 2014. The beans were evacuated from the pods and allowed to ferment for 5 days while being shielded from sunlight. Fermentation of cocoa bean was done by using the conventional heap fermentation method<sup>[32]</sup>. The wet beans were heaped on layers of plantain (*Musa paradisiaca*) leaves and covered with the same material to retain the heat generated during the fermentation process. On the third day and fifth day, the beans were quickly and thoroughly remixed by using a wooden spade and covered once again. Next, the fermented beans were sun-dried for 10 days till constant weight was achieved. A 50 g sample of the beans was pulverized by using Thomas-Willey milling machine (ASTM D-3182, India), after which the ground samples were stored in air-tight plastic bottles with screw caps until being used to compound the rat diets.

### 2.2. Animal diets

The RCBP was mixed with sucrose at the ratio of 10:1 w/w to sweeten it. The PCB-BBs were three brands of cocoa beverages (OT = cocoa beverage 1, BV = cocoa beverage 2, MO = cocoa beverage 3) commonly consumed in Nigeria, and

were purchased from a grocery shop. Also, PCB-BB and RCBP were compounded separately with pelletized standard guinea feed (PSGF) at the ratio of 10:1 w/w to obtain the test diets, whereas the control diet was composed of PSGF only. The PSGF, the product of a subsidiary of United African Company Nigeria Plc., Jos, Nigeria) was purchased at the Relief Market, Owerri, Imo State, Nigeria.

### 2.3. Animal handling

The present study was approved by the Ethical Committee on the use of animals for the research, Department of Biochemistry, Federal University of Technology, Owerri, Nigeria. The rats were obtained from the Animal House of the Department of Biochemistry, Federal University of Technology, Owerri, Nigeria. Female Albino (Wistar) rats were maintained at room temperatures of (28 ± 2) °C, 30%–55% of relative humidity on a 12-h light/12-h dark cycle, with an access to water and PSGF *ad libitum* for 2-week acclimatization period. Handling of the rats was in accordance with the standard principles of laboratory animal care of the United States National Institutes of Health.

### 2.4. Experiment design

A total of 30 female Wistar rats (90 days old) with average weight of (106 ± 2) g were allotted into 5 groups of 6 rats in each. The rats were deprived of food and water for additional 16 h before commencement of feeding as described elsewhere<sup>[33]</sup>. The rat groups were designated on the basis of experimental diets which were given for 28 days.

For Wistar rats in Group 1 (WR-PSGF), PSGF and water were given *ad libitum*.

For Wistar rats in Group 2 (WR-RCBP), RCBP and water were given *ad libitum*.

For Wistar rats in Group 3 (WR-OT), OT and water were given *ad libitum*.

For Wistar rats in Group 4 (WR-BV), BV and water were given *ad libitum*.

For Wistar rats in Group 5 (WR-MO), MO and water were given *ad libitum*.

At the end of the feeding period, blood samples were drawn from the orbital sinus of 12-h post-fasted rat groups for renal and hepatic function tests<sup>[34]</sup>. Also, renal and hepatic tissues were excised from the various rat groups for histological examinations.

### 2.5. LFT

#### 2.5.1. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

Measurement of serum AST and ALT activities was conducted according to the methods of Reitman and Franke<sup>[35]</sup>.

#### 2.5.2. Bilirubin

Serum total bilirubin concentration was measured by using diazotized sulphanilic acid methods as previously described<sup>[36]</sup>.

#### 2.5.3. Total protein

Serum total protein concentration was measured by using the biuret method as described by Gornall *et al.*<sup>[37]</sup>.

### 2.5.4. Albumin

Measurement of serum albumin concentration was conducted by the method described by Doumas *et al.*<sup>[38]</sup>.

## 2.6. Renal function test

### 2.6.1. Urea

Serum urea concentration was measured by using the rapid method as described by Fawcett and Scott<sup>[39]</sup>.

### 2.6.2. Creatinine

Measurement of serum creatinine concentration was conducted according to the methods as described by Bonsnes and Taussky<sup>[40]</sup>.

## 2.7. Histopathological examinations

Organ histology test was performed according to the methods described by Bancroft and Stevens<sup>[41]</sup>. Autopsy samples were taken from the renal and hepatic tissues in different animal groups, fixed in 10% formal-saline (pH = 7.2) for 24 h and washed with continuous flow of distilled water. The specimens were cleared in xylene and embedded in paraffin in hot air oven at 56 °C for 24 h. Paraffin bee wax tissue blocks were prepared for sectioning at 4 mm thickness by using a semi-automated rotatory microtome.

The obtained tissue sections were collected on glass slides, dehydrated by immersing in serial dilutions of ethyl alcohol-water mixture, cleaned in xylene and embedded in paraffin wax. Next, the specimens were deparaffinized and stained with hematoxylin and eosin (H&E) dye for histopathological examinations. Photomicrographs of the tissue sections were captured by using chare-couple device camera under light microscope (Olympus BX51TF; Olympus Corporation, Tokyo, Japan) at 400 magnification power.

## 2.8. Statistical analysis

The results were expressed as mean  $\pm$  SEM, and statistically analyzed by One-way ANOVA followed by Dunnett's test, with level of significance set at  $P < 0.05$ .

## 3. Results

Figure 1 showed that levels of serum activities of the two enzymes in LFT were in the order: ALT > AST. However, serum ALT activities in WR-PSGF, WR-RCBP, WR-OT, WR-BV and WR-MO showed no significant difference ( $P > 0.05$ ) and were within a relatively narrow range of (32.17  $\pm$  4.98) IU/L to (41.00  $\pm$  10.85) IU/L.

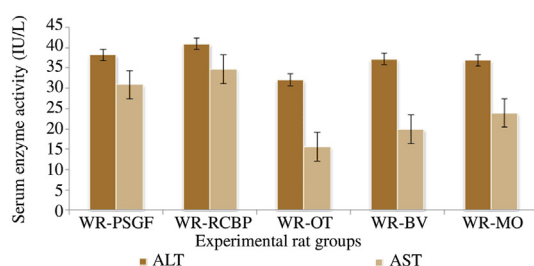


Figure 1. Serum AST and ALT concentrations in experimental rat groups.

Conversely, serum AST activities in WR-PSGF, WR-RCBP, WR-OT, WR-BV and WR-MO gave wide variation, which was within the range of (15.67  $\pm$  2.13) IU/L to (34.83  $\pm$  8.31) IU/L with  $P < 0.05$ . Specifically, WR-OT exhibited the lowest serum AST activity, whereas WR-RCBP gave the highest serum AST activity.

Generally, serum total bilirubin concentrations in WR-PSGF, WR-RCBP, WR-OT, WR-BV and WR-MO were less than 1 mg/dL. WR-RCBP gave a peak value of (0.85  $\pm$  0.18) mg/dL, which was over 2 folds higher than that in WR-OT with serum total bilirubin concentration value of (0.39  $\pm$  0.04) mg/dL (Figure 2).

Figure 3 showed that serum total protein concentration and serum albumin concentration in WR-PSGF, WR-RCBP, WR-OT, WR-BV and WR-MO varied within a relatively narrow range. Furthermore, serum total protein concentration and serum albumin concentration in WR-PSGF were not significantly different ( $P > 0.05$ ) from those in WR-RCBP. Finally, serum total protein concentration and serum albumin concentration in WR-OT, WR-BV and WR-MO were comparatively higher than those in WR-PSGF and WR-RCBP.

Figure 4 showed that WR-RCBP gave the highest serum urea concentration, which was significantly different ( $P < 0.05$ ) from those in other four experimental rat groups.

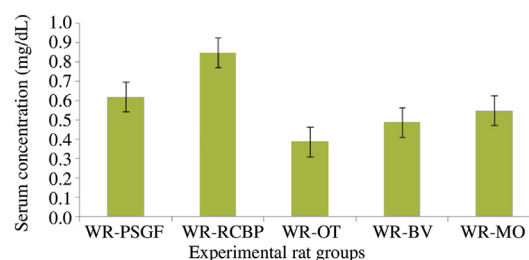


Figure 2. Serum total bilirubin concentration in experimental rat groups.

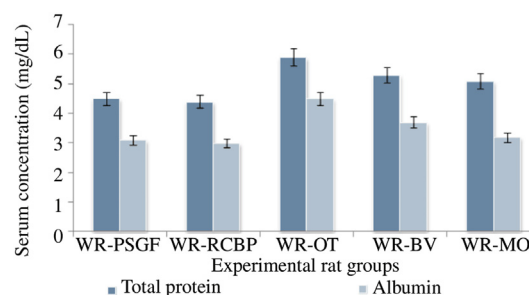


Figure 3. Serum total protein concentration and serum albumin concentration in experimental rat groups.

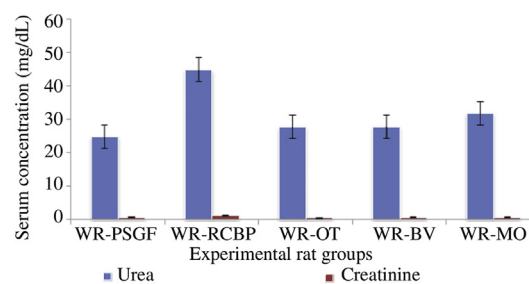


Figure 4. Serum concentrations of renal function indicators in experimental rat groups.

Conversely, serum urea concentration in WR-PSGF, WR-OT, WR-BV and WR-MO showed no significant difference ( $P > 0.05$ ). Additionally, serum creatinine concentration was significantly lower ( $P < 0.05$ ) than serum urea concentration in WR-PSGF, WR-RCBP, WR-OT, WR-BV and WR-MO. Finally, serum creatinine concentrations in the five experimental rat groups were within a relatively narrow range of ( $1.35 \pm 0.23$ ) mg/dL to ( $0.72 \pm 0.03$ ) mg/dL with  $P > 0.05$ .

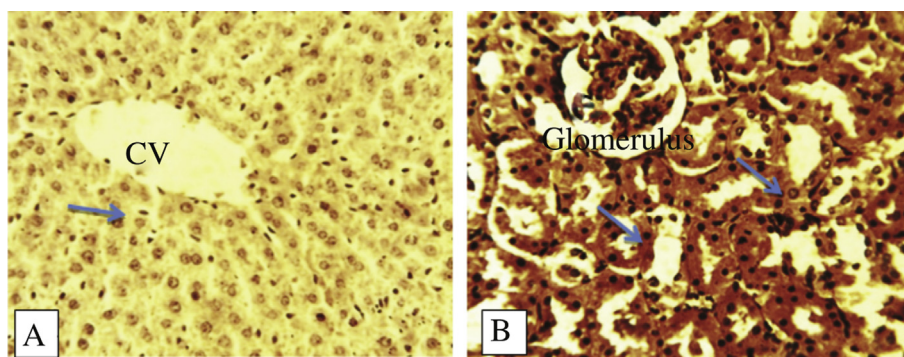
Hepatic parenchyma in WR-PSGF showed several hepatic lobules separated from each other by delicate connective tissue septa that served as repositories to the portal triad. Additionally, the hepatic lobules consisted of thin-walled central vein (CV) surrounded by hepatic cords with irregular blood spaces lined by endothelial cells and Kupffer cells. The nuclei appeared densely stained (Figure 5A). Also, renal tissues in WR-PSGF showed normal histology of renal corpuscles and tubules. The renal

corpuscles consisted of tuft of blood capillaries surrounded by the Bowman's capsule (Figure 5B).

The hepatocytes in WR-RCBP appeared vacuolated with moderate degenerative changes, whereas the nuclei appeared pyknotic and densely stained (Figure 6A). Renal tissues in WR-RCBP showed evidence of slight loss of cellular architecture with pronounced venous congestion (Figure 6B).

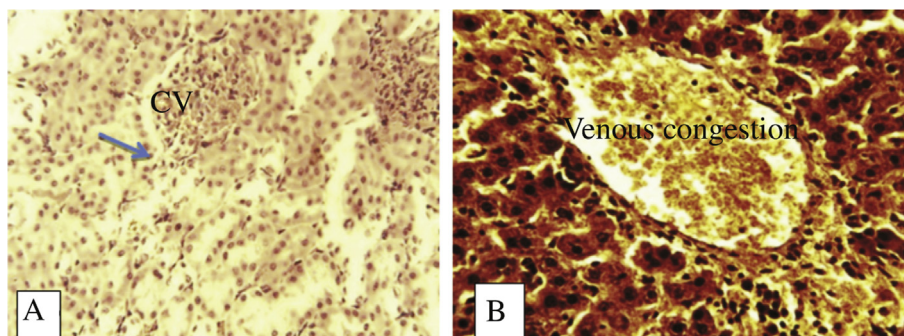
The architecture of hepatic parenchyma in WR-OT showed evidence of moderate degenerative changes and disarrangement with generalized dilatation and congestion involving hepatic arteries, lymph vessels, CVs and sinusoids (Figure 7A). Renal tissue in WR-OT showed evidence of degeneration of renal tubules and glomerular tuft as well as absence of brush borders (Figure 7B).

Sections of hepatocytes from WR-BV showed vascular degenerations (Figure 8A). Likewise, renal tissue in WR-BV



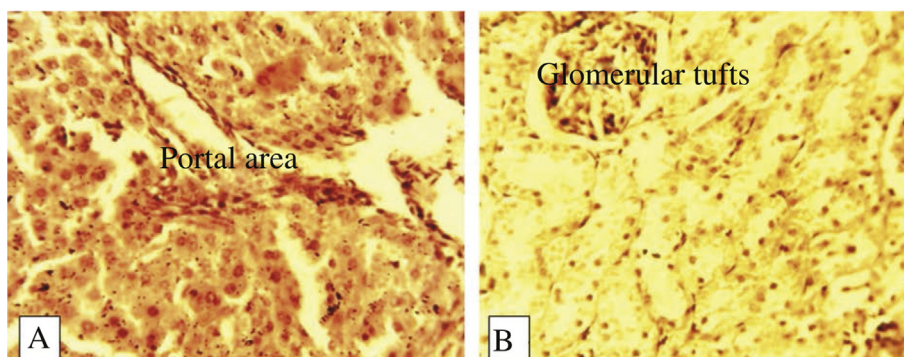
**Figure 5.** Photomicrograph of sections of organs from WR-PSGF (H&E,  $\times 400$ ).

A: Normal hepatic tissue with CV and Kupffer cells along the sinusoids as shown by blue arrow; B: Renal tissue showing normal glomerulus and renal tubules as shown by blue arrows.



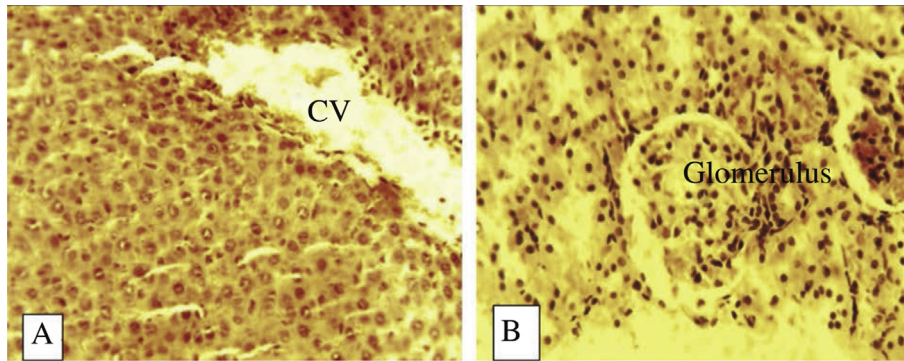
**Figure 6.** Photomicrograph of sections of organs from WR-RCBP (H&E,  $\times 400$ ).

A: Hepatic tissue with its CV and Kupffer cells along the sinusoids (blue arrow); B: Renal tissue showing venous congestion.



**Figure 7.** Photomicrograph of sections of organs from WR-OT (H&E,  $\times 400$ ).

A: Hepatic tissue showing the portal area with remarkable histologic change; B: Renal tissue with moderate degenerated tubules and glomerular tufts.



**Figure 8.** Photomicrograph of sections of organs from WR-BV (H&E,  $\times 400$ ).

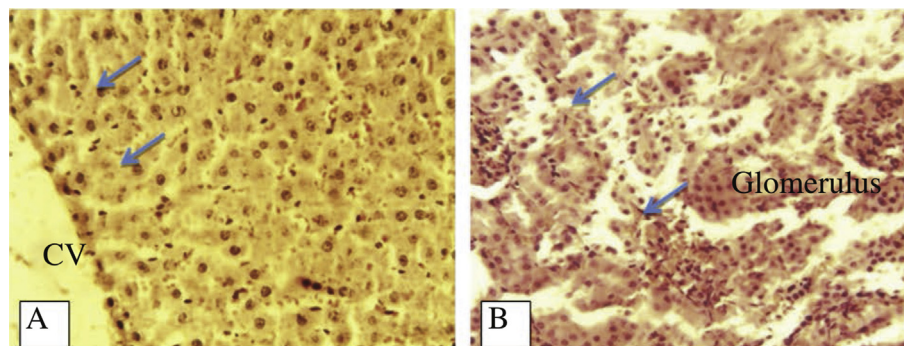
A: Hepatic tissue showing CV and moderate disarrangement of hepatocytes; B: Renal tissue showing moderate hypercellularity of the glomerulus.

showed evidence of degeneration of renal tubules and glomerular tuft (Figure 8B).

Hepatic parenchyma in WR-MO showed normal architecture with thin-walled CV surrounded by hepatic cords. Additionally, the nuclei appeared densely stained (Figure 9A). Renal tissue in WR-MO showed evidence of loss of cellular architecture (Figure 9B).

hyperbilirubinemia<sup>[52,53]</sup>. The underlying causative factors leading to the development of hyperbilirubinemia are as follows<sup>[52]</sup>:

(a) The production of more bilirubin than the normal liver can excrete; (b) Failure of damaged liver to excrete bilirubin produced in normal amounts; (c) Obstruction of the excretory ducts of the liver in the absence of hepatic damage, thereby preventing the excretion of bilirubin<sup>[52]</sup>. Studies have shown that



**Figure 9.** Photomicrograph of sections of organs from WR-MO (H&E,  $\times 400$ ).

A: Hepatic tissue showing the CV and normal plates of hepatocytes (blue arrows); B: Renal tissue showing moderate congestion of the glomerulus and interstitium as shown by blue arrows.

#### 4. Discussion

Clinical surveys and animal model experiments have revealed that raised levels of ALT and AST activities are indicative of organ damage, specifically, in pathologic and toxicologic events leading to cardiac and hepatic necrosis<sup>[29,42–44]</sup>. Precisely, earlier studies had associated raised serum ALT activity with non-diabetic non-alcoholic fatty liver disease and insulin resistance<sup>[45–50]</sup>. In the present study, whereby serum ALT was more active than AST (Figure 1), the ratio of the activities of two aminotransferases in serum, was an indication that the two non-functional plasma enzymes were of hepatic origin rather than the cardiac tissues<sup>[48,51]</sup>. Accordingly, the pattern of ALT activity being more active than AST one in serum appeared to correlate with the extent of disarrangement of hepatic tissue architecture following the consumption of PCB-BB- and RCBP-containing diets by corresponding experimental rat groups. It is worthwhile to note that the remarkable histologic alterations in hepatic tissue architecture in WR-RCBP, WR-OT, WR-BV and WR-MO (Figures 6A–9A) were reflections of the significant ( $P < 0.05$ ) differentials in activity between their ALT and AST values (Figure 1).

Previous reports have noted that human serum total bilirubin concentration between 1.0 mg/dL and 1.2 mg/dL was diagnostic of

ingestion of certain toxic food substances or compounds can provoke rapid erythrocyte hemolysis and interfere with normal functions of the liver<sup>[54–59]</sup>. Accordingly, the peculiar proximate composition of RCBP as described elsewhere<sup>[2,7,8,60–62]</sup> may have caused the comparatively raised serum total bilirubin concentration in WR-RCBP following the consumption of RCBP-containing diet by the corresponding experimental rat groups. Nevertheless, the results of the present study appeared to suggest the absence of hyperbilirubinemia in WR-RCBP, WR-OT, WR-BV and WR-MO in spite of the moderate histological changes in their hepatic tissues (Figures 6A–8A). It is worthwhile to note that bilirubin and its derivative, biliverdin, by virtue of their anti-oxidant activity, protect mammals against nephropathy, stroke, atherosclerosis and vasculitis<sup>[53,63–65]</sup>, and bilirubin at micromolar concentrations efficiently scavenge peroxy radicals *in vitro*<sup>[53]</sup>.

Although certain plasma proteins have their origins from the endothelial and plasma cells, most proteins biosynthesized in the hepatocytes eventually find their ways in plasma. Therefore, a compromised hepatic function engenders absence or low circulating levels of plasma proteins with attendant pathophysiologic conditions. The plasma proteins have been studied extensively in both humans and animals and the relationship between serum total protein concentration/serum albumin concentration and the

nutritional status of humans is well established, as typified in cases such as marasmus and kwashiorkor<sup>[66]</sup>. Comparative assessments of serum total protein concentration and serum albumin concentration (Figure 3) suggested no incident of poor nutritional status in the various experimental rat groups. Specifically, experimental rat groups fed with PCB-BB-containing diets exhibited relatively higher serum total protein concentration and serum albumin concentration than those fed with RCBP-containing diet and PSGF.

By implication, feeding experimental rats with PCB-BB- and RCBP-containing diets satisfied the minimum physiologic nutritional standards required by the rats. Additionally, the PCB-BB- and RCBP-containing diets induced moderate changes in hepatic tissues and histology of corresponding experimental rat groups did not substantially interfere with the capacity of the hepatocytes to biosynthesize plasma proteins.

Under normal physiologic conditions, urea is the primary vehicle for the excretion of metabolic nitrogen, whose sources are, for the most part, traceable to dietary constituents and body protein turnover<sup>[62,67]</sup>. Urea is a low threshold substance, which is why it is rapidly cleared from vascular system by the renal system. Therefore, raised level of urea nitrogen concentration in blood is diagnostic of renal dysfunction. The comparatively raised level of serum urea concentration in WR-RCBP (Figure 4) correlated with the structural alteration of the renal tissues as exemplified by the noticeable venous congestion of the tissue section (Figure 6B). However, the moderate degenerated tubules and glomerular tufts in WR-OT and WR-BV as well as moderate congestion of the glomerulus and interstitium in WR-MO did not profoundly affect the functionality of the renal tissues, since the serum urea concentrations in the corresponding experimental rat groups were comparable with that in the WR-SGFP, whose renal tissue histology revealed normal glomerulus and renal tubules. Likewise, moderate disarrangement of renal tissues in the various experimental rat groups did not adversely affect the capacity of their renal tissues to clear the blood creatinine. Although the present study showed that serum urea concentration was greater than serum creatinine concentration in the experimental rat groups as described elsewhere<sup>[68–72]</sup>, previous reports have shown that measurement of serum creatinine concentration offers a more reliable diagnostic parameter than serum urea concentration for confirmation of renal dysfunction<sup>[73,74]</sup>. Furthermore, Kang *et al.*<sup>[75]</sup>, had earlier noted significant elevation of serum urea concentration against marginal alterations of serum creatinine concentration in streptozotocin-induced diabetic rats that exhibited renal dysfunction, which conformed to the present findings (Figure 4).

Creatinine is sourced from the muscle protein turnover, and urinary creatinine concentration is proportionate to muscle mass and remains relatively constant. Accordingly, the approximate equal body weights of the various experimental rat groups indicated the corresponding comparable serum creatinine concentrations in WR-PSGF, WR-RCBP, WR-OT, WR-BV and WR-MO. However, increase in serum creatinine concentration can result from increased ingestion of cooked meat<sup>[67]</sup>.

The pattern of activity of ALT being more active than that of AST in serum appeared to correlate with the extent of disarrangement of hepatic tissue architecture. The experimental rat groups did not exhibit hyperbilirubinemia. Also, PCB-BB- and RCBP-containing diets did not substantially interfere with the capacity of the hepatocytes to biosynthesize plasma proteins and the functionality of renal tissues.

## Conflict of interest statement

The authors report no conflict of interest.

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