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Functional Assessments and Histopathology of Hepatorenal Tissues of Wistar Rats Infused with Carbon Tetrachloride and Treated with Raw and Hydrothermal Processed Herbs

Okey Alphonsus Ojiako¹, Paul Chidoka Chikezie^{2,*},
Doris Ifeyinwa Ukairo¹, Chiedozi Onyejiaka Ibegbulem¹,
Reginald Nwazue Nwaoguikpe¹

¹Department of Biochemistry, Federal University of Technology, Owerri, Nigeria

²Department of Biochemistry, Imo State University, Owerri, Nigeria

Email address

p_chikezie@yahoo.com (P. C. Chikezie)

*Corresponding author

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Abstract

The present study ascertained the functional integrity of hepatic and renal tissues, concurrently with blood lipid patterns, of Wistar rats infused with CCl₄ and treated with raw and hydrothermal processed herbs, namely, *Monodora myristica*, *Chromolaena odorata*, *Bucchozia coriacea* and *Sphenostylis stenocarpa*. Measurement of phytochemical contents of the herbs was according to standard methods. The rats were randomly designated on the bases of diets and treatments received for 28 consecutive days. Fibrosis was induced in the Wistar rats by single dose intra-peritoneal injection of CCl₄ for 2 consecutive days. Liver and kidney function tests and serum lipid profile were measured using spectrophotometric methods. Renal and hepatic tissues were subjected to histopathological examinations. The concentrations of alkaloids in the four herbal extracts were within the range of $4.83 \pm 0.03 - 31.33 \pm 0.29$ mg/100 g sample, whereas the concentrations of saponins varied within a relatively narrow range: $0.33 \pm 0.09 - 4.33 \pm 0.02$ mg/100 g dry sample; $p > 0.05$. The activity ratios of AST to ALT of the rat groups were generally less than 1.0 unit. Atherogenic indices of fibrotic rats were within the following ranges: TAG/HDL-C ratio ($3.59 \pm 0.03 - 6.76 \pm 0.06$), TC/HDL-C ratio ($3.72 \pm 0.02 - 6.94 \pm 0.05$) and LDL-C/HDL-C ratio ($2.00 \pm 0.01 - 4.59 \pm 0.02$). Losses in phytochemical contents following hydrothermal processing of the herbs did not substantially affect their overall therapeutic scores against morphological and functional impairments of hepatic and renal tissues following CCl₄ intoxication of the rats.

1. Introduction

The metabolic concerns of hepatic tissues, among several physiologic functions, ensure the detoxification of endogenously formed toxic molecules and xenobiotics through concerted processes of functional enzyme systems and facilitated by adaptable

anatomical architecture [1, 2]. Additionally, administered drugs and metabolic waste products are eliminated by a combination of hepatic metabolism and renal excretion [3]. The kidneys, which are located in the posterior abdominal wall, are the principal organs for osmoregulation, electrolytes balance and regulation of blood pH levels as well as elimination of detoxified xenobiotics from the vascular system [4].

Biochemical investigations using hepatic and renal functional indices, the so-called liver function test (LFT) and kidney function test (KFT), coupled with histopathological studies have revealed that dysfunctional and morphological damage to hepatic tissues predispose animal models to developing acute renal failure when exposed, in acute way, to chemical toxicants [5-9]. Carbon tetrachloride (CCl_4) is a chemical agent noted to be toxic to hepatorenal tissues in experimental animals and human subjects [5, 6, 8, 10-13]. Reports showed that CCl_4 toxicity is elicited by microsomal *CYP2E1*-induced formation of reactive trichloromethyl ($\text{CCl}_3^\cdot$) radical and the highly reactive oxygenated derivative, trichloromethylperoxyl ($\text{Cl}_3\text{COO}^\cdot$) radical, which initiates lipid peroxidation and accumulation of lipid peroxidation products, engendering inflammation and fibrotic changes to hepatic and renal tissues via activation of the macrophages [1, 9, 11-14].

Herbal extracts have been reported to ameliorate hepatic and renal disorders in humans and animal models [2, 9, 13, 15]. There are vast array of Nigerian indigenous medicinal plants that are used for the treatment and management of pathologic conditions linked with overwhelming levels of reactive metabolic radicals [16, 17]. *Monodora myristica*, also called calabash Nutmeg, is an edible plant of the Annonaceae family. Phytochemical screening showed that the plant is rich in flavonoids, saponins and sterols but with minimal anti-nutrients such as cyanogenic glycosides, tannins, oxalates and phytates [18]. The medicinal usefulness of *M. myristica* has been reported elsewhere [19-22]. *Chromolaena odorata* (Linn) is commonly referred to as Siam weed, 'Elizabeth', 'Independence leaf', whereas in South-Eastern Nigeria, it is called 'Enugu plantation weed' and 'Awolowo' [23]. The proximate composition and phytochemical contents as well as the medicinal usefulness of the herb have been reported elsewhere [23-26]. *Bucchozia coriacea*, commonly called 'Wonderful Kola', is frequently use in traditional medicine practice [27]. The phytochemical contents and proximate composition as well as the medicinal usefulness of *B. coriacea* have been reported elsewhere [27-31]. *Sphenostylis stenocarpa* is commonly referred to as African yam bean. The nutraceutical usefulness of the African yam bean is associated with its antioxidant and low hepatotoxic properties [32, 33]. Furthermore, Ndidi *et al.*, [34] reported the proximate, antinutrients and mineral composition of raw and processed African yam bean.

Studies have shown that CCl_4 infusion or inhalation elicits chemical-induced multiple organ lesions such as fibrosis, cirrhosis and hepatocarcinoma [12, 13, 35, 36], and by

extension, renal dysfunction in animal models [5, 6, 12]. Organ lesion and ensuing pathology elicit wide variations in concentrations of metabolites and elevation of non-functional plasma enzymes in blood samples of experimental animals. Specifically, because plasma lipoproteins, for the most part, are biosynthesized in the hepatocytes, chemical and biotic agents that impact negatively on liver functional integrity distort plasma lipid patterns [2, 37, 38]. Serum lipid profile (SLP) describes the propensity to developing atherogenic plague [2, 39]. Accordingly, functional assessments of hepatic and renal tissues, using blood indicators in conjunction with inspection of tissue morphology and integrity tests using histopathological technique, describe the level of chemical-induced organ lesions and measure of success of therapeutic interventions.

There are growing interests in the quest for multi-dimensional applications of herbs; particularly those commonly used among traditional herbal medicine practitioners, for the alleviation of pathologic conditions [13, 17, 40]. Thus, ethno-botanical bio-prospecting offers another strategy for new herbal remedy discoveries, including those used for alleviation of co-existing liver and kidney pathologic conditions. Additionally, in ethno-medicinal practices, herbs are either administered raw or subjected to hydrothermal processing prior their application. The present study sought to ascertain the functional integrity of hepatic and renal tissues, concurrently with blood lipid patterns, of Wistar rats infused with CCl_4 and treated with raw and hydrothermal processed Nigerian indigenous herbs, namely, *M. myristica*, *C. odorata*, *B. coriacea* and *S. stenocarpa*.

2. Materials and Methods

2.1. Collection and Preparation of Samples

High-grade raw seeds of *M. myristica*, *B. coriacea* and *S. stenocarpa* were purchased from Relief and Obazu-Mbieri Markets located in Owerri Capital Territory, Imo State, Nigeria. Fresh leaves of *C. odorata* were harvested from a private garden in Amakihia, Owerri-North Local Government Area, Imo State, Nigeria. The samples were transported to the laboratory, identified and authenticated by Dr. E. S. Willie at the Herbarium of the Department of Agronomy, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria. All samples were collected between the months of February and March, 2015. Voucher specimens were deposited at the Herbarium for reference purposes.

The various samples were washed separately in continues flow of distilled water for 15 min and allowed to dry at laboratory ambient temperature ($T = 24 \pm 5^\circ\text{C}$). The samples were divided into two portions on equal weight basis and designated as follows:

Group R: Raw samples

Group H: Hydrothermal processed samples

Appropriate separate quantities of Group R samples were

pulverized using Thomas-Wiley milling machine (ASTM D-3182, India). The ground samples were transferred into corresponding vacuum desiccators and allowed to dry at laboratory ambient temperature until a constant weight was achieved. Appropriate separate quantities of Group H samples were boiled in distilled water in corresponding conical flasks (sample/water ratio = 1:4 w/v). According to local traditional medicine practice, hydrothermal processing of the seed samples was in the following durations: *M. myristica* = 10 min, *B. coriacea* = 1 h and *S. stenocarpa* = 1.5 h, whereas leaves of *C. odorata* were subjected to hydrothermal processing for 5 min. Next, the Group H samples were dried separately in an oven (Gallenkamp Oven 300 plus series, England) at 50°C until a constant weight was achieved. Finally, Group H samples were ground using the Thomas-Wiley milling machine (ASTM D-3182; India), after which the samples were stored in separate air-tight plastic bottles with screw caps pending extraction.

2.2. Extraction of Samples

Extraction of Group R and Group H samples was according to the methods previously described [15]. Portion of 10 g each of the ground and dried Group R and Group H samples were subjected to repeated soxhlet extraction cycles for 2 h using 96% CH₃OH (BDH, U.K) as solvent to obtain final volume of 250 mL of corresponding extracts. The volumes of the extracts were concentrated and recovered in a rotary evaporator (Büch Rotavapor R-200) for 12 h at 50°C under reduced pressure. The extracts were dried in vacuum desiccators for 24 h, wrapped in aluminum foil and stored in air-tight plastic bottles with screw caps at ≤ 4 °C. The yields were calculated to be as follows:

- a) Extract R1; Raw seeds of *M. myristica* = 8.94% (w/w).
- b) Extract R2; Raw leaves of *C. odorata* = 6.22% (w/w).
- c) Extract R3; Raw seeds of *B. coriacea* = 8.07% (w/w).
- d) Extract R4; Raw seeds of *S. stenocarpa* = 14.02% (w/w).
- e) Extract H1; Hydrothermal processed seeds of *M. myristica* = 6.41% (w/w).
- f) Extract H2; Hydrothermal processed leaves of *C. odorata* = 4.39% (w/w).
- g) Extract H3; Hydrothermal processed seeds of *B. coriacea* = 7.12% (w/w).
- h) Extract H4; Hydrothermal processed seeds of *S. stenocarpa* = 13.51% (w/w).

Portion of the each extract was measured for phytochemical contents. Also, the each extract was reconstituted in phosphate buffered saline (PBS) solution that was osmotically equivalent to 100 g/L PBS (90.0 g NaCl, 17.0 Na₂HPO₄·2H₂O and 2.43 g NaH₂PO₄·2H₂O) and appropriate dose was administered to corresponding experimental animals.

2.3. Phytochemicals

Quantitative compositions of some phytochemicals, namely, alkaloids, flavonoids, tannins and saponins were measured using standard methods. The concentration of

alkaloids and saponins were measured using the methods of Harborne, [41]. The flavonoids content was according the methods of Boham and Kocipal, [42]. The concentration of tannins was measured using the methods of Van-Burden and Robinson, [43] as reported by Belonwu *et al.*, [44].

2.4. Experimental Animals

Healthy male Wistar rats (90 days old) weighing between 150-260 g were maintained at laboratory ambient temperature of 30–55% relative humidity on a 12-h light/12-h dark cycle, with access to water and standard commercial feeds (SCF) (Ewu Feed Mill, Edo State, Nigeria) *ad libitum*, for 2 weeks acclimatization period. The Institutional Review Board of the Department of Biochemistry, Federal University of Technology, Owerri, Nigeria, granted approval for this study. The care and handling of the animals conformed to the standard principles of laboratory animal care of the United States National Institutes of Health (NIH, 1978).

2.5. Infusion of Carbon Tetrachloride/Experimental Design

A total of 72 male Wistar rats were allotted into 12 groups of 6 rats each. Fibrosis was induced in the Wistar rats by single dose intra-peritoneal (i.p) injection of CCl₄ in paraffin oil as vehicle {1:1 v/v; dose = 1.0 mL/kg body weight (b.wt.)} for 2 consecutive days [45]. The animals were deprived of feeds only for additional 16 h before commencement of treatment as described [1, 46]. The rats were randomly designated on the bases of diets and treatments (dose = 250 mg/kg b.wt.; i.p. of the extracts and silymarin or otherwise 1.0 mL/kg b.wt.; i.p. of PBS, paraffin oil and CCl₄/paraffin oil mixture) received for 28 consecutive days. Silymarin (Medical Union Pharmaceuticals Company) was used as the standard drug for reference treatment of fibrotic rats [1].

- a) Group 1: Normal rats received SCF + water *ad libitum* + PBS.
- b) Group 2: Normal rats received SCF + water *ad libitum* + paraffin oil.
- c) Group 3: Fibrotic rats received SCF + water *ad libitum* + CCl₄/paraffin oil mixture.
- d) Group 4: Fibrotic rats received SCF + water *ad libitum* + silymarin.
- e) Group 5: Fibrotic rats received SCF + water *ad libitum* + Extract R1.
- f) Group 6: Fibrotic rats received SCF + water *ad libitum* + Extract R2.
- g) Group 7: Fibrotic rats received SCF + water *ad libitum* + Extract R3.
- h) Group 8: Fibrotic rats received SCF + water *ad libitum* + Extract R4.
- i) Group 9: Fibrotic rats received SCF + water *ad libitum* + Extract H1.
- j) Group 10: Fibrotic rats received SCF + water *ad libitum* + Extract H2.
- k) Group 11: Fibrotic rats received SCF + water *ad libitum* + Extract H3.

- l) Group 12: Fibrotic rats received SCF + water *ad libitum* + Extract H4.

At the end of the feeding and treatment period, the rats were subjected to fasting for 12 h, after which the animals were sacrificed and blood samples were drawn from the orbital sinus [47] and measured for serum biomolecules of interest. Autopsy samples of the renal and hepatic tissues were excised for histopathological examinations.

2.6. Liver Function Test (LFT)

2.6.1. Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) Activities

Measurement of serum AST and ALT activities were according to the methods of Reitman and Frankel, [48].

2.6.2. Alkaline Phosphatase (ALP) Activity

Serum ALP activity was assayed by the methods described by Glogowski *et al.*, [49], but with minor modifications according to Njoku *et al.*, [50].

2.6.3. Bilirubin

Serum total bilirubin concentration (TBC) was measured using diazotized sulphanilic acid methods as previously described [51].

2.6.4. Total Protein

Serum total protein concentration (TPC) was measured using the biuret methods as described by Gornall *et al.*, [52].

2.6.5. Albumin

Measurement of serum albumin concentration (AIC) was by the methods described by Doumas *et al.*, [53].

2.7. Kidney Function Test (KFT)

2.7.1. Creatinine

Measurement of serum creatinine concentration (CrC) was according to the methods as described by Bonsnes and Taussky, [54].

2.7.2. Urea

Estimation of serum urea concentration (UrC) was by the rapid methods as described by Fawcett and Scott, [55].

2.8. Lipid Profiling

Blood samples were obtained from the various experimental animal groups and measured for SLP according to the methods previously described [56]. Serum total cholesterol (TC), triacylglycerol (TAG) and high-density lipoprotein cholesterol (HDL-C) concentrations were measured using commercial kits (Randox Laboratory Ltd., UK). Serum low-density lipoprotein cholesterol (LDL-C) concentration was estimated according to the formula of Friedewald *et al.*, [57]: $LDL-C = TC - (HDL-C) - (TAG/5)$, as reported by Shaker *et al.*, [1]. Atherogenic index (AI) was

calculated as previously reported [58].

2.9. Hepatorenal Tissue Morphology

Organ histology was according to the methods described by Banchroft *et al.*, [59] as previously reported [15, 60]. Autopsy samples of the renal and hepatic tissues of the different animal groups, fixed in 10% formolsaline (pH = 7.2) for 24 h and washed with continuous flow of distilled water. The specimens were cleared in xylene embedded in paraffin in hot air oven at 56°C for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4-mm thickness 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 using chare-couple device (CCD) camera under light microscope (Olympus BX51TF; Olympus Corporation, Tokyo, Japan) at $\times 400$ magnification power.

2.10. Statistical Analysis

The data collected were analyzed by the analysis of variance procedure while treatment means were separated by the least significance difference (LSD) incorporated in the statistical analysis system (SAS) package of 9.1 version, (2006).

3. Results

Table 1 showed that the concentrations of alkaloids in the four herbal extracts were within the range of $4.83 \pm 0.03 - 31.33 \pm 0.29$ mg/100 g sample, in which Extract R1 gave the highest concentration of alkaloids. The concentration of alkaloids in Extract R2 was approximately 1.9 fold lower than that of Extract R1; $p < 0.05$. Furthermore, the concentration of alkaloids in Extract R3 was 2.36 folds lower than that of Extract R2; $p < 0.05$. The Extract R4 gave the lowest concentration of alkaloids and was not significantly different ($p > 0.05$) from that of Extract R3.

The concentration of alkaloids in Extract H1 was significantly different ($p < 0.05$) from that of Extract H2. Conversely, the concentration of alkaloids in Extract H3 was not significantly different ($p > 0.05$) from that of Extract H4. The concentrations of alkaloids in Extract H3 and Extract H4 were significantly different ($p < 0.05$) from those of Extract H1 and Extract H2. An overview of Table 1 showed that the concentrations of alkaloids in Extracts (R1-R4) were significantly higher ($p < 0.05$) than those of Extracts (H1-H4), except the corresponding concentrations of alkaloids between Extract R4 and Extract H4; $p > 0.05$.

Table 1. Some phytochemical contents of raw and hydrothermal processed herbal extracts.

Extract	Concentration (mg/100 g dry sample)			
	Alkaloids	Flavonoids	Tannins	Saponins*
R1	31.33 ± 0.29 ^a	14.17 ± 0.10 ^b	13.28 ± 0.11 ^a	1.67 ± 0.04
R2	16.50 ± 0.10 ^{b,c}	21.00 ± 0.18 ^a	12.63 ± 0.09 ^{a,b}	2.23 ± 0.02
R3	7.00 ± 0.09 ^{d,e}	6.33 ± 0.08 ^{d,e}	7.39 ± 0.07 ^{c,d}	2.33 ± 0.03
R4	4.83 ± 0.03 ^{d,e,f}	6.23 ± 0.07 ^{d,e,f}	8.10 ± 0.12 ^c	2.83 ± 0.03
H1	20.01 ± 0.21 ^b	6.50 ± 0.07 ^d	0.62 ± 0.01 ^e	1.33 ± 0.09
H2	7.33 ± 0.07 ^d	12.33 ± 0.09 ^{b,c}	0.42 ± 0.01 ^{e,f,g,h}	1.00 ± 0.01
H3	1.50 ± 0.01 ^{e,f,g,h}	4.33 ± 0.03 ^{d,e,f,g}	0.46 ± 0.01 ^{e,f,g}	1.67 ± 0.02
H4	2.17 ± 0.03 ^{e,f,g}	3.17 ± 0.03 ^{d,e,f,g,h}	0.52 ± 0.01 ^{e,f}	2.33 ± 0.02

The mean ($\bar{X}$) ± S.D. of six ($n = 6$) determinations. Means in the column with the same letter are not significantly different at $p > 0.05$ according to LSD.

*Concentrations of saponins showed no significant difference $p > 0.05$ according to LSD.

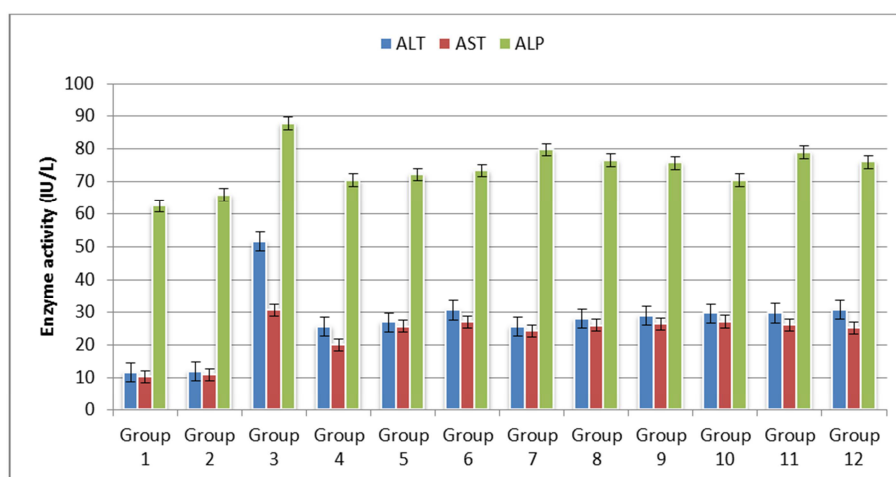
Table 1 showed that Extract R2 gave the highest concentration of flavonoids, which was significantly different ($p < 0.05$) from that of Extract R1. The concentrations of flavonoids in Extract R3 and Extract R4 showed no significant difference ($p > 0.05$). In a corresponding manner, the concentration of flavonoids in Extract H2 was significantly higher ($p < 0.05$) than that of Extract H1. Likewise, the concentration of flavonoids in Extract H3 was not significantly different ($p > 0.05$) from that of Extract H4.

An overview of Table 1 showed that the concentrations of flavonoids in Extracts (R1-R4) were significantly higher ($p < 0.05$) than those of Extracts (H1-H4), except the corresponding concentrations of flavonoids in Extract R3 and Extract H3 as well as Extract R4 and Extract H4.

The concentration of tannins in Extract R1 was not significantly different ($p > 0.05$) from that of Extract R2. However, the concentration of tannins in Extract R3 was 1.79 fold lower than that of Extract R1; $p < 0.05$. The Extract R4

gave marginal higher concentration of tannins than that of Extract R3; $p > 0.05$. Table 1 showed that the concentrations of tannins in Extracts (H1-H4) were significantly lower ($p < 0.05$) than their corresponding Extracts (R1-R4). Additionally, the concentrations of tannins in Extracts (H1-H4) was within a relatively narrow range of $0.42 \pm 0.01 - 0.62 \pm 0.01$ mg/100 g dry sample; $p > 0.05$.

The concentrations of saponins in the herbal extracts varied within a relatively narrow range: $0.33 \pm 0.09 - 4.33 \pm 0.02$ mg/100 g dry sample; $p > 0.05$. Although the concentrations of saponins in Extracts (R1-R4) were comparatively higher than those of Extracts (H1-H4), the values showed no corresponding significant difference ($p > 0.05$). Finally, a general survey of Table 1 showed that the concentrations of the phytochemicals analyzed in the raw and hydrothermal processed herbs were in the order: alkaloids > flavonoids > tannins > saponins.

**Figure 1.** Serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities of experimental rat groups.

Serum ALT activity of Group 1 was not significantly different ($p > 0.05$) from that of Group 2 (Figure 1). However, Group 3 exhibited comparatively raised level of serum ALT activity, which represented 4.44 folds increase in the enzyme activity compared with that of Group 1; $p < 0.05$. Conversely, serum ALT activities of Groups (4-12) were significantly lower ($p < 0.05$) than that of Group 3, but significantly higher ($p < 0.05$) than those of Group 1 and Group 2. Specifically, serum ALT activities of Group 6, Group 10, Group 11 and

Group 12 were significantly higher ($p < 0.05$) than that of group 4, whereas serum ALT activities of Group 5, Group 7, Group 8 and Group 9 were comparable with that of Group 4; $p > 0.05$. Serum AST activities of the various experimental rat groups followed the same pattern with their serum ALT activities (Figure 1). However, the activity ratios of AST to ALT of the various experimental rat groups were generally less than 1.0 unit.

Serum ALP activity of Group 1 was not significantly

different ($p > 0.05$) from that of Group 2 (Figure 1). Serum ALP from Group 3 gave the highest activity, which was significantly different ($p < 0.05$) from other experimental rat groups. Additionally, serum ALP activity of Group 3 was

comparable with those of group 5, Group 6 and Group 10 ($p > 0.05$), whereas serum ALP activities of Group 7, Group 8, Group 9, Group 11 and Group 12 were significantly higher ($p < 0.05$) than that of Group 3.

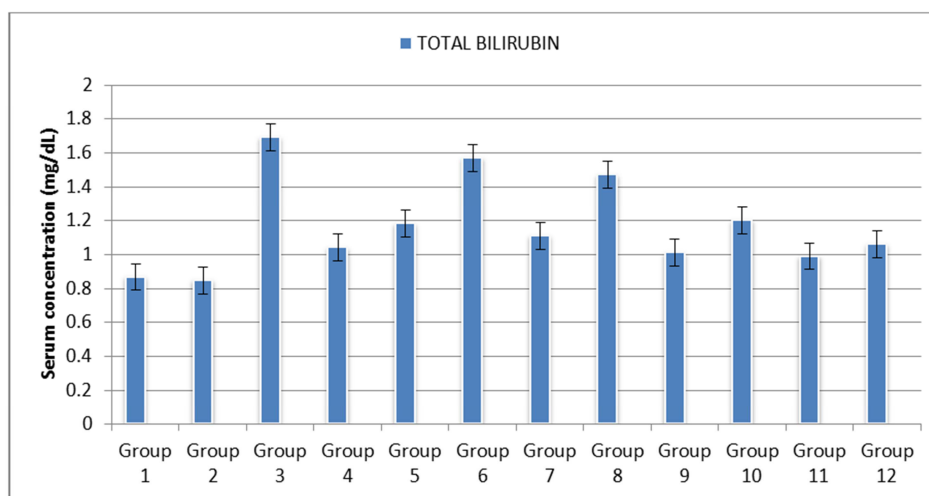


Figure 2. Serum total bilirubin concentration of experimental rat groups.

Figure 2 showed that serum TBCs of Group 1 and Group 2 were less than 1.0 mg/mL and showed no significant difference ($p > 0.05$). Contrary, serum TBCs of other experimental groups, except Group 11, were above 1.0 mg/dL. Specifically, Group 3 gave the highest serum TBC,

which was significantly different ($p < 0.05$) from other experimental rat groups. Furthermore, serum TBC of Group 11 was comparable with those of Group 4, Group 7, Group 9 and Group 12; $p > 0.05$.

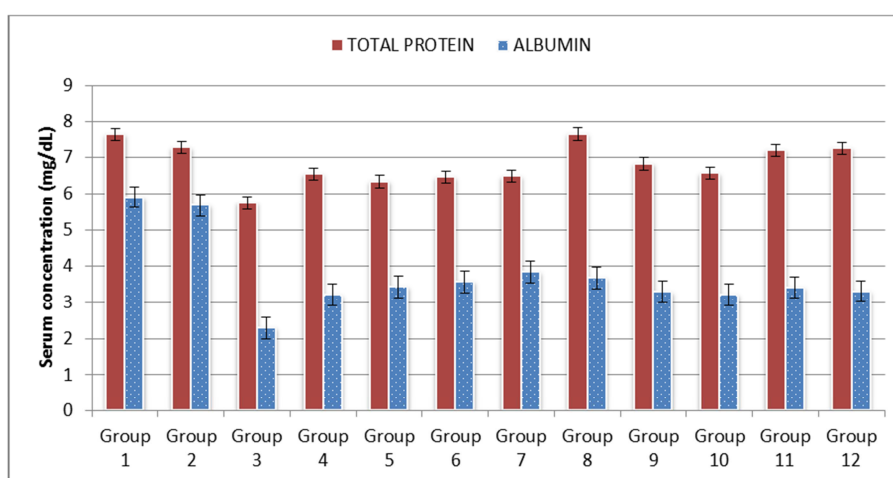


Figure 3. Serum total protein and albumin concentrations of experimental rat groups.

Figure 3 showed that serum TPC of Group 1 was not significantly different ($p > 0.05$) from that of Group 2. Additionally, serum TPCs of Group 4, Group 5, Group 6, Group 7 and Group 10 were comparable; $p > 0.05$. Likewise, serum TPCs of Group 11 and Group 12 were comparable with that of Group 2; $p > 0.05$. However, serum TPC of Group 3 was significantly lower than other experimental rat groups; $p < 0.05$. A cursory look at Figure 3 showed that serum AICs of the various experimental rat groups exhibited similar pattern with serum TPCs, exemplified by the fairly strong correlation coefficient ($r = 0.651662$) between their serum TPCs and serum AICs.

Figure 4 showed that serum CrCs and serum UrCs of Group 1 and Group 2 did not show significant difference ($p > 0.05$). Conversely, serum CrC of Group 3 was significantly higher ($p < 0.05$) than those of Group 1 and Group 2. Additionally, serum CrC of Groups (4-12) varied within a narrow range of $0.61 \pm 0.03 - 0.66 \pm 0.05$ mg/dL; $p > 0.05$.

Serum UrC of Group 3 significantly higher ($p < 0.05$) than those of Group 1 and Group 2 as well as other experimental rat groups- Groups (5-12). The correlation coefficient ($r = 0.898134$) between serum CrC and serum UrC of the experimental rat groups indicated a strong positive correlation.

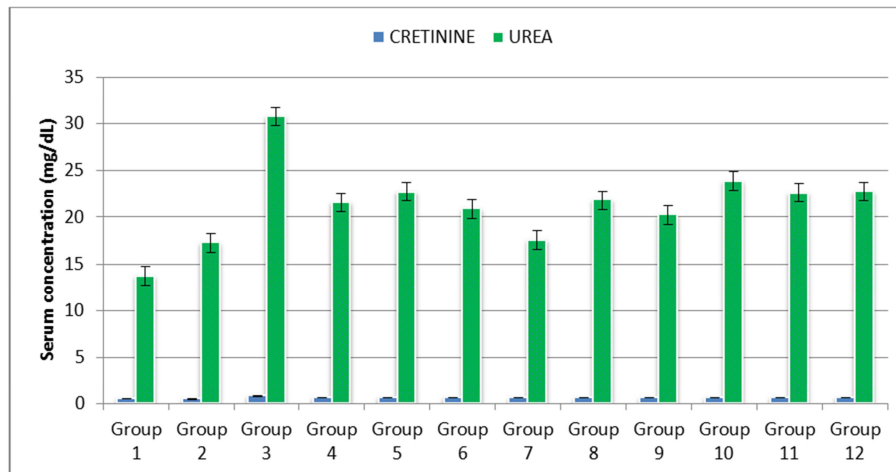


Figure 4. Serum creatinine and urea concentrations of experimental rat groups.

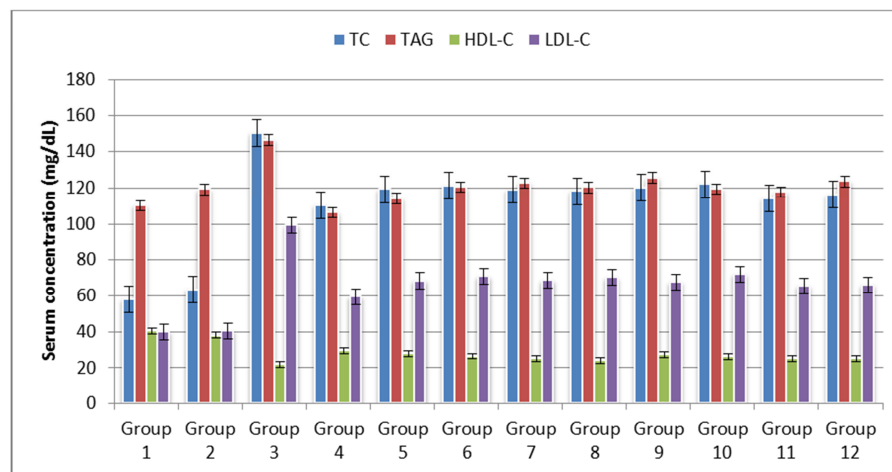


Figure 5. Serum lipid profile of experimental rat groups.

Figure 5 showed that SLP of Group 1 exhibited comparable pattern with that of Group 2 as typified by their atherogenic indices (Table 2). Specifically, serum TC, TAG and LDL-C concentrations of Group 3 were significantly raised ($p < 0.05$) compared with Group 1 and Group 2. Conversely, Group 3 exhibited the lowest serum HDL-C

concentration compared with other experimental rat groups. Generally, serum TC, TAG and LDL-C concentrations of Groups (5-12) were higher than that of Group 4, and in turn, those of Group 4 was higher than those of Group 1 and Group 2; $p < 0.05$.

Table 2. Atherogenic indices of experimental rat groups.

Atherogenic indices			
Groups	TAG/HDL-C	TC/HDL-C	LDL-C/HDL-C
Group 1	2.74 ± 0.02	1.44 ± 0.01	0.99 ± 0.001
Group 2	2.99 ± 0.02	1.59 ± 0.01	1.01 ± 0.01
Group 3	6.76 ± 0.06	6.94 ± 0.05	4.59 ± 0.02
Group 4	3.59 ± 0.03	3.72 ± 0.02	2.00 ± 0.01
Group 5	4.07 ± 0.03	4.26 ± 0.02	2.44 ± 0.01
Group 6	4.56 ± 0.03	4.59 ± 0.02	2.68 ± 0.01
Group 7	4.91 ± 0.03	4.76 ± 0.02	2.78 ± 0.02
Group 8	5.40 ± 0.03	4.91 ± 0.02	2.91 ± 0.01
Group 9	4.59 ± 0.03	4.39 ± 0.02	2.47 ± 0.01
Group 10	4.59 ± 0.03	4.69 ± 0.02	2.77 ± 0.02
Group 11	4.69 ± 0.02	4.54 ± 0.02	2.61 ± 0.01
Group 12	4.96 ± 0.02	4.66 ± 0.02	2.65 ± 0.01

The mean ($\bar{X}$) ± S.D of six ($n = 6$) determinations. Reference values: TAG/HDL-C ratio [61, 62], TC/HDL-C ratio < 1.66 and LDL-C/HDL-C ratio < 1.06 [39]. Castelli risk indices I (TC/HDL-C) and II (LDL-C/HDL-C) [63].

An overview of Table 2 showed that atherogenic indices of Group 1 and Group 2 were below the critical values, whereas those of Groups (3-12) were within the following ranges: TAG/HDL-C ratio ($3.59 \pm 0.03 - 6.76 \pm 0.06$), TC/HDL-C ratio ($3.72 \pm 0.02 - 6.94 \pm 0.05$) and LDL-C/HDL-C ratio ($2.00 \pm 0.01 - 4.59 \pm 0.02$).

The histopathological features of hepatic tissue sections of the experimental rat groups are presented in Figure 6. Photomicrograph section of hepatic tissues of Group 1 showed normal histology and was comparable with that of Group 4; except for slight localized distortion in hepatic parenchyma of Group 4. Additionally, hepatic section of

Group 2 showed normal histology with minimal cytoplasmic vacuolization. Conversely, hepatic section of Group 3 showed evidence of massive disarrangement and distortions of tissue architecture, accompanied with infiltration of fatty deposits as well as centrilobular necrosis comparable with that of Group 5. Figure 6 showed that hepatic sections of Group 8, Group 9, Group 10, Group 11 and Group 12 showed evidence of fatty deposits and fibrotic changes of hepatic parenchyma. Specifically, hepatic tissues sections of Group 9 and Group 10 were also associated with cytoplasmic vacuolization.

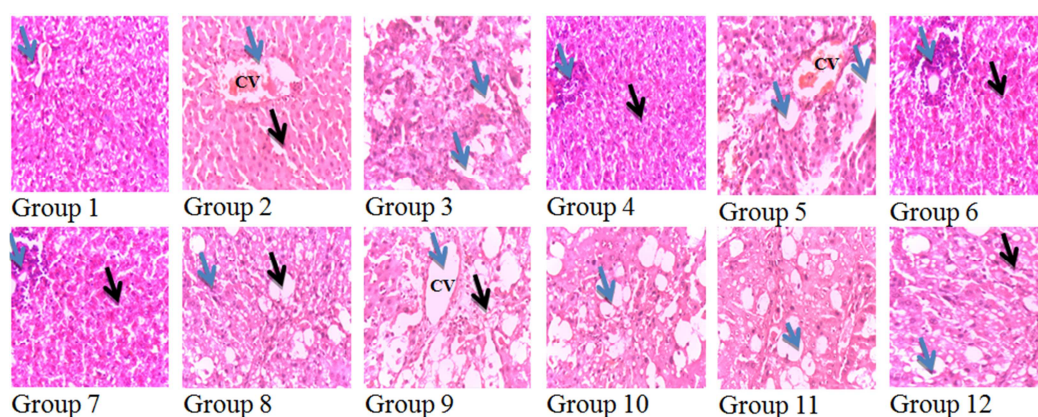


Figure 6. Photomicrograph sections of hepatic tissues (H&E x400).

Group 1: Normal histology showing normal central vein (blue arrow). Group 2: Normal histology showing pronounced normal central vein (CV) (blue arrow) and Kupffer cells along the sinusoids (black arrow). Group 3: Massive tissue necrosis and fibrotic changes of hepatic parenchyma with centrilobular vacuolization and extensive fatty deposits (blue arrows). Group 4: Localized minimal distortion of hepatic parenchyma architecture (blue arrow) with infiltration of mononuclear cells around portal area and adjoining hepatic tissues (black arrows). Group 5: Massive tissue necrosis and fibrotic changes of hepatic parenchyma with extensive fatty deposits (blue arrows). Group 6 and Group 7: Localized minimal distortion of tissue architecture (blue arrow) with massive number of apoptotic hepatocytes (black arrow). Group 8: Fibrotic changes (blue arrow) with fatty deposits (black arrow). Group 9: Fibrotic changes with fatty deposits (black arrow) as well as CV congestion (blue arrow). Group 10: Fibrotic changes with fatty deposits (blue arrow). Group 11: Fibrotic changes of hepatic parenchyma with fatty deposits (blue arrow). Group 12: Fibrotic changes with fatty deposits (blue arrow) with cytoplasmic vacuolization (black arrow).

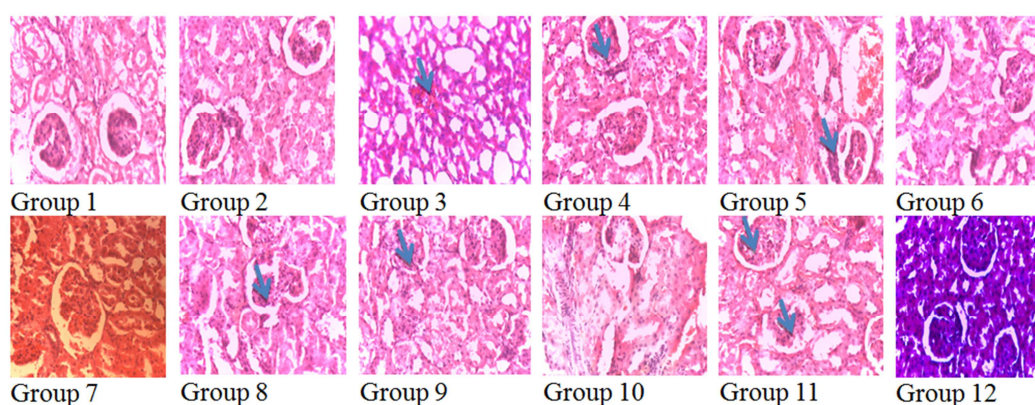


Figure 7. Photomicrograph sections of renal tissues (H&E x400).

Group 1 and Group 2: Normal histology showing normal tubular and glomeruli architecture. Group 3: Pronounced degenerated and distorted tubular and glomeruli architecture as well as evidence of distorted renal parenchyma integrity (blue arrow). Group 4 and Group 5: Minor distorted glomeruli architecture and infiltrated mononuclear cells around the glomeruli (blue arrow). Group 6: Distorted tubular and glomeruli architecture as well as necrosis of renal parenchyma. Group 7: Minor distorted glomeruli architecture but degenerated and distorted tubular morphology. Group 8 and Group 9: Major distorted glomeruli architecture and infiltration of mononuclear cells around the glomeruli (blue arrow). Group 10: Dispersed mononuclear cells around the glomeruli and renal parenchyma. Group 11: Minor distorted glomeruli architecture and infiltrated mononuclear cells around the glomeruli (blue arrows). Group 12: Normal glomeruli architecture but degenerated and distorted tubular morphology.

The histopathological features of renal tissue sections of the experimental rat groups are presented in Figure 7. Photomicrograph section of renal tissues of Group 1 showed normal histology, which was comparable with that of Group 2. The tubular and glomeruli configurations were absent in renal tissues of Group 3, whereas those of Group 4 and Group 5 were present but distorted. Renal tissue section of Group 6 showed evidence of parenchyma necrosis with distorted glomeruli architecture. The degenerated and distorted tubular morphology of renal tissues of Group 7 was comparable with that of Group 11. There were evidence of infiltrated mononuclear cells around the glomeruli (Group 8, Group 9 and Group 11) and dispersed in renal parenchyma (Group 10).

4. Discussion

Hydrothermal processing of herbs brings about changes in their physical characteristics and biochemical compositions [64, 65]. Expectedly, the present study showed evidence of losses in phytochemical contents following hydrothermal processing of the herbal samples. Most of these phytochemicals have been demonstrated to possessing antioxidant properties using both *in vitro* and *in vivo* evaluation models [1, 9, 66-68]. However, the effects of hydrothermal processing on the antioxidant contents and activities of herbs are still been controversially discussed [69-71]. Antioxidant phytochemicals function by modulation of nuclear factor- κ B (NF- κ B), p38 mitogen-activated protein kinase (MAPK), NH₂-terminal Jun kinases/stress-activated protein kinases (JNK/SAPK) and hexosamines mediated cellular stress-sensitive pathways [72-76] as well as transforming growth factor- β (TGF- β)-Smad3 signaling pathway [77]. Previous reports had associated the loss in phytochemical content of hydrothermal processed herbs to leaching and low thermal stability of most phytochemicals [78]. For instance, hydrothermal processing of broccoli, choy-sum and cabbage resulted to losses in their total phenolic contents [79]. Additionally, cooking methods and storage temperature of raw vegetables influenced their phytochemical contents and antioxidant capacities *in vitro* [64, 66]. Specifically, report showed that hydrothermal processing caused 19% loss in the phytochemical contents of cauliflower [80]. It is worthwhile to note that losses in phytochemical contents following hydrothermal processing of herbs may compromise their radical scavenging potential and the capacities of such products to exert health benefits [66, 81]. Nevertheless, hydrothermal processing serves as one of several measures for the elimination of inherent cytotoxic phytochemicals from raw medicinal herbs and vegetables [82-85].

Measurements of hepatic enzymes in serum of animal models have been used in previous studies as basis for ascertaining the capacities of herbal remedies to ameliorate chemically induced liver damage [8, 9, 86-88]. Elevated levels of serum AST, ALT and ALP activities are diagnostic

of fibrotic changes of the liver, myocardial infarction, bone disorders and other related pathologic conditions associated with organ necrosis [89]. Serum ALT activity is more specific for hepatic injury than an increase in serum AST activity and may reflect fatty changes in the liver as in the case of non-alcoholic fatty liver disease (NAFLD) [90-93]. Specifically, the raised level of serum ALT activity of Group 3 (Figure 1) was obvious indication of fibrotic changes of hepatic tissues of the experimental rats as corroborated in previous findings [12, 13, 94]. However, fibrotic rats treated with raw and hydrothermal processed herbs showed evidence of amelioration of CCl₄-induced liver injury as indicated by their comparatively lower serum ALT activities, which concurred with previous findings [13, 77, 94, 95]. The present findings showed that the capacities of the raw and hydrothermal processed herbs to reverse elevated levels of serum AST and ALT activities were comparable with that of silymarin, which was used as reference treatment of fibrotic rats. Furthermore, it appeared that the raw herbs exhibited greater capacities to lower serum ALT activities of fibrotic rats than those of corresponding hydrothermal processed herbs, which was an indication of greater capacities of the raw herbs to ameliorate CCl₄-induced liver injury than those of corresponding hydrothermal processed herbs. Likewise raised level of serum ALP activity of Group 3 was a reflection of hepatobiliary disorder and fibrosis [87], which paralleled the patterns of serum AST and ALT activities of other experimental rat groups and conformed with the outcome of related investigations [1, 12, 13, 45, 96]. Overall, serum AST, ALT and ALP activities of fibrotic rats treated with raw and hydrothermal processed herbs as well as the standard herbal drug-silymarin were profoundly higher those of normal rat groups (Group 1 and Group 2), which by implication showed that the various herbal remedies did not possess the capacities to offer full therapeutic benefits within the experimental 28 days of treatment.

The factors that elicit raised level of bilirubin in serum are multifaceted and serum bilirubin concentration greater than 1.0 mg/dL is diagnostic of hyperbilirubinemia [97, 98]. Hyperbilirubinemia occurs due to rapid haemolysis such that the production of bilirubin far exceeds the capacity of normal hepatocytes to conjugate and excrete bilirubin. In the event of hepatic damage, the capacities of the hepatocytes to conjugate and excrete bilirubin are compromised, even when bilirubin is produced in normal quantities. Furthermore, hepatobiliary obstruction prevents the excretion of bilirubin engendering the accumulation of bilirubin in the liver, which eventually diffuses into the blood system.

Previous studies have noted that reactive CCl₃[•] and Cl₃COO[•] radicals generated from CCl₄ metabolism promoted membrane lipid peroxidation, in which the erythrocytes [99] and hepatorenal tissues were vulnerable [1, 9, 11-14] and consequently, prompted rapid haemolysis and hepatic necrosis with attendant hyperbilirubinemia in the experimental rats (Groups 3-12). The results of the present study (Figure 2) indicated that fibrotic rats, regardless of the

type of herbal treatment administered, presented evidence of hyperbilirubinemia. However, the hydrothermal processed herbs appeared to exhibit relatively higher tendencies to lower the severity of hyperbilirubinemia than those of corresponding raw herbs. In that regard, the results appeared to suggest that hydrothermal processing of the herbs caused the elimination or reduction in the amount of cytotoxic components in the herbs that probably interfered with the capacity of the herbs to ameliorate hyperbilirubinemia.

The plasma proteins (e.g. albumin) are synthesized by the liver and therefore low circulating level of plasma proteins indicates hepatic dysfunction. Previous studies have shown that mild perturbation of hepatic tissues integrity did not profoundly affect the capacity of the liver to biosynthesize plasma proteins [58, 100]. The present study showed that serum total protein and albumin concentrations of Group 3 were lower than those of Group 1 and Group 2 (Figure 3), which confirmed compromised hepatic function. However, the administration of raw and hydrothermal processed herbs caused limited improvement in the capacity of hepatocytes of fibrotic rats to biosynthesize plasma proteins.

The renal tissues are primarily concerned with the clearance of nitrogenous waste products and other blood low threshold substances. Creatinine is mainly the catabolic waste product from tissue protein turnover, whereas urea is derived from oxidative deamination of dietary amino acids [1, 101, 102]. Elevated blood creatinine and urea concentrations are diagnostic of impaired renal function. The present study showed evidence of renal dysfunction following infusion of the experimental rats with CCl_4 , exemplified by comparatively raised serum creatinine and urea concentrations of Group 3 (Figure 4). The present findings confirmed previous research outcomes, which noted that acute chemical intoxication that caused morphological and functional damages to hepatic tissues predisposed animal models to developing acute renal dysfunction [1, 5, 6, 8, 9]. Fibrotic rats treated with raw and hydrothermal processed herbs showed evidence of limited amelioration of impaired renal function, typified by their lowered serum creatinine and urea concentrations compared to that of Group 3. In a related study, Elgazar and AboRaya, [103], using serum urea and creatinine concentrations as biomarkers, noted that single and combinatorial formulations of *Petroselinum sativum*, *Eruca sativa* and *Curcuma longa* ameliorated gentamicin-induced renal tubular necrosis in adult male Sprague Dawley rats.

The metabolic concerns of the liver, amongst other functions, are to regulate the mobilization, biosynthesis and catabolism of lipoproteins in vertebrates. Previous reports showed that CCl_4 interferes with the metabolism of lipoproteins in hepatic smooth endoplasmic reticulum, engendering alterations in SLP patterns and associated dyslipidemia [12, 104, 105], whereby blood lipid concentrations are elevated as typified by raised levels of serum LDL-C and TC of Group 3 compared to that of Group 1 (Figure 5). Additionally, the SLP patterns of fibrotic rats administered with raw and hydrothermal processed herbs were identical and therapeutic scores of the herbs were not

profoundly different from that of the standard herbal drug-silymarin. Overall, the present study showed that the herbs did not offer full therapeutic benefits to the fibrotic rats, in terms of their capacities to ameliorate dyslipidemia. The predisposition of the experimental rat groups to arteriosclerosis and associated cardiovascular morbidity and mortality were defined by their atherogenic indices (Table 2). According to the TAG/HDL-C and LDL-C/HDL-C ratios of the present study, adjustments of SLP patterns in the fibrotic rats, irrespective of the type of herbal treatment they received, indicated incidences of atherogenicity as defined elsewhere [39, 58, 61-63].

The massive disarrangement of hepatic tissues architecture of untreated fibrotic rats (Group 3) correlated with the levels of alterations of serum indicators, namely, serum ALT, AST and ALP activities as well as serum TBC as previously described [12, 13, 98, 99]. Additionally, photomicrograph section of hepatic tissues of fibrotic rats (Figure 6) revealed and confirmed CCl_4 -induced necrosis and steatosis as previously described [12, 13, 88, 106]. The localized distortions in hepatic architecture, persistence of hepatic steatosis and hydropic degenerations in fibrotic rats following treatment with raw and hydrothermal processed herbs were indications, which confirmed limited capacities of the herbs to ameliorate CCl_4 -induced morphological and functional impairments of hepatic tissues within the experimental period of 28 days. According to Sokol *et al.* [107], manifestation of hepatic steatosis was as a result of low availability of tissue α -tocopherol and ascorbic acid, rather than glutathione (GSH), which also predisposed the liver to oxidative injuries as exemplified by the presence of hepatic necrosis in tissue sections of the present report.

Rincón *et al.* [5] suggested that the effect of CCl_4 on kidney tissue morphology and function depended on the functional state of the liver. Similar to the histopathological status of the liver, photomicrograph sections of renal tissues (Figure 7) showed that the distortions in renal tissue architecture correlated with the levels of alterations in their blood indicators; serum creatinine and urea concentrations as previously described [60]. Furthermore, the tissue architecture of fibrotic rats administered with raw and hydrothermal processed herbs confirmed the limited capacities of the herbs to ameliorate renal dysfunction within the experimental period of 28 days.

5. Conclusion

The losses in phytochemical contents following hydrothermal processing of the herbs did not substantially affect their overall therapeutic scores against morphological and functional impairments of hepatic and renal tissues following CCl_4 intoxication of the experimental rats. However, herbal intervention against CCl_4 -induced hyperbilirubinemia showed that hydrothermal processed herbs possessed greater capacity to lower the severity of hyperbilirubinemia than their corresponding raw herbs. Nevertheless, a more rewarding bio-prospecting exercise for

the alleviation of CCl₄-induced hepatorenal impairment could be achieved by subjecting the herbs to sequential multi-solvent extraction process, which perhaps, will provide improved and better therapeutic benefits than the present outcomes.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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