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Ameliorative effects of Nigerian recipes on histopathological and immunohistochemical changes of CCl_4-induced hepatic fibrosis in male Wistar rats

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Abstract
The effects of methanol extracts of cooked *Sphenostylis stenocarpa, Buchholzia coriacea, Chromolaena odorata and Monodora myristica* on the collagen deposition of hepatic stellate cells (HSCs) in hepatic fibrotic male Wistar rats were investigated. Ninety-six adult male rats of weight 120 – 190 g were grouped into twelve (groups A-L) of eight rats each, representing normal control, olive oil, negative control, silymarin, 250 mg/kg and 500 mg/kg body weight of cooked *S. stenocarpa, B. coriacea, C. odorata and M. myristica* methanol extracts, respectively. Groups A and B received 1 ml of normal saline and 1 ml of olive oil, respectively, group C received CCl_4/olive oil mixture only and group D was treated daily with 100 mg/kg of the standard drug, silymarin, after two weeks of CCl_4 administration. Groups E - L were pre-treated with the respective extracts twice a week for four weeks by intubation before intraperitoneal intoxication with 1 ml of carbon tetrachloride (CCl_4) and olive oil (1:1 v/v) mixture per kilogram body weight of the experimental animal and then extract-treatment was continued for another four weeks. Liver specimens were procured, weighed and processed for immunohistochemical studies. Necrosis and collagen deposition were evident in the liver histology of the negative control. Treatment with methanol extracts of the cooked samples, except 250 mg/kg body weight of *C. odorata*, ameliorated the effect of CCl_4. In conclusion, methanol extracts of cooked *S. stenocarpa, B. coriacea and M. myristica* have anti-fibrotic and hepato-protective potentials against CCl_4-induced liver injury in male Wistar rats.

Keywords: CCl_4; collagen; fibrosis; histopathology; immunohistochemistry; liver

1. Introduction
Fibrosis is a hallmark histological event of chronic liver disease and a significant risk factor of hepatocellular carcinoma; the third major cause of cancer death worldwide [1, 2]. It is a common pathologic process of normal wound repair after tissue injury [3]. Liver injury occurs in response to a variety of insults caused by alcohol, viral hepatitis, steatosis, insulin resistance, autoimmune disease, excessive deposition of iron or copper on liver, congenital abnormalities, [2] drugs, [4] xenobiotics and oxidative stress [5]. Usually, after liver injury, parenchymal cells regenerate and replace necrotic and apoptotic cells following a closely regulated sequence involving inflammation, recruitment, activation and proliferation of fibroblasts, and the secretion of extracellular matrix, which culminates in healing and termination of the proliferative and secretory processes [3]. Persistence in injury eventually leads to failure in regenerative response and consequent hepatocyte substitution by abundant extracellular matrix (ECM) comprising mainly of collagen, fibronectin, elastin, laminin, and proteoglycans [6, 7]. The imbalance between the production and degradation of ECM by hepatic stellate cells (HSC), has been implicated as the critical factor in the fibrogenesis of liver fibrosis [8, 9, 7], hence it is regarded as the target of anti-fibrotic therapy [10].
The key cellular mediator of fibrosis is the myofibroblast, which when activated serves as the primary collagen-producing cell [11]. The myofibroblast is regulated by such factors as cytokines (IL-13, IL-21, TGF-β1), chemokines (MCP-1, MIP-1β), angiogenic factors (VEGF), growth factors (PDGF), peroxisome proliferator-activated receptors (PPARs), acute phase proteins (SAP), caspases, and components of the renin–angiotensin–aldosterone system (ANG II) [11]. Transcription of collagen genes increases 3–10 folds in activated fibroblasts, and increase in the stability of collagen mRNAs contributes to its high expression CF [12]. Thus, the synthesis of new collagen by the myofibroblasts exceeds the rate of45 its degradation resulting to collagen accumulation over time.
Sphonostylis stenocarpa, *Chromolaena odorata*, *Buchholzia coriacea* and *Monodora myristica*

are Nigerian indigenous plants. They were selected based on their local use, reports on their bioactive components and physiological functions [13, 14, 15, 16, 17]. Our previous report on these plants revealed the presence of phenols, flavonoids, alkaloids, saponins, tannins and other antioxidants [18] (Ojiako *et al*., 2016). Flavonoids possess anti-inflammatory, antioxidant, anti-allergic, hepatoprotective, anti-thrombic, antiviral and anti-carcinogenic activities [19, 20]. Saponins are reported to exhibit hypolipidemic, anticancer and anti-cytotoxic activities among others [21]. Tannins modulate immune response in addition to its antimicrobial and antioxidative properties [22, 23]. Despite the numerous beneficial components of the selected plants, little or no research has been carried out to elucidate the effects of the plants on carbon tetrachloride – induced hepatic fibrosis. This study investigated collagen deposition protective effect of methanol extracts of *S. stenocarpa*, *C. odorata*, *B. coriacea* and *M. myristica* on carbon tetrachloride – induced hepatic fibrosis in male Wistar rats.

2. Materials and Methods

2.1 Chemicals: Carbon tetrachloride (CCl_4) was obtained from Kermel Chemical Reagent Company Ltd., China. Silymarin was obtained from Alexandria Pharmaceutical Company, Egypt. Goya olive oil, product of Ekulo International Ltd., Lagos and normal saline manufactured by Juhel Nigeria Ltd, Awka, Nigeria were also used. Other reagents were of analytical grade.

2.2 Collection and treatment of plant materials

The leaves of *C. odorata* were obtained from surrounding garden in Amakohia, while the seeds of *S. stenocarpa*, *B. coriacea* and *M. myristica* were purchased from local markets located within Owerri metropolis, Imo State. The plants were authenticated at Michael Okpara University of Agriculture, Umudike, by Dr E. S. Willie, a taxonomist in the Department of Agronomy, College of Crop and Soil Science. The seeds of *B. coriacea, S. stenocarpa, M. myristica* and leaves of *C. odorata* were washed under running tap water, submerged in boiling water and cooked for 3 h, 1 h 30 min, 10 min and 5 min, respectively. The samples were then dried in the oven at 60°C till they attained constant weights. The dried samples were ground into flour and packaged in air-tight containers with corresponding labels.

2.3 Methanol extraction

Methanol extraction of the samples was carried out as described by Huda-Faujan *et al* [24]. Briefly, pulverized parts of the different plants were measured out and soaked in methanol at the ratio of 1 g of sample to 6 ml of methanol for 3 days with constant shaking before filtering with Whatman No 1 filter paper. The methanol was completely removed using rotary evaporator set at 50°C resulting in viscous masses. The crude extracts were weighed and stored at -4°C for analysis.

2.4 Animals

Ninety-six (96) adult male Wistar rats, aged about 3 months and weighed 120 – 190 g, were used for the experiment. The animals were maintained *ad libitum* on clean water and grower's mash (Vital Feeds, Grand Cereals Ltd. Jos, Plateau State) and allowed to acclimatize for one week. All the animals were treated in accordance with the recommendations of National Institutes of Health (NIH) guidelines for the care and use of laboratory animals [25].

2.5 Experimental protocol

The male Wistar rats were grouped into twelve of eight rats each based on a weight range of ± 7.0 g between each group. Group A served as normal control, group B served as olive oil control (vehicle for CCl_4), group C received CCl_4/olive oil mixture and group D was treated daily with 100 mg/kg of standard drug, silymarin, after two weeks of CCl_4 administration. Groups E to L were fed with 250 mg or 500 mg of methanolic extracts of cooked (C) *S. stenocarpa*, *B. coriacea, M. myristica* and C. odorata per kilogram body weight of animal by intubation for eight (8) weeks. Similarly, groups A and B were given 1 ml of normal saline and 1 ml of olive oil, respectively, for the same duration. After 28 days of feeding, all the groups, except groups A and B, were injected intraperitoneally with 1 ml/kg of CCl_4/olive oil mixture (1:1 v/v) twice a week for 28 days concurrently with extract treatment.

2.7 Organ Weights

The animals were all sacrificed 12 h after the last treatment with silymarin or extract, by cervical dislocation under anesthesia using dichloromethane (CH_2Cl_2) vapour. The liver of each rat was excised, observed and washed in normal saline, blotted dry using filter paper and weighed. The relative organ weight (ROW) of each rat was calculated according to the method of Salawu *et al* [26].

ROW = {Absolute organ weight (g) / Body weight (g) of rats on sacrifice day} x 100

2.8 Histopathological examination of the liver and kidney tissues

The liver organs that were fixed in formalin were processed according to the method of Baker and Silverton [27]. The fixed tissues were cut into bits (about 3-5 mm thick and 1 cm long) and re-fixed in 10% formalin prior to processing. The tissues were thereafter dehydrated in an ascending concentrations of ethanol (70 %, 80 %, 90 % and 100 %), followed by clearing through changes (thrice) in xylene for 1 hr 30 min each. The tissues were embedded in paraffin, sectioned transversally at 4-6 µm and mounted on glass slides. The section was de-paraffinized with xylene and rehydrated in descending concentrations of ethanol. It was rinsed in water and stained in hematoxylin for 5 min. Thereafter, it was quickly dipped in 1 % acid alcohol for 3 seconds to de-stain and rinsed in water. Excess water from the slide was blotted out and counter stained with eosin for 2 min. After rinsing with water, the section was dehydrated in ascending grades of ethanol, and cleared in xylene. A xylene-based cover slip was placed on the slide and mounted on a di-n-butyl phthalate in xylene (DPX – mounting medium) and viewed under a microscope (Leica 1000 DM) at ×400 magnification.

2.9 Immunohistochemistry

Some portions of the liver fixed in 10 % formalin were analysed according to the immunohistochemical technique of Matos *et al.* [28] using cytokeratin cocktails AE1 & AE3 kit manufactured by Bio SB, USA. Cytokeratin AE1/AE3 is a mixture of two different clones of anti-cytokeratin monoclonal antibodies, AE1 and AE3 capable of detecting certain high and low molecular weight keratins [29]. Similar to the histopathological protocol, the liver tissues were dehydrated with alcohol, de-alcoholed with xylene, embedded in paraffin and sectioned. Paraffin sections were deparaffinised in xylene and rehydrated in ethanol and water. The sections were incubated for 10 min in 0.1 % H_2O_2 thus

avoiding endogenous peroxidase activity. After washing with phosphate-buffered saline (PBS), the sections were incubated in immuno DNA retriever containing EDTA. The set up was immersed for 30 minutes in a water bath set at 95˚C and later cooled to room temperature. In order to avoid background activity, sections were incubated in 10 % normal mouse serum for 20 minutes and subsequently with the primary antibodies and mouse secondary antibody for 30 minutes at room temperature. The sections were then washed in PBS, stained with diaminobenzidine tetrahydrochloride solution (DAB), and counter-stained with hematoxylin. The negative controls were made for each primary antibody by labeling the tissues only with secondary antibody and staining with DAB.

3. Results

3.1 Clinical signs

No adverse clinical manifestations were observed within four weeks of pre-treatment with extracts, but mild sedative effects, yellow skin colouration, weight loss and black stool were observed few days after CCl_4 intoxication. At sacrifice, the gross appearance of the livers excised from the normal control (Fig 1.0A) and olive oil control (Fig 1.0B) showed normal appearance of smooth, tan brown, glistening capsule with no granularity while that of the negative control was enlarged, had pale, coarsely granular, yellowish-brown surface with macronodules and high fatty liver (Fig 1.0C). The CCl_4-intoxicated groups had enlarged livers of varying sizes, pallor, nodularity and consistency (Fig 1.0D).

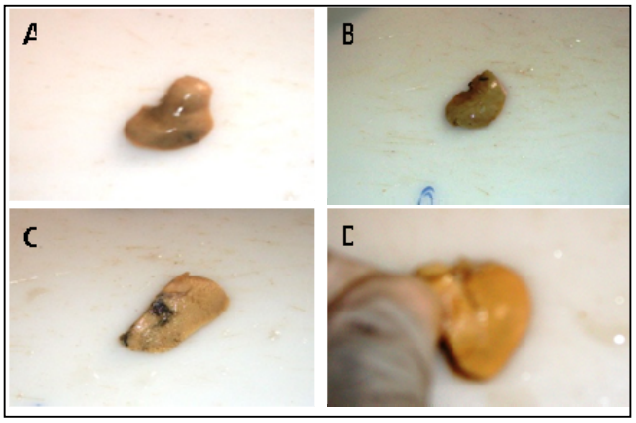

Fig 1: Gross appearance of liver extracted from groups;
A (Control) –showing smooth, shinny and tan brown liver;
B (olive oil) – smooth, shinny and yellowish-brown capsule;
C (Negative control) – granular yellowish liver with macronodules;
D (Positive control) – yellowish liver without surface granularity.

3.2 Effect of the extracts and CCl_4-intoxication on liver weights

CCl_4-intoxication significantly ($p<0.05$) increased the liver weights in comparison with the normal control and olive oil control (Fig 2.0). Treatment of the intoxicated animals with silymarin and cooked plant extracts except high concentrations of *C. odorata* and *B. coriacea* caused significant ($p<0.05$) dose-dependent decrease in the liver weights. Reductions caused by treatment with the extracts did not significantly ($p>0.05$) differ from the group treated with silymarin.

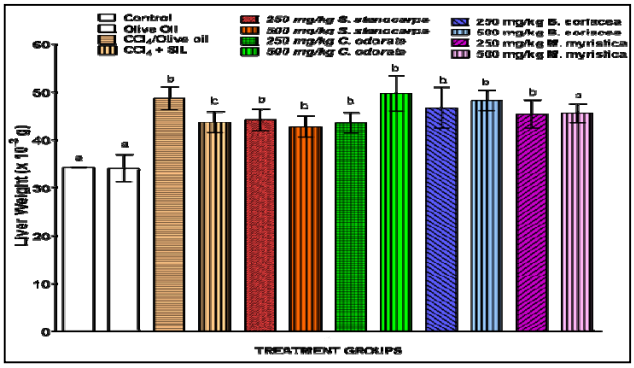

Fig 2: Effect of treatments on relative organ weights of animals. Bars are mean ± standard deviation. Bars with different letters per block of treatment are statistically significant ($p<0.05$).

3.3 Histopathological and immunohistochemical effect of the extracts on hepatic tissues

The results of the histopathological and immunohistochemical analyses of hepatic tissues of rats treated with cooked plant extracts are presented in Fig 3.0 – 10.0.

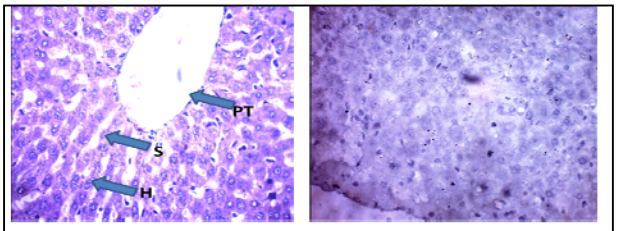

Fig 3.0: Histological and Immunohistochemical Sections of the Control (A): showing normal outlay of liver cells with binucleated hepatocytes, prominent nucleoli, intact sinusoids and central veins as well as intact endothelial cells lining the sinusoid. No collagen fibres and fatty deposits were observed.
PT= Portal tract, S = Sinusoid, H= Hepatocyte

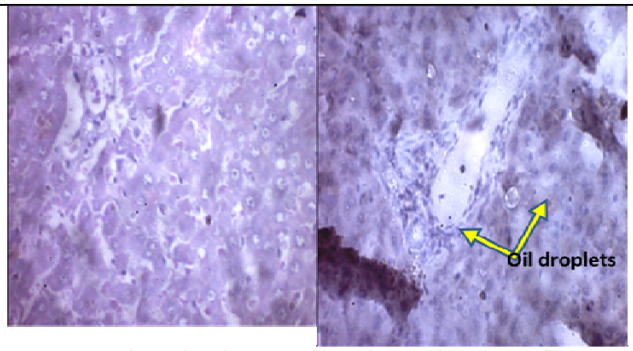

Fig 4.0: Histological and Immunohistochemical Sections of liver tissues extracted from rats treated with Olive oil only (group B)
Photomicrograph shows binucleated hepatocytes, prominent nucleolus, intact sinusoids and central vein. No collagen fibres were evident in the liver parenchyma but oil droplets were observed.

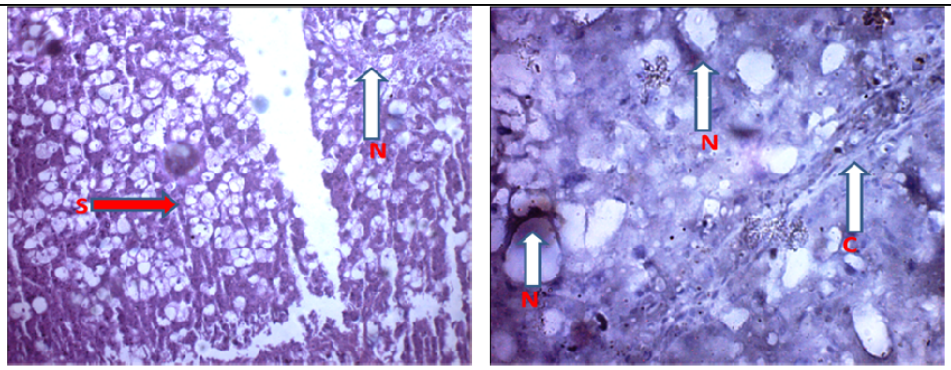

Fig 5.0: Histological and immunohistochemical results of representative liver tissues from the negative control (group C) intoxicated with CCl_4/olive oil mixture: Histological section shows extensive changes in liver morphology, severe fatty degeneration of the hepatocytes and areas of broad bands of necrosis. Small dark brown stained lines of collagen deposition within the liver parenchyma and the portal tracts of the immunohistochemical section. Liver cell death was more evident in the immunohistochemistry than in the haematoxylin and eosin stains.
S = fat deposits, N = necrosis, C = collagen fibres

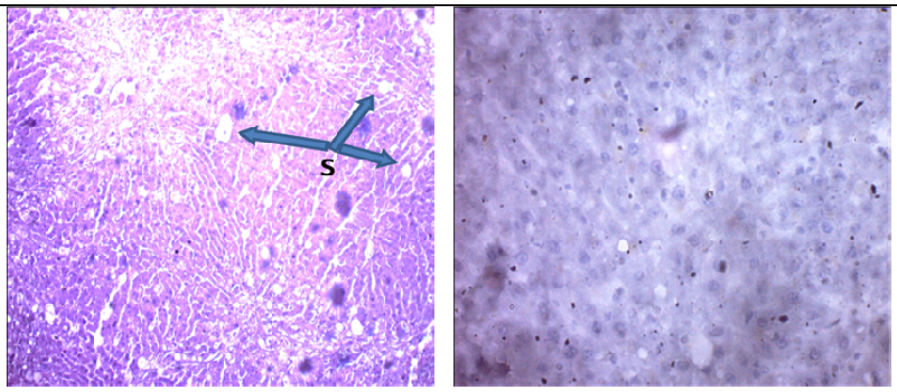

Fig 6.0: The histological and immunohistochemical results of the representative liver tissues of group D treated with Silymarin: showing pleomorphic nuclei, prominent nucleolus, very minimal fatty changes and features lined with inflammatory changes. No area of collagen deposition or fibrosis. **S = fatty cells**

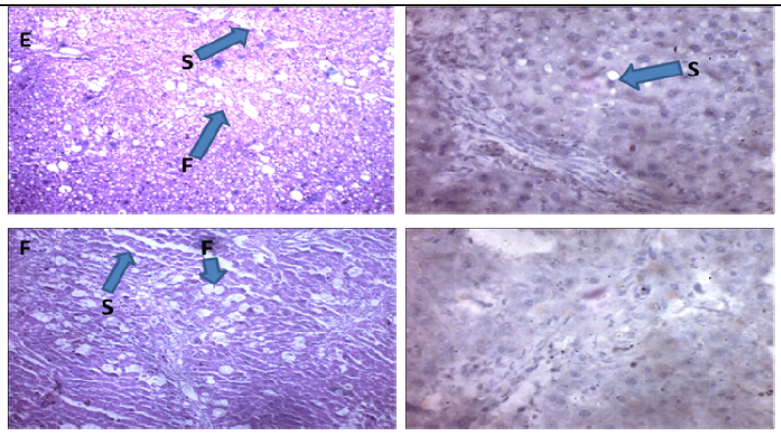

Fig 7.0: The histological and immunohistochemical results of the representative liver tissues of rats from groups E and F treated respectively with 250 and 500 mg of methanol extracts of cooked *Sphenostylis stenocarpa* per kg body weight of the animal. Sections E show hepatocytes with dilated sinusoids, moderate fatty change but no apparent fibrosis. Sections F show mild hepatic fat accumulation, inflammatory cell infiltration into the portal tracts and no evidence of fibrosis. **S = sinusoid, F = fatty cells**

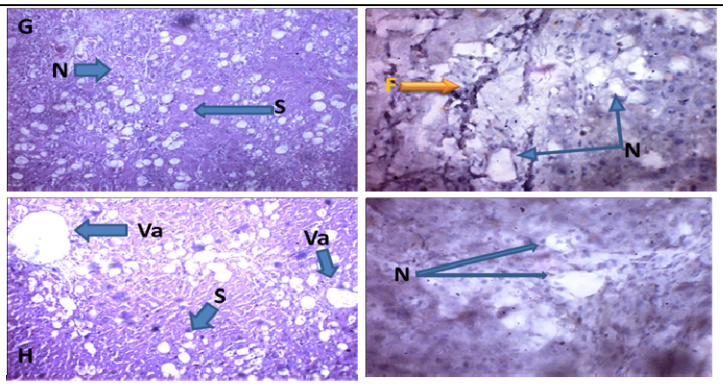

Fig 8.0: Histological and immunohistochemical results of the representative liver tissues of groups G and H that received 250 mg and 500 mg of methanol extracts of cooked *Chromolaena odorata* per kilogram body weight of the animals respectively: Sections of group G show necrosis of liver cells, toxic inflammation and moderate fatty changes with dark lines of significant small discontinous bundles of collagen deposition. Vacuolated hepatocytes, focal liver bridging necrosis and mild fatty changes with no evidence of fibrosis were observed in sections H. **Va = vacuolation S = fat deposits, N = necrosis.**

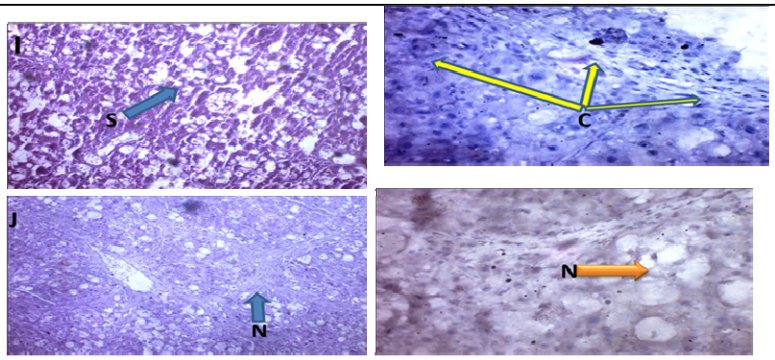

Fig 9.0: Histological and immunohistochemical sections of representative liver tissues from groups I and J that received 250 mg and 500 mg of methanol extracts of cooked *Bulcchozia coriacea* per kilogram body weight of the animals respectively: Sections I show severe fatty changes in the hepatocytes, vacuolations, necrotic cells and bundles of non confluent dark stained fibroblasts laying down fresh collagen fibres. Broad bands of well developed necrotic tissues in the liver parenchyma, mild fatty changes but no collagen fibres were seen in sections J. **S - fat deposits, N - necrosis, C - collagen fibres**

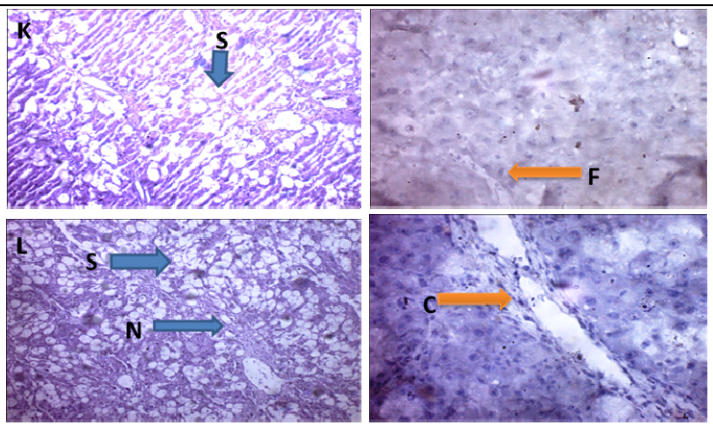

Fig 10.0: Histological and immunohistochemical sections of representative liver tissues from groups K and L that received 250 mg and 500 mg of methanol extracts of cooked *Monodora myristica* per kilogram body weight of the animals respectively: Sections K indicate extreme steatosis, inflammatory changes with with small bands of fibrous tissue separating hepatocytes, no fibrosis. Sections L show severe lipid accumulation in hepatocytes and mild deposition of collagen fibres in the portal tract regions.
S = fat deposits, N = necrosis, C = collagen fibres, F = fibrous tissue

4. Discussion

The liver is a major target organ in safety assessment of preclinical toxicity and oncogenicity studies with rodents, [30] while CCl_4-induced liver fibrosis is a well-established model of hepatotoxicity [31, 32, 33]. Hence, this study evaluated the histopathological and immunehistochemical effects of some Nigerian traditional medicinal plants on CCl_4-induced hepatic fibrosis in rats. Results of the study revealed that the liver excised from the normal control rats consisted of soft, smooth, tan-brown and encapsulated tissue, while irregularities in the surface, colour, size and consistency were observed on liver organs extracted from the intoxicated animals. Surface nodularity, heterogeneous enhancement and caudate lobe enlargement are well known features of liver cirrhosis [34]. Cirrhosis is defined as a diffuse process characterized by fibrosis and conversion of liver into structurally abnormal nodules that affect the whole organ [35]. Increase in the relative liver weight indices of the intoxicated animals is an indication of the negative effect of the CCl_4 toxin on the internal organs of the animals. According to Harlita *et al.* [36] body and organ weight changes serve as sensitive indicators of the general health status of animals. The enlargement observed could be as a result of inflammatory cells, sinusoidal dilation and congestion, nodular regenerative hyperplasia or haemorrhages in the liver.

Histological examination is regarded as the golden standard for evaluating treatment-related pathological changes in tissues and organs [37]. The mild to severe alterations in the hematoxylin and eosin sections of liver tissues extracted from intoxicated rats when compared with normal control suggested varying degrees of disturbance in physiological parameters of the tissues. The alterations included pleomorphic nuclei, dilated sinusoids, vacuolated hepatocytes, inflammatory cell infiltrations into portal tracts, fat accumulations and cellular degenerations. These findings are in agreement with the report of Ki *et al.* [32] and other such investigations where CCl_4 was injected into adult rats [38]. It has been revealed that sinusoidal dilation may be a non-specific feature of impaired portal venous blood inflow or that of severe systemic inflammatory reaction syndrome as a result of toxin ingestion [39]. Vacuolated hepatocytes are cellular adaptive measures beneficial to host organisms rather than hydropic degenerative change [40]. Consistent in the intoxicated groups was the presence of fatty liver, indicating the induction of non-alcoholic fatty liver damage (NAFLD) by CCl_4 within the twenty-eight days of intoxication. NAFLD represents the accumulation of triacylglycerols within hepatocytes in excess of 5% of liver weight [41]. It is reported to be the most common cause of chronic liver disease in developing and industrialized countries [41, 42]. NAFLD consists of broad spectrum of disorders ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which can progress to fibrosis, cirrhosis and finally to hepatocellular carcinoma [43]. NAFLD has been associated with metabolic syndrome such as dyslipidaemia, insulin resistance and hypertension [44, 41]. Reduced fatty accumulations observed in the liver photomicrographs of animals treated with silymarin, methanol extracts of *S. stenocarpa* (SS), *C. odorata* (CO) and higher concentration of *B. coriacea* (BC) could be through the mechanism of reduced lipid synthesis or enhanced hepatic mitochondrial β-oxidation.

A key defining feature of progressive liver damage is inflammation which presents signs such as severe hepatocellular degeneration, necrosis and mononuclear cellular infiltration [45, 33]. The presence of portal inflammation has been strongly correlated with severity and stages of fibrosis as well as ductular reaction in NAFLD [46]. The mechanism of CCl_4 toxicity requires a hepatic microsomal CYP450 (CYP2E1) [47]. The bioactivation of CYP450 following CCl_4 administration generates trichloromethyl (CCl_3) radical and other cascades of free radicals capable of initiating lipid peroxidation and oxidative stress [48]. Continued oxidative stress could lead to chronic inflammation which in turn mediates other chronic diseases such as fibrosis [49]. The increased immune response (necrosis and lymphatic infiltration) and tissue alterations presented by the negative control group are clear indication of oxidative stress and inflammatory damage. Variations in immune responses observed in the histological results of the treatment groups showed dose-dependent ability of the plants in ameliorating the deleterious effects of CCl_4.

Collagen, a fibrous, transmembrane, triple helix and structural protein accounting for 25% of body protein mass, is a key marker of liver fibrosis [50, 51, 35]. The marked expression of collagen fibres in the negative control showed that intraperitoneal injection of CCl_4-olive oil mixture induced liver fibrosis. Excessive accumulation of collagen and other extracellular matrix occurs as a result of increased expression of transforming growth factor (TGF-β) by activated HSC [3]. The over expression of TGF-β, has been correlated with the degree of fibrosis in both animal models and human diseases [52]. During liver inflammation due to paracrine signals from immune cells consequent to injury, reactive oxygen species (ROS) sensitive cytokines get activated and contribute to hepatic stallate cell (HSC) activation and responsiveness to platelet derived growth factor (PDGF) and TGF-β [53]. The potent mitogen-PGDF and fibrogenic factor-TGF-β through their signaling pathway release more ROS in the system, thus inducing more stress due to oxidation, enhancing fibrosis [53].

Reduced accumulation of collagen in the silymarin and cooked extract-treated groups (except cooked *C. odorata* at 250 mg/kg b. wt) indicated that silymarin and methanol extracts of cooked *S. stenocarpa, B. coriacea, M. myristica* and 500 mg/kg of *C. odorata* interfered with the mechanism involved in hepatotoxicity. Thus inhibiting or reducing the trans-activation of HSC, and antagonizing the ability of CCl_4 to increase levels of TGF-β and PGDF [32]. The deposition of collagen in rats treated with 250 mg/kg body weight of cooked *C. odorata* showed increase in the concentration of the extract resulted to increase in biologically active components which were effective in ameliorating the effects of CCl_4. The reverse effect observed in the treatment with M. myristica could be related to lipid accumulation in the hepatocytes with increase in extract dosage. Lipid accumulation in liver cells is regarded a manifestation of metabolic syndrome and lipid overload which predisposes organisms to overproduction of ROS, endoplasmatic reticulum stress, lipotoxicity and autophagy [54]. Hyperactivation of autophagy induces necrosis [54]; this is evidenced by the severity of necrosis in the representative photomicrograph of the group (L) that received 500 mg of M. myristica per kilogram body weight of animal. Lipid accumulation in the hepatocyte is also reported to induce the release of factors that accelerate the activation and proliferation HSCs [55]. The process of boiling, traditionally used to improve extraction of oil may also alter its quality and stability. These could lead to defective lipid metabolism, cumulating to non-alcoholic fatty liver and lipid peroxdation. Nevertheless, boiling is reported to increase antioxidant activities by liberating antioxidant compounds from insoluble

portions of foods and valuable phytochemicals found to remain in water during cooking process [56, 57]. Therefore, anti-fibrotic and hepatoprotective effects of the extracts involved mechanisms such as anti-inflammatory reactions, immunosuppressive activities, protection against oxidative stress and inhibition of fibrotic gene expression [55]. The data obtained in this study is in agreement with the report of Malaguarnea *et al*. [31] who stated that the main pathogenic mechanisms responsible for functional damage caused by solvents such as CCl_4 are inflammation, dysfunctional cytochrome P450 and oxidative stress.

Conclusion

Treatment of rats that had liver fibrosis with silymarin and methanol extracts of S. stenocarpa, B. coriacea and M. myristica and high concentration of C. odoratata ameliorated histological alterations induced by CCl_4 intoxication. The possible mechanisms underlying their protective actions were inferred to be enhanced lipid metabolism, antioxidation and anti-inflammation. These actions could be attributed to phytochemical components of the extracts. Hence, this study is a proof in support of the potentials of S. stenocarpa, B. coriacea and M. myristica seeds as anti-fibrotic and hepatoprotective agents.

Disclosure of Conflict of Interest

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

Acknowledgement

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