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Amelioration of Chemical Induced Hepatic Injury by *Vitex agnus castus* Extract

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Authors' contributions

All the authors participated actively in the study. Author RSA conceptualized and designed the study, supervised the experiment, did the final analysis and interpretation of the results prepared the manuscript. Author NAA performed the experiment, collated the results of the various parameters and performed initial analysis of the data. Authors TAJ and BOE handled the estimations of the biochemical parameters and histology aspect of the study. Literature search was by authors RSA and NAA. Authors RSA, NAA, TAJ and BOE read and approved the manuscript. Author RSA is the guarantor of the study. All the four authors read and approved the final manuscript.

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ABSTRACT

Objective: The liver is the major organ of detoxification of ingested materials such as food, beverages and drugs, thus it is prone to toxicity with attendant pathologies. We studied the ability of *Vitex agnus castus* plant extract to ameliorate the biochemical and structural alterations in Wistar rats with carbon tetrachloride-induced liver injury.
Methods: Forty adult male Wistar rats were allotted into eight equal groups. Group1 was normal control (NC); Group 2 Liver injury without extract (LI). The remaining six groups were paired composite group of varying dosage of the plant extract (200 mg/kg, 400 mg/kg and 600 mg/kg). Only one member of each pair had induced liver injury. Consequently, the groups were Low extract without liver injury (LE), Low extract with liver injury (LEL); Medium extract without liver injury(ME) Medium extract with liver injury (MEL) and High extract without liver injury (HE),High extract with liver injury (HEL).The biochemical parameters evaluated were the liver function test {Total protein

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plus globulin and albumen fractions; liver enzymes- alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase}. Oxidative stress was evaluated by measuring the activities of antioxidants namely; Superoxide dismutase (SOD), Catalase, Malondialdehyde (MDA) and Glutathione Peroxidase (GPx).Histology of the harvested liver specimens was used to assess the structural alterations.

Results: The mean liver weight was highest in the HEL group and significantly different from that of its control (HE) and NC.The mean serum total protein of all the groups were significantly higher than that of the NC.The mean aspartate amino transferase levels of the LEL, ME and MEL groups were significantly lower than that of the control while that of the HEL was significantly higher than those of the LEL and MEL. The results of alanine amino transferase were similar to those of aspartate amino transferase. The alkaline phosphatase levels in all the experimental groups were significantly depressed when compared with the control. Amongst the experimental groups, the serum alkaline phosphatase level was significantly raised than those of the LEL, and MEL groups.
The glutathione (GSH) activities of LE, HE and HEL were significantly lower. While the glutathione peroxidase (GPX) activity of the control was significantly lower to those of groups LEL, ME, MEL and HEL.Histopathology of the liver showed preservation of the liver architecture with normal hepatocytes in all the groups.

Conclusion: The ethanolic extract of *Vitex agnus castus* was able to reduce the severity of carbon tetrachloride induced liver injury in wistar rats.

Keywords: Vitex agnus castus; carbon tetrachloride liver toxicity.

1. INTRODUCTION

The human liver is the largest glandular organ in the body weighing between 1400 to 1600 grams in adults. All the venous blood from the derivatives of the fore, mid and part of the hind gut flow into the liver via the portal vein. Although the liver receives arterial blood from the paired hepatic arteries, the portal vein source is responsible for more than 70 % of blood flow into the liver [1]. Potential sources of toxins to the liver include some components of ingested food, beverages especially alcoholic, herbs and herbal preparations, psychoactive substances, chemically polluted ground water and metabolites of drugs. Thus the liver is highly prone to toxic injuries whose manifestations vary from inflammatory to malignancy. Mechanisms of hepatotoxicity include inflammation, immunomodulation and oxidative stress [2]. Most liver pathologies are of insidious onset and more worrisome is the fact that they progress and most times regrettably terminal. All the previously enumerated sources of toxins are largely avoidable consequent on dietary indiscretion, malnutrition, environmental pollution and degradation and industrialization. One of the ways of reducing the burden of liver disease is by reducing the severity of hepatotoxicity this may be achieved by medicinal plant supplement capable of counteracting mechanisms of actions of liver toxins.

Vitex agnus castus, a deciduous shrub that grows naturally in several countries including Nigeria has been elucidated to have dopaminergic components, essential oil and flavonoids [3]. The plant is said to be efficacious against irregular menstruation, infertility and cyclical mastalgia [4]. Although the medicinal applications of V*. agnus castus* in the management of human ailments and diseases have been investigated and documented. Availability of literature on the relevance of the plant in the management of liver pathologies is scanty or even nonexistent thus the need to investigate the plausible role of the plant in the management of liver pathologies becomes pertinent. As stated earlier toxins and toxicology play significant role in the aetiopathogenesis of liver diseases especially those of chronic varieties. This informed the decision to conduct this interventional plant-based experimental study.

2. MATERIALS AND METHODS

2.1 Plant Materials

2.1.1 Plant collection and authentication

Vitex agnus castus plant was sourced from a herbal garden located in Aladja, Delta State, South-South Region of Nigeria. Botanical identification and specie confirmation were done at the Herbarium Unit of the Department of Botany, University of Ibadan, Nigeria. For reference purpose, a sample of the plant was banked with the Herbarium with voucher number UIH-22953.

2.1.2 Extract preparation

The flowering stems of *V.agnus castus* plant were initially washed under running water and then allowed to dry under ambient temperature till moisture content was zero. The dried sample was subsequently milled into fine powder 1.2 kg of which was used for the ethanolic extraction with a 9.6 % yield. A portion of the powdery sample was used for phytochemical analyses.

2.2 Animals

Forty adult male Wistar rats weighing 190 to 290 g were sourced from the Central Animal house of the College of Medicine, University of Ibadan. They were acclimatized for three weeks in a well ventilated and illuminated environment with optimal ambient temperature (26±2°C, 12 hours light / dark cycle) that was conducive for the study. The animals were fed liberally with locally sourced but standard pelletized rat feed and had unrestricted water intake.

2.3 Design of the Experiment

The confounding factors of the study were induction of liver injury and dosage of extract administered consequent upon which eight groups with five animals each were created. The details of the groups were;

(1) Normal control (NC)- liver injury not induced
(2) Liver injury (LI)- extract not administered
(3a) Low extract without liver injury (LE)
(3b) Low extract with liver injury (LEL)
(4a) Medium extract without liver injury(ME)
(4b) Medium extract with liver injury (MEL)
(5a) High extract without liver injury (HE)
(5b) High extract with liver injury (HEL)

2.4 Induction of Liver Injury

Based on empirical evidence from available literature, liver injury was induced by single intraperitoneal administration of carbon tetrachloride at a dose of 1.6mg /kg [5,6].

2.5 Conduct of the Experiments

The ethanolic extract of the plant was administered once daily via oral steel canula to the following groups- low extract (LE &LEL), medium extract (ME & MEL) and High extract (HE & HEL) at respective dosage of 200 mg/kg, 400 mg/kg and 600 mg/kg for 21 days. The normal control (NC) and the liver injury (LI) groups had only normal rat chow and water for the same period.

On day 22 of the study, venous blood was collected through intraocular puncture from the animals. The animals were subsequently sacrificed by cervical dislocation with prior light sedation for the purpose of organ harvesting. The biochemical parameters evaluated were the liver function test (Total protein plus globulin and albumen fractions; liver enzymes- alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase).Oxidative stress was evaluated by measuring the activities of antioxidants namely; Superoxide dismutase (SOD), Catalase, Malondialdehyde (MDA) and Glutathione Peroxidase (GPx).The harvested liver specimens were initially washed in buffered saline and thereafter stored in 10 % formaldehyde solution for subsequent light microscopy.

2.6 Data Analysis and Processing

The numerical aspects of the results were analyzed with Statistical Package for the Social Sciences (SPSS) version 24 and expressed as percentages, means plus standard deviation of means (SD). The student t- test was used for inter group comparison and level of significance was set at $p<0.05$.

3. RESULTS

3.1 Phytoconstituent Analysis

Only cardiac glycosides were not detected in the sample of V.*agnus castus* used for the study. Alkaloids, terpenoids and flavonoids constituted about 25% each of the phytochemicals of the plant, while anthraquinones, tannins, steroids and phenol accounted for less than 10% (Table 1).

3.2 Organ Weights

The mean liver weight was highest in the HEL group, this was significantly higher than those of its control (HE) and the non-injured, non-extract group (NC). (Table 2)

3.3 Biochemical Parameters

The mean serum total protein of all the groups were significantly higher than that of the NC

group. However, only the medium dose extract liver-injured group (MEL) had a significantly higher total protein than its corresponding control (ME). The albumin levels of groups liver-injured without extract (LI), low dose extract (LE), low dose extract with liver injury (LEL) and ME were significantly lower than that of the normal control (NC). However, the HEL group had a significantly higher level of serum albumin than those of groups HE, MEL and LEL. The globulin levels were essentially similar across the groups. Only the albumin / globulin ratio of the ME group was significantly lower than that of the control (NC). The serum aspartate amino transferase levels of the LEL, ME and MEL groups were significantly lower than that of the control. However, the aspartate amino transferase of the HEL was significantly higher when compared with those of the LEL and MEL. The serum alanine amino transferase level of the control group was significantly higher than those of the LEL, ME and MEL groups. While the HEL group had a significantly higher alanine amino transferase level than those of the LEL, ME and MEL groups. The alkaline phosphatase levels in all the experimental groups were significantly depressed when compared with the control. Amongst the experimental groups, the serum alkaline phosphatase level was significantly raised than those of the LEL, and MEL groups (Table 2).

3.4 Oxidative Stress Parametric Evaluation

The glutathione (GSH) activities of all the experimental groups were lower than that of the control (NC); those of significance were groups LE, HE and HEL. However, the glutathione peroxidase (GPX) activity of the control was significantly lower to those of groups LEL, ME, MEL and HEL. Amongst the experimental groups, only the low dose extract with liver injury group (LEL) had significantly different GPX activity from its non-injured counterpart (LE).The control group expressed a significantly higher catalase activity than the LEL and HEL groups however, that of ME was higher than those of control, LEL, MEL, HE and HEL. Also the high dose extract group had a significantly higher catalase activity than its liver injured counterpart (HEL). The superoxide dismutase (SOD) activity of the control was significantly lower to those of LE and LEL, while that of LE was significantly higher than those of LI, MEL and HE. Also the SOD activity of the LEL group was significantly higher than those of MEL, HE and HEL groups. For malondialdehyde (MDA), its activity in group LI was significantly higher than those of groups NC, LEL, ME, MEL, HE and HEL (Table 3).

3.5 Histopathology

Histopathology of the liver showed preservation of the liver architecture with normal hepatocytes in all the groups. However, some hepatocytes had reduced cytoplasmic content as suggested by the observed vacuolation (Plate 1).

4. DISCUSSION

Although the high extract dose liver injured group (HEL) had significant liver enlargement as evidenced by its mean liver weight being significantly greater than that of the control, the relative liver weight did not show any significant difference across the groups. Hepatic enlargement is one of the clinical features of inflammatory process triggered by toxicity from drugs, food, beverages, chemicals etc.[7] From the result of the relative liver weight, one could infer that the ethanolic extract of the plant ameliorated this clinical feature at low and medium doses even in rats with pre-existing chemical poisoning of the liver.

Following an acute insult or injury to a tissue, structure or organ; an immediate inflammatory response usually occurs with increased extravasation of leukocytes and their derivable cells such as macrophages and phagocytes. Prominent cells of the liver include hepatocytes, kupffer cells, stellate cells, macrophages and lymphocytes. The hepatocytes have synthetic functions (enzymes, protein and coagulation factors) and metabolic functions (ammonia, vitamin D etc). The kupffer cells are phagocytic while the stellate cells are responsible for scar formation following injury by potentiating myofibroblasts. The hepatocellular enzymes are present in the cytosol and their quantification serves as determinant of hepatocyte integrity.

Following irreversible hepatic injury, death of hepatocytes could occur either by necrosis or apoptosis. In hepatocyte necrosis, there is swelling of the cell with consequent release of the cytosol enzymes into the extracellular fluid this results in the elevation of the serum levels of the hepatic enzymes. The mean serum levels of aspartate amino transferase (AST) of the low and medium doses extract groups with liver injury (LEL & MEL) and medium extract without liver

injury (ME) were significantly lower than the control value while those of the high extract dose with liver injury (HEL) and lower dose extract without injury (LE) were higher but not significant. This observation may suggest that the ethanolic extract of *V. angus castus* is capable of reducing hepatocyte necrosis following injury at low and medium doses but potentiates it at higher dose. Our results also showed that the serum AST of HEL group was significantly higher than those of LEL and MEL groups; this reinforced the assertion that the ethanolic extract of *V. angus castus* reduced the severity of hepatocyte necrosis following carbon tetrachloride poisoning.The non-injured liver extract group (LE) had a slightly elevated alanine amino transferase (ALT) than the control. However, the mean ALT values for LEL, ME and MEL were significantly lower than the control. Also the HEL had a significantly higher ALT value than that of the MEL.The pattern for alkaline phosphatase levels across the groups was similar to those of AST and ALT. As previously mentioned these three enzymes are normally located in the cytosol of the hepatocytes. The values of these enzymes provide the evidence that the ethanolic extract of *V.angus castus* was capable of reducing or reversing the hepatocyte necrosis following CCL_4 administration.

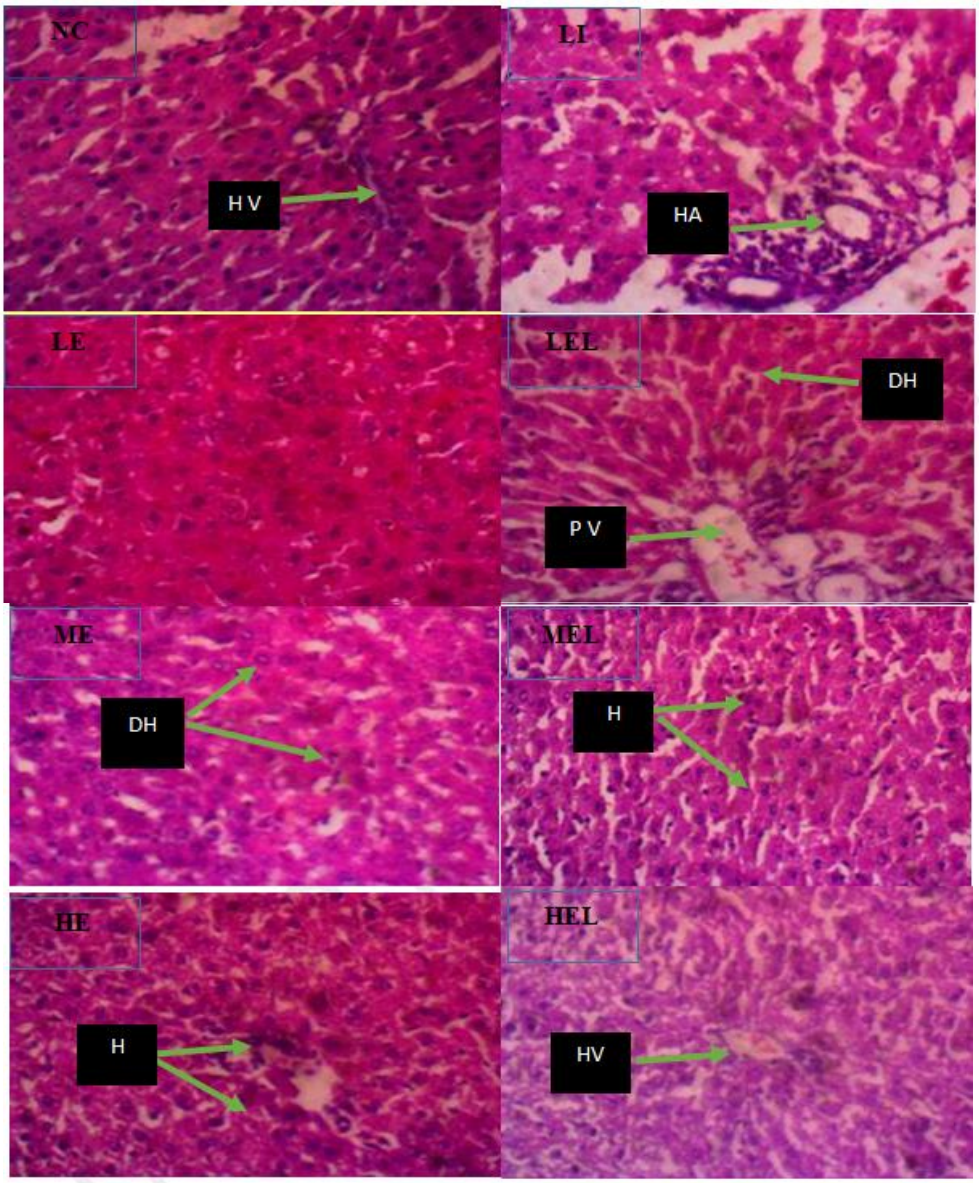

Plate 1. Photomicrographs obtained from the Liver specimens of the groups (H & E x 400).
*Legend: **H**- hepatocytes,DH-Degenerated hepatocytes**HV-**Hepatic vein, **PV**- Portal vein and **S**- Sinusoid*

Table 1. Phytochemical components of *Vitex agnus castus*

Saponins	Tannins	Flavonoids	Cardiac glycosides	Anthraquinones	Terpenoids	Steroids	Alkaloids	Phenol
+	+	+	-	+	+	+	+	+

+ (present) and – (absent)

Table 2. Liver function parameters (mean values)

Parameter	NC	LI	LE	LEL	ME	MEL	HE	HEL
Liver weight (g)	$6.35\pm0.41^{\alpha}$	5.58±0.99	7.1±0.01	6.93±0.72	7.2±0.63	7.44±0.88	$6.1\pm0.27^{\beta}$	$8.52\pm0.85^{\alpha\beta}$
Relative liver weight (g/100g body wt)	2.6±0.03	2.4±0.07	3.3±0.00	2.92±0.04	3.16±0.04	2.62±0.05	3.05±0.02	3.04±0.04
Total Protein (g/dl)	6.7 ± 3.4^{A}	7.7 ± 0.3^{A}	7.8 ± 0.0^{A}	7.6 ± 0.2^{A}	7.4 ± 0.3^{AB}	8.0 ± 0.4^{AB}	7.8 ± 0.6^{A}	8.3 ± 0.5^{A}
Albumin (g/dl)	3.3 ± 0.2^{C}	3.0 ± 0.2^{C}	3.0 ± 0.0^{C}	2.7 ± 0.2^{CD}	2.8 ± 0.2^{C}	3.2 ± 0.3^{D}	2.9 ± 0.5^{CD}	3.5 ± 0^{D}
Globulin (g/dl)	4.9±0.2	4.8±0.3	4.8±0.0	4.8±0.2	4.6±0.4	4.8±0.4	4.9±0.2	4.8±0.1
Alb/Glo ratio	0.7 ± 0.5^{E}	0.6±0.1	0.6±0.0	0.5 ± 0.5^{E}	0.6± 0.1	0.6± 0.1	0.6± 0.1	0.7±0.7
Aspartate aminotransferase (u/l)	46.2 ± 2.2^{F}	45.4± 1.5	47.0±0.0	42.5 ± 1.7^{FG}	42.8 ± 2.2^{F}	43.0 ± 1.6^{FGH}	46.2 ± 2.3	48.3 ± 2.1^{GH}
Alanine amino transferase (u/l)	34.6 ± 1.7^{J}	32.8± 2.0	35.0±0.0	30.8 ± 2.2^{JK}	30.8 ± 1.0^{J}	32.0 ± 0.7^{JL}	34.8± 1.5	35.5 ± 1.3^{KL}
Alkaline phosphatase (10^2u/l)	1.2 ± 0.0^{M}	1.1 ± 0.1^{M}	1.2± 0.1	1.1 ± 0.1^{MN}	1.1 ± 0.0^{M}	1.1 ± 0.1^{MP}	1.1 ± 0.1^{M}	1.2 ± 0.1^{NP}

$^{\alpha,\beta}$; A,B ; C,D ; E ;F,G,H ; J,K,L; M,NP. Values with significant difference with regards to the Liver weight, Total protein, Albumin, Albumin / Globulin ratio, Aspartate amino transferase, Alanine aminotransferase and Alkaline Phosphatase respectively.

Table 3. Antioxidant parameters (mean values)

	TP (g/dL)	GSH (µmol/mg)	GPX (µmol/mg	CAT (µmol/mg)	SOD (µmol/mg)	MDA (µmol/mg)
NC	6.7±3.4	5.3±0.7^{a}	3.7±0.3^{a}	2.2±0.4^{a}	0.3±0.01^{a}	0.3±0.01^{a}
LI	7.7±0.3	3.9±0.2	5.2±0.1	2.5±0.8	0.5±0.15^{b}	0.7±0.01^{abc}
LE	7.8±0.0	3.3±0.1^{a}	5.6±0.1^{b}	1.9±0.2	1.2±0.2^{ab}	0.4±0.02
LEL	7.6±0.2	4.1±0.7	7.1±0.6^{ab}	0.6±0.2^{ab}	0.7±0.2^{acd}	0.1±0.02^{bc}
ME	7.4±0.3	4.1±0.9	5.7±0.3^{a}	3.6±0.6^{ab}	0.5±0.1	0.1±0.01^{bc}
MEL	8.0±0.4	4.1±0.5	6.3±0.4^{a}	1.9±0.1^{b}	0.2±0.01^{bcd}	0.1±0.04^{bc}
HE	7.8±0.6	3.2±0.9^{a}	5.3±0.8	2.5±0.9^{bc}	0.2±0.01^{bcd}	0.1±0.02^{bc}
HEL	8.3±0.5	3.4±0.2^{a}	6.8±1.4^{a}	0.6±0.3^{abc}	0.4±0.02^{c}	0.1±0.03^{bc}

Legend: Total protein (TP), Glutathione (GSH), Glutathione peroxidase (GPX), Catalase (CAT), Superoxide dismutase (SOD) and Malondialdehyde (MDA). Values with superscript "a" are of significant difference from the control (NC) of the respective parameter. While experimental groups with same superscript had significant difference in the values of the respective parameter.

In the absence of pre-existing chronic disease, following acute liver insult, the synthetic functions of the liver are usually unaltered this is due to the large reserve of the hepatocytes. Thus the plasma levels of total protein and its components ie albumin and globulin of the experimental groups would have been expected to be similar to those of control. The results of our study contradicted this assumption as all the liver injured groups had significantly higher plasma total protein levels than the control. A plausible explanation for this contradictory observation was leakage of protein from the dead hepatocytes into the blood. The liver-injured high and medium dose groups had higher total plasma protein level than their respective non-injured group (HEL vs HE and MEL vs ME). For plasma albumin, the liver injured (LI), low extracts (LE & LEL), medium and high dose non-injured groups (ME & HE) had significantly lower levels than the control while the liver-injured medium and high doses groups (MEL & HEL) had albumin level similar to that of the control. This might suggest the ability of the ethanolic extract of *V.angus castus* at reducing necrosis of hepatocytes following acute liver insult. The globulin level across the experimental groups was essentially similar to that of the control. Although there are other specific proteins in the plasma besides albumin and globulin; these two account for over 90 % of blood protein, hence their quantification is a reliable index of hepatic synthetic function.

Reactive oxygen species (ROS) are a group of highly reactive oxygen containing chemicals that are generated during the various metabolic processes. They are of two broad types namely free oxygen radicals such as superoxide andhydrogen peroxide; and non- radical ROS [8]. The intracellular level of ROS is kept under control by antioxidants such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase. A disruption of this oxidant-antioxidant equilibrium results in oxidative stress [9] which plays pivotal role in diverse human pathologies [10]. This may be through cell necrosis, apoptosis or production of degradative enzymes [7].

Our results showed the occurrence of oxidative stress sequel to chemical induced liver injury as evidenced by significant differences in enzymatic activities of antioxidants between the control and experimental groups. For some, the control was higher (GSH, SOD and MDA) while in some groups, the experimental were higher (GPX, CAT and SOD). Glutathione has been described to be the most ubiquitous antioxidant powerfully protecting cells against oxidative injury [11] and it has been reported to be present in large quantity in the liver [12]. Also, acute stress in rodents had been documented to increase oxidative stress and decreased activities of GSH, CAT and SOD [13]. Irrespective of the pattern of difference in the level of enzymatic activities, an inference that could be drawn was the existence of disequilibrium between oxidation-antioxidation dynamics. Also the administration of the extract did not appear to confer any ameliorating effect as there was no reasonable difference in the enzymatic activities of same-dose extract pair. Histopathological examination of liver sections from all the groups revealed the preservation of the liver micro architecture with relatively abundant hepatocytes. Hepatic necrosis as evidenced by the vacuolation seen in some hepatocytes from some of the experimental groups especially the medium and high –dose groups was also observed but noted to be

minimal. Severity of hepatic degeneration is dependent upon the degree of the insults and its duration. Thus the mild hepatic degeneration observed in this study was likely to be due to the duration of the study. Also this minimal hepatic degeneration might suggest the ability of *V. angus castus* extract at reducing the severity of cellular necrosis following acute liver insult. One of the early events following insult to the liver is inflammation which induces the activation of Kupffer and hepatic stellate cells. This cellular activation leads to production of pro-inflammatory cytokines with resultant liver damage [14]. Although cytokines quantification was not done in this study, reducing production of cytokines may be a plausible mechanism of action of *V.angus castus* extract in ameliorating severity of chemical- induced liver damage. Flavonoid containing plants have been documented to ameliorate carbon tetrachloride induced liver injury via many pathways that include free radical scavenging [15]. Phytochemical analysis of *V. angus castus* in this study revealed the presence of flavonoid thus scavenging of free radicals might be a plausible mechanism via which the plant under investigation ameliorated the severity of the acute liver damage.

5. CONCLUSION

The structural and biochemical alterations occasioned by chemical (carbon tetrachloride)-induced hepatic injury were of reduced severity following post-injury administration of ethanolic extract of leaves of *Vitex angus castus* plant. The plant may thus have role in the management of non-traumatic liver injury. This is a natural growing plant thus accessibility will not be a hindrance in its utilization in complementary and alternative medicine.

CONSENT

It is not applicable.

ETHICAL APPROVAL

In the conduct of this study, the animals were handled in accordance to the guidelines as prescribed by the ethical conduct of animal research of the University of Ibadan. Also, the principles of laboratory animal care as contained in the 8th edition (2011) of the Guide for the Care and Use of Laboratory Animals by the National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals were observed [16].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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