Phyllantus amarus aqueous extract as antidote to alcoholic liver injury

ABSTRACT

Objective: In most human communities, the consumption of alcoholic beverages is unregulated. The liver is one of the major organs that bear the brunt of regular and or heavy consumption of alcohol. This study set out to elucidate the modulation of alcohol induced liver injury in wistar rat by aqueous extract of *Phyllanthus amarus* plant.

Methodology: Five groups with six animals each were used for the study. Group CN was the control. The alcohol only group (ALC) had 1ml / 100g body weight (b.w) of 43% ethanol. The Extract only group (EXT) had 200mg/ Kg b.w of *P.amarus* aqueous extract. The Low Extract plus Alcohol group (LEA) had concomitant administration of 1ml/100g b. w of 43% ethanol with 200 mg/ Kg of the extract. The High Extract plus Alcohol group (HEA) had concomitant administration of 1ml/100g b. w of 43% ethanol and the extract at 400 mg/ Kg. The alcohol and extract were administered once daily for fourteen days. Thereafter, blood samples were collected for biochemical analyses, the animals sacrificed and livers harvested for histopathological analyses.

Results: Group HEA had the highest mean body weight. The mean liver weight of group EXT was significantly higher ($p<0.05$) than those of other groups. Both the total protein and its globulin fraction of the ALC group were significantly lower($p>0.05$)than those of others. The liver enzymes (Alanine and Aspartate transaminases) levels were significantly low in the ALC group. However, those of the LEA and HEA groups were comparable with the EXT group. The glutathione peroxidase superoxide dismutase activities of the LEA and HEA groups were significantly higher than that of ALC. Lipid peroxidation was most severe in the ALC group as evidenced by the significantly high malondialdehyde level. Histopathological sections of the liver revealed preserved hepatic architecture with pronounced steatosis in the ALC group.

Conclusion: Aqueous extract of *Phyllanthus amarus* considerably reduced the severity of alcohol induced liver injury.

Key words: Phyllantus amarus, alcohol, liver injury

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INTRODUCTION

Excessive alcohol consumption is a global healthcare problem [1]. According to the World Health Organization, the total alcohol per capita consumption globally in people aged 15 years and above rose from 5.5 litres of pure alcohol in 2005 to 6.4 litres in 2010 and remained same as of 2016. The highest levels of per capita alcohol consumption are observed in countries of the WHO European Region.

Whereas in the WHO African Region, the Region of the Americas and the Eastern Mediterranean Region alcohol per capita consumption remained rather stable, in the European Region it decreased from 12.3 litres in 2005 to 9.8 litres in 2016.

The increase in per capita alcohol consumption is observed in the WHO Western Pacific and South-East Asia regions.

Current drinkers consume on average 32.8 grams of pure alcohol per day, and this is some 20% higher (40.0 g/day) in the African Region and about 20% lower (26.3 g/day). in the South-East Asia Region. Drinkers increased their alcohol consumption since2000 in almost all regions except the WHO European Region.

One quarter (25.5%) of all alcohol consumed worldwide is in the form of unrecorded alcohol – i.e. alcohol that is not accounted for in national official statistics on alcohol taxation or sales as it is usually produced, distributed and sold outside the formal channels under governmental control.

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Worldwide, 44.8% of total recorded alcohol is consumed in the form of spirits. The second most consumed type of beverage is beer (34.3%) followed by wine (11.7%). Worldwide there have been only minor changes in beverage preferences since 2010.

Prevalence of heavy episodic drinking (defined as 60 or more grams of pure alcohol on at least one occasion at least once per month) has decreased globally from 22.6% in 2000 to 18.2% in 2016 among the total population, but remains high among drinkers, particularly in parts of Eastern Europe and in some sub-Saharan African countries (over 60% among current drinkers).

Worldwide, 26.5% of all 15–19-year-olds are current drinkers, amounting to 155 million adolescents. Prevalence rates of current drinking are highest among 15–19-year-olds in the WHO European Region (43.8%), followed by the Region of the Americas (38.2%) and the Western Pacific Region (37.9%).

Until 2025, total alcohol per capita consumption in persons aged 15 years and older is projected to increase in the Americas, South-East Asia and the Western Pacific.

In 2016, the harmful use of alcohol resulted in some 3 million deaths (5.3% of all deaths) worldwide and 132.6 million disability-adjusted life years (DALYs) – i.e. 5.1% of all DALYs in that year. Mortality resulting from alcohol consumption is higher than that caused by diseases such as tuberculosis, HIV/AIDS and diabetes.

In 2016, of all deaths attributable to alcohol consumption worldwide, 28.7% were due to injuries, 21.3% due to digestive diseases, 19% due to cardiovascular diseases, 12.9% due to infectious diseases and 12.6% due to cancers. About 49% of alcohol attributable DALYs are due to non-communicable and mental health conditions, and about 40% are due to injuries [1].

The liver being the primary site of ethanol metabolism bears the brunt of heavy alcoholic consumption. Sequelae of hepatic liver injury include steatosis (fatty liver), hepatitis, cirrhosis and carcinoma [2,3]. Unfortunately, liver cirrhosis and cancer are end stage pathologies with the available management options being ameliorative rather than curative.

Phyllanthus amarus of the family of *Euphorbiaceae* is a small and erect perennial plant that grows largely as weed. Quite a large number of phytochemicals are attributable to its leaves, stem and roots with a wide range of health importance [4]. It is known as Eyin òlòbè in South West Nigeria and have other native names in other geo political zones of Nigeria. Herbal remedies of *P.amarus* may be of importance in the management of alcoholic liver diseases hence the justification of this study.

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MATERIALS and METHODS

2.1Plant Materials

2.1.1 Plant collection and authentication

Fresh samples of *Phyllanthus amarus* plant were harvested from Government Forest Reserve in Ado-Ekiti, South West Nigeria and authentication was at the Herbarium Unit of the Department of Botany, University of Ibadan, Nigeria.

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2.1.2Extract preparation

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The aerial portion of *P. amarus* was used for the aqueous extraction. After initial washing under potable running water, 600 g of the plant was room dried at an ambient temperature of 26^{o} C two weeks and subsequently grinded into fine textured powder. This was use used to produce an aqueous extract of 55.01 g giving a 9.2 % yield.

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2.2 Animals

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Thirty adult Wistar rats with a weight range of 100-150 g were sourced from the Research Animal Holding facility of the College of Medicine, University of Ibadan. They were acclimatized for two 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.

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2.3 Design of the Experiment.

The determinants of group allotment were alcohol and dose of the plant extract administered. Consequently, five groups with 6 animals each were used for the experiments. Details of the groups are as stated below;

(1) Control (CN)- neither alcohol nor *P.amarus* extract was administered.
(2) Alcohol only (ALC)
(3) Extract only (EXT)
(4) Low Extract plus Alcohol (LEA).
(5) High Extract plus alcohol (HEA).

2.4 Conduct of the Experiments

The control group had normal rat feed and water while the Alcohol group had 1ml / 100g body weight (b.w) of 43% ethanol. The Extract only (EXT) group had 200mg/ Kg b.w of *P.amarus* aqueous extract. The Low Extract plus Alcohol (LEA) group had concomitant administration of 1ml/100g b. w of 43% ethanol with 200 mg/ K b. w of aq. extract of *P.amarus*.

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The High Extract plus alcohol (HEA) group had concomitant administration of 1ml/100g b. w of 43% ethanol with 400 mg/ Kg b. w of aq. extract of *P.amarus*. The alcohol and extract were administered once daily for fourteen days via oral steel cannula.

On day 15, venous blood was collected through intraocular puncture from the animals. Thereafter, they were sacrificed by cervical dislocation with prior light sedation for the purpose of organ (liver) harvesting. Both the blood samples and liver homogenates were used for the biochemical analyses and oxidative stress parameters. The liver function tests were-Total protein plus globulin and albumen fractions; liver enzymes- alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase. The oxidative stress was evaluated by the determination of the activities of antioxidants namely; Superoxide dismutase (SOD), Catalase, Malondialdehyde (MDA) and Glutathione Peroxidase (GPx). Portions of the harvested liver specimens were initially washed in buffered saline and thereafter stored in 10 % formaldehyde solution for subsequent light microscopy.

2.5 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

3.1Quantitative parameters

The mean body weight dropped significantly in the alcohol associated groups at the termination of the study this was most evident in the Alcohol only group. The group that received 400mg/kg of the *P. amarus* aqueous extract (HEA) had the highest mean body weight. The mean liver weight of the control group was significantly higher than those of the Alcohol only and Low extract dose groups but lower than that of Extract group. Samples from both the serum and the liver homogenate in the ALC group were significantly lower in total protein with reference to the control. However, no significance difference was observed amongst the other groups. The albumin fraction was similar in all the groups. The Extract only group had the highest mean serum globulin fraction and significantly higher than those of the other groups. While the

globulin fraction obtained from homogenized liver was highest in the Control group and even significantly higher than those of other groups. The ALC group had the least mean globulin fractions. The liver enzymes (Alanine and Aspartate transaminases) levels were significantly low in the ALC group. However, those of the LEA and HEA groups were comparable with the EXT group. The serum Alanine transaminase level of the ALC was significantly lower than those of other groups while value obtained from homogenized liver sample of the ALC was significantly higher than those of others. The Aspartate transaminase values were of similar pattern to those of the alanine transaminase. The glutathione peroxidase (GPX) and superoxide dismutase activities of the LEA and HEA groups were significantly higher than that of ALC. Lipid peroxidation was most severe in the ALC group as evidenced by the significantly high malondialdehyde level.

The GPX activities as measured from the serum and homogenized liver of the ALC group were significantly lower than those of the other groups respectively. The catalase of the ALC was significantly higher than those of the other groups. The catalase activities of Extract only, LEA and HEA were significantly lower than that of the control. The superoxide dismutase (SOD) activity of the control group (as determined from serum) was significantly higher than those of the other groups while the activity for the ALC group was significantly lower than others. While SOD activity as estimated from homogenized liver of the LEA group was significantly higher than the other groups. There was no significant difference in the extent of lipid peroxidation amongst the groups as evidenced by the malondialdehyde activities (Table 1).

Histopathological examinations of the sections of the liver samples from the group revealed preserved liver architecture with cytoplasmic fatty infiltration of hepatocytes in some of the groups. This steatosis was most pronounced in the Alcohol only group but very mild in the High extract alcoholic group. Considerable number of macrophages were observed in the liver specimen obtained from the alcohol group. (Plate 1).

Table 1: Morphological, biochemical and antioxidants parameters (mean values)

PARAMETERS	CONTROL	ALC ONLY	EXTRACT ONLY(EXT)	LEA	HEA
Total Body Weight (g)	105.1 ± 3.86	110.8 ±2.72	117.1 ±1.45	112.2 ± 1.77	127.5 ± 3.19
Liver weight(g)	3.90 ± 0.30	3.50 ± 0.20	5.34±0.26	2.90 ± 0.08	3.73 ± 0.21
Total protein (g/dl)	5.14 ±0.84*	0.51 ±0.87*	6.93 ±0.00*	5.82 ± 1.42*	6.19 ± 1.03*
	27.8 ± 3.59^{+}	5.08 ± 0.18^{+}	24.2 ± 1.08^{+}	24.1 ± 0.95^{+}	24.2 ± 0.71^{+}
Albumin (g/dl)	2.58±0.17*	2.43±0.04*	2.41±0.02*	2.41±0.02*	2.52±0.04*
	2.27 ±0.03^{+}	2.32±0.09^{+}	2.08±0.02^{+}	2.27±0.11^{+}	2.40±0.081^{+}
Globulin (g/dl)	44.8 ±12.6*	29.1 ±6.55*	66.8 ±0.01*	40.1± 10.8*	59.4 ± 10.4*
	13.4 ± 0.13^{+}	3.77 ± 0.88^{+}	4.12 ± 1.57^{+}	4.53 ±0.79^{+}	6.22 ± 1.10^{+}
Alanine Transaminase	97.3 ± 5.95*	57.3 ±3.54*	93.3 ±1.54*	86.8± 2.65*	88.1 ±4.48*
(U/L)	27.1± 0.27^{+}	88.9 ± 1.08^{+}	48.6 ± 1.87^{+}	43.7 ± 2.88^{+}	49.1 ± 0.99^{+}
Aspartate transaminase	165 ±2.27*	137 ± 2.09*	174 ± 2.24*	172 ± 3.74*	167 ± 1.55*
(U/L)	122 ± 20.2^{+}	144 ± 14.6^{+}	137 ± 7.96^{+}	128 ± 9.09^{+}	131 ± 17.5^{+}
Glutathione Peroxidase	70.7± 2.94*	27.2 ±7.29*	78.1 ±13.4*	88.0 ± 3.85*	81.0 ± 9.01*
(U/mg Protein)	0.08±0.01^{+}	0.37± 0.02^{+}	0.09 ±0.00^{+}	0.09±0.00^{+}	0.09±0.00^{+}
Catalase (µM/min/mg	19.7 ± 3.58*	36.1 ±10.9*	1.80 ±0.20*	2.06±0.457*	2.14 ± 0.32*
Protein)	0.13 ±0.00^{+}	0.05 ±0.04^{+}	0.002±0.00^{+}	0.09 ±0.06^{+}	0.02 ±0.01^{+}

Superoxide Dismutase	135 ±0.44*	63.3 ±12.7*	108 ± 12.0*	79.6 ± 8.09*	73.3 ± 1.33*
(µmg /protein)	36.18±10.02^{+}	50.67±15.00^{+}	39.73±13.66^{+}	77.33±6.13^{+}	50.67±29.09^{+}
Malondialdehyde	0.18 ± 0.05*	0.20 ± 0.06*	0.07 ± 0.01*	0.04 ± 0.01*	0.08 ± 0.01*
(Mmol/L)	0.98 ± 0.11^{+}	9.39 ± 0.49^{+}	0.16 ± 0.08^{+}	0.14 ± 0.01^{+}	0.88 ± 0.21^{+}

Legend: ALC (Alcohol only). **EXT** (Extract only @200g/Kg. **LEA** (Low dose extract-200g/Kg). **HEA (**High dose extract-400g/Kg). *Blood serum as sample. $^{+}$Liver homogenate as sample.

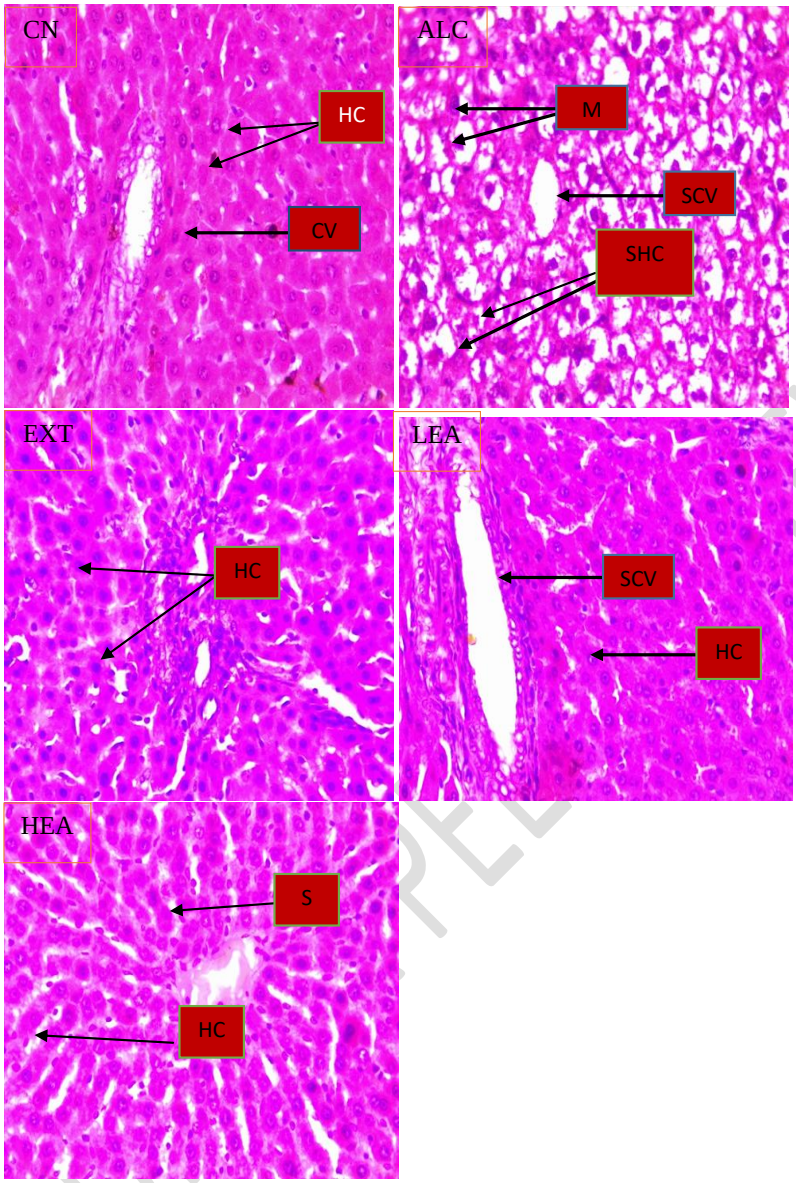

PLATE 1: Photomicrographs of the Liver specimens (H & E x 400).
Legend: **HC**- hepatocytes, **CV**- Central vein, **M**-Macrophages, **SHC**- Hepatocyte with steatosis, **SCV**- Central vein with steatosis. and **S**- Sinisoid

4. DISCUSSION

The affectation of the body weight by alcohol consumption depends on several factors such as frequency and volume of consumption. For people of low to moderate frequency and volume, there will be increased appetite with consequent weight gain. For high frequency and volume consumers, there is usually associated altered sensorium such as lethargy, somnolence and comatose state. Alcoholics in this category invariably develop progressive weight loss arising from reduced food intake, anorexia or increased production of catabolic metabolites. Chronic heavy alcoholics have increased tendency of overweight or obesity manifesting as higher body mass index and increased waist circumference [5,6]. However, some clinical studies have shown that moderate drinking assists in the maintenance of normal body weight with lower prevalence of obesity [7,8]. In both human and animal models, chronic heavy drinking has been shown to induce pancreatic β-cell dysfunction [9,10]. This may be the mechanism via which it increases the body weight. The duration, frequency and amount of alcohol used in this study might explain the higher body weights in the four experimental groups relative to the control. The HEA group that had the double dose of *P.amarus* extract had the overall highest mean body weight. This observation could infer that the plant has anabolic property that may result in weight gain. In human subjects classified as binge drinkers they drink large quantity of alcohol within a short duration usually less than two hours, weight gain may not be obvious on short term basis [11]. The ability of alcohol to shrink the liver was evident in the pattern of the mean liver weight as all the three alcoholic groups namely ALC, LEA and HEA had lower liver weight than that of the control. However, the Extract only group had significantly higher liver weight than the others. In humans, one of the sequelae of chronic alcoholic abuse is liver cirrhosis which is characterized by progressive loss of liver parenchymal due to fibrosis. The liver weight results of this study thus provided additional evidence of negative impact of alcohol on the liver. The protein –building capability of *P.amarus* was evident from the serum total protein levels of the extract only and HEA groups being significantly higher than that of the control and that of the Alcohol only being markedly depressed. The serum levels of the albumin fraction were similar across the groups while those of the globulin fraction followed the total protein pattern. This might be due to the immunologic function of globulin being much more diverse than the plasma oncotic pressure function of albumin. This might explain the compromised immune status usually seen in patients with alcoholic liver diseases. The ability of alcohol to depress the synthetic function of the liver was evident from the results of the two liver enzymes-ALT and AST that were significantly low in the ALC group. The serum levels of ALT and AST of groups LEA and HEA were similar to those of the control thus aqueous extract of *P.amarus* was able to reverse the depressive action of alcohol on liver enzyme synthesis. Both ALT and AST are contained within the hepatocytes thus high blood levels of these enzymes are indicative of necrosis of hepatocytes. In humans, alcohol is said to cause direct toxic injury on the liver leading to necrosis of hepatocytes with consequent elevated levels of the enzymes. The results of ALT and AST in this study appeared contrary. Could this be due to quantity of alcohol, duration of administration? Also, could this imply that the magnitude hepatocyte necrosis was very mild or not severe enough? The values of liver enzymes are known sensitivity and specificity for alcoholic liver diseases as they are also altered in non-alcoholic fatty liver disease and ALT has been reported to have the least sensitivity amongst them [12]

Glutathione peroxidase (GPx) is the most ubiquitous antioxidant powerfully protecting cells against oxidative injury [13] and is present in large quantity in the liver [14]. Alcohol causes oxidative stress as evidenced by the glutathione peroxidase value of the ALC group that was significantly lower than that of the control. The GPx levels of LEA and HEA groups were significantly higher than that of the control thus it would not be out of place to conclude that

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aqueous extract of *P.amarus* is an antioxidant. In this study, alcohol generated superoxide as evidenced by the significantly depressed activity of superoxide dismutase (SOD) of the ALC being significantly lower than that of the control and the extract only groups. However, its activity in the LEA and HEA was not as depressed in the ALC. Thus the aqueous extract of *P.amarus* appeared to offer protection against oxidative cellular and tissue damage. Sodium dismutase is one of first line antioxidants and the most powerful and slows down the ageing process in humans [15]. It has been documented that over expression of SOD protects against oxidative damage thereby extending life span [16,17] while a decreased activity makes cells more vulnerable to oxidative stress with resultant mortality [18].

Catalase is an enzyme that mediates the breakdown of hydrogen peroxide (an oxidant) to water and oxygen. The decomposition of hydrogen peroxide by catalase proceeds according to first-order kinetics, the rate being proportional to the hydrogen peroxide concentration [19,20]. Thus high levels connote high tissue oxidation activity. In fact, catalase deletion has been reported to promote prediabetes phenotype in mice by increasing serum and hepatic triglycerides [21]. In this study, the catalase level of ALC was about twenty fold of those of EXT, LEA and HEA. From the foregoing it could be concluded that (i) habitual and or heavy alcohol consumption has a high risk of oxidative stress and (ii) this associated oxidative stress high risk was significantly ameliorated by concurrent administration of aqueous extract of *P.amarus*.

Malondialdehyde (MDA) is a major end-product of oxidation of polyunsaturated fatty acids and its estimation is commonly used as indicator of lipid peroxidation and oxidative stress in vivo [22]. It is highly toxic and when it interacts with DNA and proteins could be mutagenic and atherogenic [23]. The ALC had the highest level of MDA while those of Extract only, LEA and HEA were significantly lower. Thus chronic consumption of alcohol is capable of causing significant lipid peroxidation leading to increased cardiovascular morbidity and mortality. In view of the significantly low values of MDA from the extract groups, the aqueous extract of *P.amarus* is likely to be a good nutritional supplement that protects against lipid peroxidation and oxidative stress.

Histology of the liver sections from all the groups revealed the preservation of the liver architecture with abundant hepatocytes though in the alcohol group, fatty infiltration of hepatocytes was evident. This observation could be due to the total volume of alcohol administered and the duration of the study. In humans, the liver has the ability to regenerate as the hepatocytes are of the stable class of cells which have regenerative capability in response to stimuli such as injury and increased functional demand.

Oxidative modification of low density lipoprotein (LDL) stimulates LDL intracellular accumulation of lipid [24]. Thus the considerable number of macrophages seen in the sections prepared from the liver specimen of the alcohol group could explain the associated steatosis observed in the same sections. Adipose tissue can serve as a major source of proinflammatory factors that contribute to liver injury. Adipocytes have the ability to recruit immune cells such as lymphocytes and macrophages and prime them to proinflammatory cytokines such as interleukine 1β, interleukine 6, interleukine 12 and tumour necrosis factor-α thus promoting liver inflammation and eventual injury [25].

Considering the very high global mortality from hepatic diseases with about 35 million reported three years ago [26,27] ; slowing down progression from inflammation to fibrosis is crucial to reduction in the number of deaths associated with chronic liver diseases.

5.CONCLUSION

Extract of *P.amarus* markedly increased the globulin fraction of plasma protein.

P amarus extract maintained the integrity of the hepatocytes as evidenced by elevated levels of liver enzymes but depressed by alcohol.
Aqueous extract of *P.amarus* offered protection against oxidative cellular and tissue damage as evidenced by elevated level of glutathione peroxidase (the ubiquitous antioxidant) initially depressed by alcohol. Malondialdehyde, an indicator of lipid peroxidation was markedly depressed by concomitant administration of *P.amarus* aqueous extract. The precursor of liver injury is inflammation thus from the results of this study, *P.amarus* protected the liver from inflammatory process triggered by alcohol
From the foregoing, *P.amarus* as beverage or drink becomes relevant in health care. This is of paramount importance in persons with either alcohol dependency or addiction that require micro nutritional supplementation or correction.

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 [28].

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