

Research paper

The protective effect of combined leave extracts of *Gongronema latifolium* and *Nauclea latifolia* on hepatotoxicity in rats.

Effiong Grace^{1*}, Udoh Imoh¹, Mbagwu Henry², Udo Nsikan² Wilson Lucy³ Eyong Ubana⁴, Ebong Patrick⁴

¹Department of Clinical Pharmacy & Biopharmacy, Faculty of Pharmacy, University of Uyo, Nigeria.

²Department of Pharmacology & Toxicology, Faculty of Pharmacy, University of Uyo, Nigeria.

³Department of Chemistry, Akwa Ibom State College of Arts and Science, Nung Ukim Ikono, Nigeria.

⁴Department of Biochemistry, Faculty of Basic Medical Sciences, University of Calabar, Nigeria.

*Corresponding author: Grace Sylvester Effiong, Department of Clinical Pharmacy & Biopharmacy, Faculty of Pharmacy, University of Uyo, Nigeria.

Tel: *2348063222797

E-mail: graceffiong2003@yahoo.com, graceffiong2007@yahoo.com

ABSTRACT:

Objective: The protective effect of the combined ethanolic leave extracts of *Gongronema latifolium* (GL) and *Nauclea latifolia* (NL) on acetaminophen-induced liver toxicity was studied in rats in comparison with only GL or NL treatment. **Methods:** Liver marker enzymes and antioxidant status were assayed in serum using standard methods. Histopathology was also studied and Silymarin was the standard drug. **Results:** Administration of only toxicant showed that the ALT and AST levels were significantly ($P < 0.05$) increased by 3.19 and 3.01 fold respectively while the levels of total protein and albumin were decreased when compared to the normal control. Treatment with extracts of GL or NL only, GL and NL combined, and silymarin for 21 days decreased the elevated levels of the transaminases and restored the normalcy of total protein and albumin significantly and was most impacted in the combined extract treatment group, indicating a positive synergy. In only acetaminophen treated rats, catalase (CAT), glutathione Peroxidase (GPx) and superoxide dismutase (SOD) were decreased significantly but treatment with extracts of GL or NL and in combination caused a significant increase ($p < 0.05$) in these antioxidants activities. Acetaminophen treatment showed severe liver damage, pretreatment with only NL or GL showed an improvement in histological section of the liver but the protective effect was more pronounced in the combined extract treatment group against the hepatocellular damage. **Conclusions:** These results suggested that NL and GL leaves extract may have a significant role in alleviating liver damage, with the combined extracts synergistically improving the levels of these indices more, thus suggesting a better amelioration of hepatotoxicity.

Key words: Hepatotoxicity, *Nauclea latifolia*, *Gongronema latifolium*, combined extracts, Acetaminophen, Silymarin, antioxidants.

1. Introduction:

Herbal therapy is a fundamental aspect of traditional medicine that primarily uses medicinal plants preparations to prevent and improve health and for the management of ailments [1]. Most of our traditional societies in Africa and elsewhere have always used herbs and other plant parts to promote healing [2]. In the recent past, people who are sick consulted physicians for treatment, however this is gradually changing now. According to [3] more and more people around the world are turning to herbal medicine because of the following reasons: Herbal medicines are natural, potent and have minimum aftermath effect; herbal medicines are relatively cheap and affordable to many people; the medicinal plants are readily available around us and treatments with herbal medicines in most cases are long lasting or permanent. However a number of these plants are yet to be scientifically evaluated for their efficacy and safety. The consensus guidelines for research on complementary and alternative systems have recommended that traditional systems of medicine be in their intact form as clinically used (rather than as isolated components) for clinical efficacy and overall safety [4].

Gongronema latifolium (Asclaiadaceae) is a perennial edible plant with soft and pliable stem. It is widely used in the West African sub-region for a number of medicinal and nutritional purposes [5].

In Eastern states of Nigeria, the plant locally known as 'utazi' is a popular spice. The leaves are used to prepare food for mothers that have recently put to bed where it is believed to stimulate appetite, reduce post-partum contraction and enhance the return of the menstrual cycle [6].

On the other hand, *Nauclea latifolia* is from the family *Rubiaceae*. The plant is commonly known as Pincushion tree. *Nauclea latifolia* is used profusely by traditional medicine practitioners. The bark and root of *N. latifolia* are used in the treatment of malaria in Ghana [7]. The leaves are also used in the treatment of malaria in East Africa [8] and in Nigeria [9]. The crude extract of the roots have been shown to have anti-hypertensive effect [10].

Human beings are exposed on a daily basis to certain toxic chemicals and pathogens, which cause certain serious health problems. Certain chemicals and reagents that were thought to be health friendly have been proved to have serious adverse effects on health. Amongst these toxic substances is acetaminophen.

Acetaminophen, a most commonly used analgesics, effectively reduces fever and mild-to moderate pain, and is considered to be safe at therapeutic doses. However, acetaminophen overdose causes severe hepatotoxicity that leads to liver failure in both humans and experimental animals [11,12]. The concern here is the adverse effect of acetaminophen to the liver, having visualized the prominent functions of the liver for survival.

Silymarin has been used for over 20 years in clinical practice for the treatment of toxic liver diseases [13]. *Silymarin* extract from the seeds of the plant *Silybum marianum*, also called "milk thistle", has been described to be an antioxidant and exhibits anticarcinogenic, antiinflammatory, hepatoprotection and growth modulatory effects [14,15]. In this study, *silymarin* was used as a positive control against the acetaminophen-induced hepatic damage in rats.

The combination of herbs or phytochemicals from more than one source has proven to be more useful and beneficial in management of various ailments including those that seem to defile conventional medication [16], as synergistic effects among individual components are considered essential to the herbal remedy approach to treatment and prevention of diseases.

There is no evidence tendered as regards the remedial effects of the leaves of NL on liver malfunction, but GL has been reported by [17] on its protective role on hepatic toxicity in Wistar rats. In view of the attributes stated for the two plants above and their antioxidant status [18,19], this work reposes on the *in vivo* determination of the effects of combined ethanolic extract of the leaves of *Nauclea latifolia* and *Gongronema latifolium* on hepatocellular damage.



2. Materials and methods

2.1. Drug



Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were obtained from Cayman Chemical Company, U.S.A. The routine assay Kits for total protein, albumin and the amino-transferases, were purchased from Randox Laboratories Ltd. United Kingdom, *Silymarin* was

purchased from Vellore, India, while acetaminophen (APAP) was obtained from Sigma Chemical (St. Louis, MO, USA).

2.2. Plant materials and extraction

Fresh but matured *Gongronema latifolium* leaves were collected from a cultivated land at Ibiaku Itam, Nigeria while *Nauclea latifolia* was collected in Calabar, Nigeria in March, 2012. They were authenticated by Dr. E. G. Amanke in the Department of Botany, University of Calabar, Nigeria and voucher specimen deposited in the Department of Botany herbarium, University of Calabar. The ethanol extract was prepared using the wet method of extraction according to [20].

2.3. Phytochemical evaluation

NL and *GL* leaves were subjected to quantitative analysis for various phytoconstituent like Cyanogenic glycosides determined by the alkaline picrate colorimetric method [21], saponins in which the method of [22] as described by [23] was employed, phenols was done according to the Folic- ciocaltean colorimetric method [24], tannins was determined by colorimetric method of [25], flavonoid was done using the ethyl acetate gravimetric method [25] while alkaloid determination was done using the alkaloid precipitation gravimetric method described by [21,23].

2.4. Experimental animals

Male Albino Wistar rats of (120-150g) obtained from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy of the University of Uyo, Nigeria were used. They were kept in clean cages (wooden bottom and wire mesh top), maintained under standard laboratory conditions (Temperature 25± 5°C, Relative humidity 50-60%, and a 12/12h light/dark cycle) and were allowed free access to standard diet (Vital Feed from Grand Cereals & Oil Mills Limited, Jos, Plateau State of Nigeria) and water *ad libitum*. Animals were acclimatized for 14 days in the animal house of the Department of Biochemistry, University of Calabar, Nigeria and then divided into groups for experimentation.

2.5. Experimental design

The design consisted of 36 rats divided into 6 groups of 6 rats each. Group 1 rats served as normal control and were given placebo treatment, Group II (intoxicated group) received orally a single dose of acetaminophen (200mg/kg, bw, p.o.) diluted with sucrose solution (40% w/v). Group III rats received acetaminophen (200mg/kg, bw,) and GL (400mg/kg b.w.), Group 4 rats received acetaminophen(200mg/kg, bw,) and NL (400mg/kg b.w.), Group 5 rats received acetaminophen(200mg/kg, bw,) and combined extracts of *GL* and *NL* (200mg/kg, bw, each), Group 6 rats received acetaminophen(200mg/kg, bw,) and Silymarin commercial drug (100 mg/kg, bw, p.o.) At the end of 21 days, food was withdrawn from the rats and they were fasted overnight but had free access to water. They were then euthanized under chloroform vapour and sacrificed. Whole blood was collected via cardiac puncture using sterile syringes and needles, emptied into plain tubes and allowed to clot for about two hours. The clotted blood was thereafter centrifuged at 3,000rpm for 10 minutes to recover serum from clotted cells. Serum was separated with sterile syringes and needles

and stored frozen until used. Prior to dissection, the liver tissue was perfused in heparinized saline (0.9% NaCl) to remove any red blood cells and clots and thereafter blotted with blotting paper. The tissue was then suspended in boun's fluid for histologic evaluation.

2.6. Biochemical analysis

Serum was used for the estimation of various biochemical parameters: ALT [26], AST [26], albumin [27], total protein [28] and antioxidants analysis using Spectrophotometer assay kit from Cayman Chemical Company; for superoxide dismutase (SOD) based on [29], catalase (CAT) based on [30] and glutathione peroxidase (GPx) based on [31].

2.7. Histopathological examination

Liver tissues were preserved in 10% formaldehyde solution. The tissues processed and embedded in paraffin wax. Sections of about 4-6 microns were made and stained with hematoxylin and eosin and photographed.

2.8. Statistical analysis

The results were analyzed by one-way ANOVA, using SPSS statistical package. All data were expressed as Mean $\pm$ SE and difference between groups considered significant at $p < 0.05$.

3. Results

3.1. Preliminary phytochemical investigation

The quantitative phytochemical investigation of the NL and GL leaves showed the content of alkaloids, polyphenols, flavonoids, saponins and hydrocyanic acid as shown on Table 1.

3.2. Serum biochemical parameters

Serum aminotransferase activities (AST and ALT) were raised significantly ($P < 0.05$) by 3.19 and 3.01 fold respectively in the hepatotoxic control (HC) relative to normal control (NC). Treatment with extracts of GL or NL only, GL and NL combined, and silymarin for 21 days caused a significant reduction of AST and ALT in relative to HC (Table 2).

Result of total protein levels and albumin concentration of serum of all hepatotoxic treated animals and HC recorded a significant decrease ($P < 0.05$) in comparison with NC. Also this result indicated a significant decrease in albumin of hepatotoxic animals treated with NL or GL alone in relative to the combined extract treated rats, but treatment with NL alone and in combination with GL indicated a significant increase in relative to silymarin treated rats (Table 2).

3.3. Hepatic oxidative stress parameters

Antioxidants analysis showed treatment with acetaminophen alone producing a significant decrease in GPx and SOD activities while CAT showed a significant increase implying hepatotoxicity imposed oxidative stress. Treatment with extracts of GL or NL only and in combination caused a significant increase ($p < 0.05$) in SOD, GPx and CAT activities compared to HC. The effect of silymarin treatment was similar with the GL or NL alone treatment in GPx activity but the combined extract treated group

indicated a significant increase ($p < 0.05$) compared to the silymarin treated group. CAT activity of only GL treated rats was significantly decreased when compared with both silymarin and combined extract treatment group. Treatment with NL alone also caused a significant decrease in CAT activity compared to its combination treatment with GL (Table 3).

3.3. Histopathological examination

Treatments with **toxicant** alone showed severe hepatic fatty change, sinusoidal dilation and congestion, mild periportal inflammation, fibrosis, severe feathery degeneration and necrosis. Pretreatment with only NL showed an improvement in histological section of the liver, GL treatment improving over NL and hepatotoxic control while the combined extract treatment group showed hepatocytes similar to the normal control rat's histology and compared well with silymarin treated group (FIG.1-5).

4.0. Discussion

In addition to their roles in human and animal nutrition, knowledge of phytochemical composition is fundamental to the understanding of the modes of action of medicinal plants in general. It is the diverse composition of these components in plants that places them at an advantaged position over and above chemotherapy [32]. The results of quantitative analysis of phytochemical compounds investigated in this study revealed high phenolics and flavonoids in the ethanol leaves extract of *G.latifolium* and *N.latifolia*. Several African medicinal plants have been shown to have hepatoprotective effects [33, 34, 35, 36]. Hepatoprotection is possibly due to flavonoids which exert a membrane-stabilizing action that protect the liver cells from injury [37, 38]. Some medicinal plants possess hepatoprotective effects. These effects are present because they contain some bioactive compounds [39]. The presence of saponins in a variety of herbal preparations administered to humans proved to be potent against cancer and hepatic cell proliferation [40]. All these facts about the roles of chemical constituents of medicinal plants suggest that *G.latifolium* and *N.latifolia* could exert their protective capacity on hepatocellular damage due to the presence of flavonoids and saponins.

The liver is a major target organ for toxicity of xenobiotics including drugs, because most of the orally ingested chemicals and drugs first go to liver where they are metabolized into toxic intermediates. With respect to acetaminophen dependent hepatotoxicity it is **generally accepted** that P450-dependent bioactivation of acetaminophen is a main cause of potentially fulminant hepatic necrosis upon administration or intake of lethal dose of acetaminophen [41,11].

The increased levels of ALT and AST in HC observed in this study was a clear indication of a kind of injury or the other, caused by acetaminophen toxicity [42] as hepatocellular necrosis or membrane damage leads to very high levels of serum AST and ALT released from liver to circulation and this was in consonance with the work of **[43,17]**.

There were reduced levels of ALT and AST in rats treated with the two extracts singly and in combination. This reduction was better impacted in the combined extract treatment group, indicating a

positive synergy and was similar to the study by [19], where GL ameliorated the increase in serum AST and ALT levels caused by acute CCl₄ induced hepatotoxicity *in vivo*. [43] also reported the same effect by GL which is in consonance with the present study.

Total protein and albumin concentrations were significantly decreased ($p < 0.05$) in acetaminophen treated group compared to the normal control and was similar with the report by [43]. Hence a mild impairment in hepatotoxicity could be inferred. Treatments of acetaminophen treated rats with only NL or GL extracts also caused significant decrease ($p < 0.05$) in total protein and albumin. These were modulated by the combined extracts treatment to values comparable to normal control and silymarin treated rats. Thus a holistic protection against hepatotoxicity in combined extracts treatment which is not feasible in the treatment with only GL or NL extracts has been established in this work.

In recent studies acetaminophen was found to induce substantial mitochondrial oxidative stress and peroxy nitrite formation [41]. This oxidative stress preceded cell injury by several hours [44] and free radical scavenger's attenuated acetaminophen induced liver injury [45]. This was also found in the present study as the antioxidants were significantly reduced in HC. Treatment with the extract of only GL or NL significantly ($p > 0.05$) increased the antioxidants activities indicating a possible attenuation of oxidant stress. The combined extracts administrations synergistically prevented the metabolism of acetaminophen into more toxic metabolite and minimized the production of free radicals and also boost the activities of the scavengers of free radicals, thus minimizing hepatocellular injury produced. This was so because a good liver function is inextricably linked to a good detoxification and antioxidant system, which is indispensable in ensuring optimum physiological function and prevention of diseases. The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects. Thus this could be attributed to the amelioration effects of the GL and NL leaf extracts on hepatocellular damage. The potency of these substances may have been amplified on account of increase concentration or build up of specific phytochemicals present in the combined extracts. The extracts of *GL* and *NL* may also act by either directly scavenging the reactive oxygen metabolites, due to the presence of various antioxidant compounds [46], or by increasing the synthesis of antioxidant molecules.

The cellular architecture and integrity of the hepatocytes were also examined in this study. Result of the untreated hepatotoxic rats showed that the lobular architecture was maintained, but there was also severe fatty change, sinusoidal dilation and congestion, mild periportal inflammation, fibrosis, severe feathery degeneration and necrosis (FIG. 1(a) HL). On the other hand, non- hepatotoxic control liver histology showed normal liver architecture with mild periportal oedema and conspicuous sinusoids with mild steatosis (FIG. 1(b) NHL). These were in consonance with the work of [19,43]. Treatment with the extract of NL showed improvement in histological structure of liver sections of hepatotoxic rats, pronounced in a normal appearance of liver lobules with strains of hepatocytes and comparing well with section of hepatotoxic control rat's liver (FIG.2 (a) HL). The portal areas showed portal veins still distended and engorged with blood. The hepatocytes exhibited some degree of

histological regeneration, reduction in fat accumulation, less sinusoids dilatation with decrease in number of Kupffer cells and less necrotic cells were observed. There was an improvement with GL leaves extract treatment compared to hepatotoxic control and treatment with only NL, their lobules were distinctly outlined together with the nuclei, implying an increase in activity of the cells. (FIG. 3 (a) HL) and this was similar with the report of [19]. Combined administration of the leaves extract of NL and GL to hepatotoxic rats showed hepatocytes similar to non- hepatotoxic control histology; Liver tissues showing normal architecture and prominent sinusoids and congested blood vessels (FIG. 4 (a) HL). These features were similar to those of Silymarin treated group (FIG. 5 (a) HL) and both compared well with the non- hepatotoxic control histology.

In conclusion, the results of this study demonstrate that GL and NL have a potent hepatoprotective action upon acetaminophen-induced oxidative stress and liver toxicity in rat. The hepatoprotective effect of both extract can be correlated directly with their ability to reduce activity of serum enzymes and enhance antioxidant defiance status. The findings of this study suggest that because the intervention with the extracts in combination produces a positive synergy in hepatotoxicity amelioration relative to only GL or NL, thereby showing the strongest modulatory effect against the hepatocellular damage induced by acetaminophen, the combined extracts of *Gongronema latifolium* and *Nauclea latifolia* may be used as a safe, cheap, and effective alternative chemopreventive and protective agent in the management of liver diseases.

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Table 1						
Quantitative phytochemical composition of leaves of <i>G.latifolium</i> and <i>N.latifolia</i>						
	Flavonoids (%)	Tannins (%)	Saponins (%)	Polyphenols (%)	Alkaloid (%)	Hydrocyanic acid (HCN) (%)
<i>G.latifolium</i>	0.54±0.02	2.04±0.02	0.66±0.03	0.33±0.00	1.97±0.04	13.29±0.02
<i>N.latifolia</i>	1.52±0.02	0.24±0.00	17.52±0.02	2.00 ± 0.01	7.07±0.02	13.2±0.02

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Table 2**Effect of treatment on serum Biochemical indices in non hepatotoxic and hepatotoxic rats.**

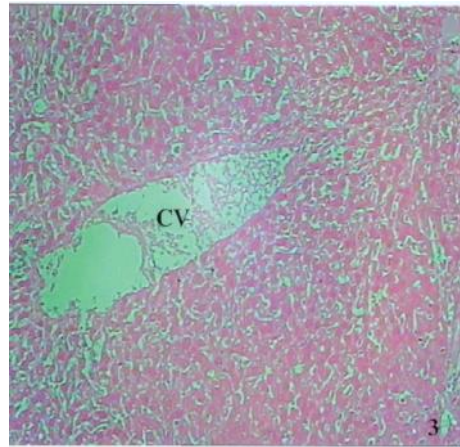
	TP(mg/L)	Alb(mg/L)	AST(U/L)	ALT(U/L)
GL _H	5.95±0.61 ^{a, b}	22.86±0.03 ^{a, b, c}	41.33±2.01 ^{a, b}	18.27±5.01 ^{a, b}
GL _{NH}	12.36±0.97 ^a	59.93±3.51 ^{a, b, c}	75.67±15.39 ^{a, b, c}	35.67±5.68 ^{a, b, c}
NL _H	7.74±0.13 ^{a, b, c}	28.71±0.08 ^{a, c}	50.33±12.23 ^{a, b}	14.33±1.38 ^{a, b}
NL _{NH}	10.31±2.03	44.84±2.30 ^{a, c}	57.33±8.34 ^{a, b, c}	49.33±2.59 ^{a, b, c}
GL +NL _H	8.65±0.08 ^{a, b}	42.10±0.11 ^{a, b}	32.67±1.17 ^{a, b}	10.00±0.73 ^{a, b}
GL+NL _{NH}	10.39±1.01	82.94±12.56 ^b	18.00±2.24	68.67±2.47 ^a
SILYMARIN _H	6.73±0.06 ^a	42.45±0.11 ^a	73.33±2.11	46.67±1.72 ^{a, b}
SILYMARIN _{NH}	12.47±0.47 ^a	57.50±8.99 ^a	12.47±0.47 ^a	57.50±8.99 ^a
HC	5.98±0.34 ^a	2.69±0.11 ^a	70.67±0.42 ^a	69.33±18.09
NC	9.24±0.92	66.05±8.38	19.17±2.52	23.00±4.02

*P<0.05 vs NC; a = P<0.05 vs HC; b = P<0.05 vs Silymarin; c = P<0.05 vs GL+NL Mean ± SE, n = 6, H = hepatotoxic rats, NH = non- hepatotoxic rats

Table 3
Effect of treatment on oxidative stress in non hepatotoxic and hepatotoxic rats.

	GPx (U/g protein)	SOD (U/g protein)	CAT (U/g protein)
GL _H	2768.51±19.78*	15.05±0.35*	531.37±27.64 ^{b, c}
GL _{NH}	7376.37±38.80* ^b	68.40±12.61	308.33±5.47
NL _H	3307.26±343.69* ^{a, b}	13.69±0.41*	475.20±50.13 ^{b, c}
NL _{NH}	7976.98±73.23 ^c	49.77±13.65	322.83±7.70
GL + NL _H	4449.59±26.48* ^{a, b}	15.69±0.60*	785.40±74.46* ^a
GL+NL _{NH}	7408.46±7.66* ^b	50.87±14.20	311.67±17.54
SILYMARIN _H	2648.67±12.47*	16.03±0.45*	884.55±7.85* ^b
Silymarin _{NH}	8016.76±97.14	54.03±14.64	337.50±8.32
HC	2720.27±33.78*	13.52±0.21*	441.47±130.95* ^a
NC	7992.09±338.28	52.56±3.96	329.67±5.18

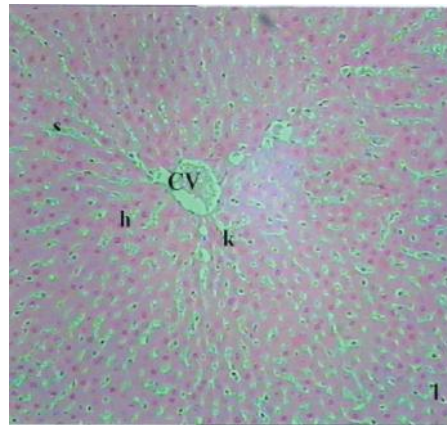
*P<0.05 vs NC; a = P<0.05 vs HC; b = P<0.05 vs Silymarin; c = P<0.05 vs GL+NL. Mean ± SE, n = 6, H = hepatotoxic rats, NH = non- hepatotoxic rats



H&E

FIG. 1 (a) HL

Section in the liver of hepatotoxic rat showing loss of the normal architecture with distended central vein (CV)

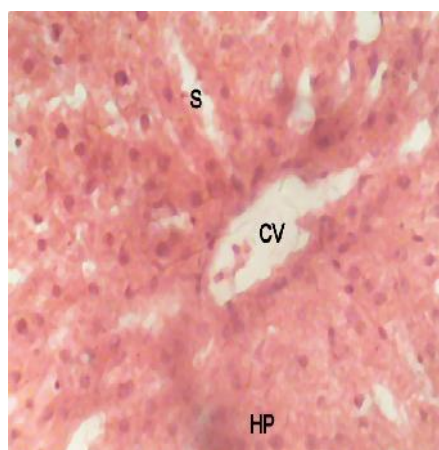


H&E

FIG. 1 (b) NHL

Section in the liver of non- hepatotoxic control rat showing hepatic cells (h), Sinusoidal spaces (s) with Kupffer cells (k) and central vein (CV)

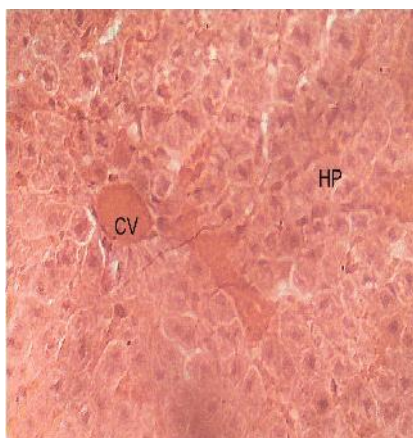
FIG. 1 (a&b): Photomicrographs of liver of hepatotoxic and non-hepatotoxic control rats given placebo (X150). H&E= Haematoxiniln and Eosin. HL = Hepatotoxic liver, NHL = non-hepatotoxic liver.



H&E

FIG.2 (a) HL

Liver tissue of hepatotoxic treated rat showing normal architecture with hepatic plates (HP), conspicuous sinusoids(S) and central vein (CV) hyperplasia



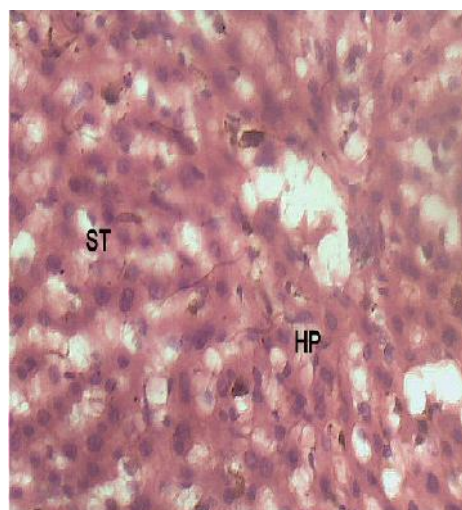
H&E

FIG.2 (b) NHL

Liver tissue showing normal hepatic plate (HP) and central vein (CV).

FIG.2 (a&b): Photomicrographs of liver of hepatotoxic and non- hepatotoxic rats treated with 400mg/kg b.w. of NL extracts (X400).

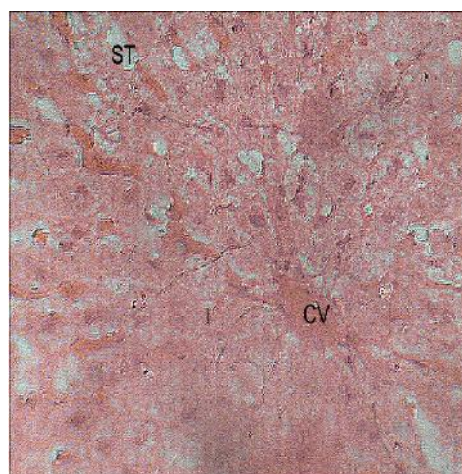
H&E=Haematoxin, HL = Hepatotoxic liver, NHL = non-hepatotoxic liver.



H&E

FIG. 3(a) HL

Liver tissue showing normal hepatic plate (HP) and moderate steatosis (ST).

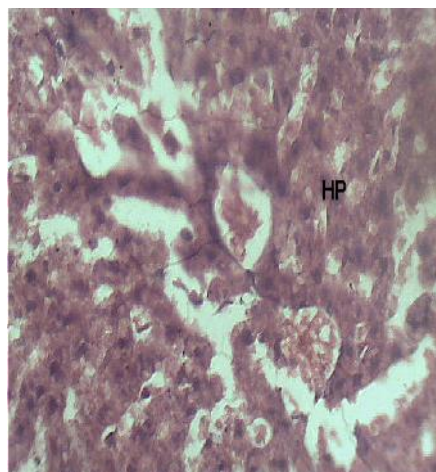


H&E

FIG.3 (b) NHL

Liver tissue showing central vein (CV) and hepatocytes radiating from the central vein. The hepatocytes show prominent steatosis (ST).

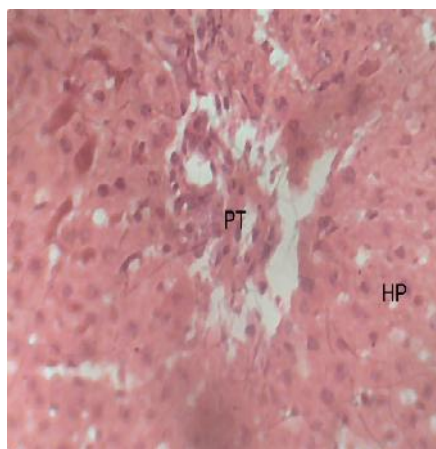
**FIG. 3 (a&b): Photomicrographs of liver of hepatotoxic and non- hepatotoxic rats treated with 400mg/kg b.w. of GL extracts (X400).
H&E=Haematoxiniln and Eosin, HL = Hepatotoxic liver, NHL = non-hepatotoxic liver.**



H&E

FIG. 4 (a) HL

Liver tissue showing normal architecture with plate of hepatocytes (HP) and congested blood vessels.

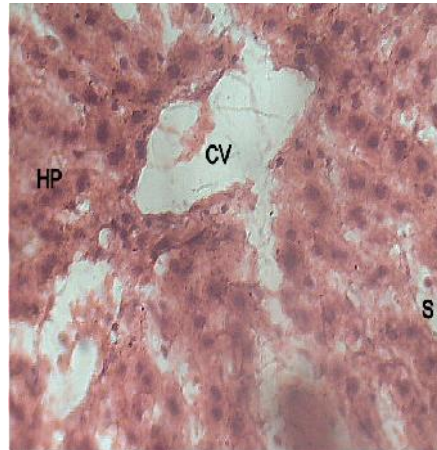


H&E

FIG.4 (b) NHL

Liver tissue showing normal hepatic plate (HP) with orderly arranged hepatocytes and portal triad (PT) as well as prominent sinusoid and steatosis

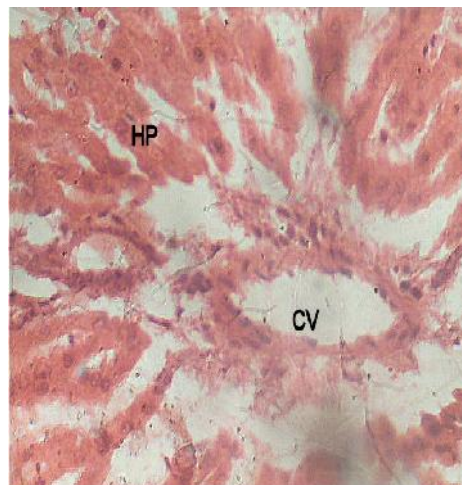
FIG. 4 (a&b): Photomicrographs of liver of hepatotoxic and non- hepatotoxic rats treated with 200mg/kg b.w. each of NL+GL extracts (X400). H&E= Haematoxilin and Eosin, HL = Hepatotoxic liver, NHL = non-hepatotoxic liver.



H&E

FIG. 5 (a) HL

Liver tissue with plates of hepatocytes (HP) radiating from the central vein (CV) and showing prominent sinusoids (S).



H&E

FIG.5 (b) NHL

Normal liver architecture showing central vein (CV) with normal hepatic plate (HP).

FIG. 5 (a&b): Photomicrographs of liver of hepatotoxic and non- hepatotoxic rats treated with Silymarin (100 mg/kg, bw, p.o.) (X400). H&E=Haematoxin and Eosin, HL = Hepatotoxic liver, NHL = non-hepatotoxic liver.

