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APOPTOTIC, HEPATOPROTECTIVE AND ANTIOXIDANT POTENTIAL OF A TRIHERBAL FORMULATION AGAINST D-GALACTOSAMINE HEPATOTOXICITY

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ABSTRACT

A triherbal formulation prepared from hydroethanolic mixture of Gongronema latifolia, Ocimum gratissimum and Vernonia amygdalina leaves (GOV) was evaluated to ascertain its heamatologic, hepatoprotective potentials, antioxidant properties and the fold increase in caspase 2, 3 and 9 activities against D-galactosamine-induced toxicity using Wistar albino rats.

Forty-nine Wistar albino rats were divided equally into seven groups. Two control experiments which included normal rats treated with D-galactosamine and normal rats that received only distilled water. Three groups were treated with different doses of GOV extract (2, 4 and 8 g kg^{-1} b. wt) while two groups received standard hepatoprotective drugs (Liv 52 and Silymarin) for 13 days prior to intoxication with D-galactosamine. The activities of serum liver enzymes, concentrations of some biochemical analytes, effect on heamatologic parameters, antioxidant status and fold increase in caspase 2, 3 and 9 activities were monitored.

HPTLC of GOV showed the presence of ascorbic acid, rutin, eugenol and β-sitosterol. Administration of GOV significantly ($p<0.05$) increased the Packed Cell Volume, Red Cell Count, Haemoglobin, White Blood Cell, platelet count, Mean Cell Haemoglobin, granulocytes and lymphocytes while the Mean Cell Volume and monocytes were significantly ($p<0.05$) depreciated dose dependently compared to the toxin control group. GOV dose dependently exhibited significant ($p<0.05$) decrease in levels of Alkaline phosphatase, Alanine aminotransferase, aspartate aminotransferase, L-γ-glutamyltransferase, Lactate dehydrogenase, cholesterol, creatinine, triglycerides, urea and Malondialdehyde. Subsequently, it significantly ($p<0.05$) increased the albumin, total protein, catalase, Glutathione Peroxidase, Reduced Glutathione, Glutathione-S-Transferase and Superoxide Dismutase levels. GOV significantly ($p\leq0.05$) attenuated the fold increase in caspase 2, 3 and 9 activities compared to the toxin control group.

The data from this study suggest that GOV possess apoptotic, hepatoprotective and antioxidant activity against D-galactosamine induced hepatotoxicity in rats, thus providing scientific rationale for its use in traditional medicine for the treatment of liver diseases.

KEY WORDS: *D-Galactosamine, HPTLC, Caspase, heamatologic, Antioxidant, hepatoprotective*

INTRODUCTION

The liver is the primary site for drug metabolism therefore hepatotoxicity is one of the most frequently reported human adverse drug reactions. Some drugs produces reactions that are similar to those of acute viral hepatitis (Mumoli *et al*., 2006). Drug-induced liver injury has become a leading cause of severe liver disease in Western countries and therefore poses a major clinical and regulatory challenge (Russmann *et al.,* 2009). Potential hepatotoxicity of some of the first-line antitubercular agents remains a problem, especially during the initial period of treatment (Shakya *et al*., 2006). Hepatic toxicity is also a common complication of anti-retroviral treatment in HIV patients, usually indicated or heralded by the elevation of liver transaminases measured in the blood. There had been reported evidence of hepatic toxicity in all the three currently approved classes of anti-retroviral drugs. However, its severity in some cases may warrant stoppage of the treatment (Akande *et al*., 2007).

The use of herbal medicines is increasing rapidly worldwide. Though the reasons for this may vary in different settings, the safety of herbal medicines is a common global concern. Medicinal plants have contributed immensely to health care in Nigeria. This is due in part to the recognition of the value of traditional medical systems, and the identification of medicinal plant from indigenous pharmacopoeias, which have significant healing power. In Nigeria, herbal agents are prescribed even when their biologically active components are unknown because of their effectiveness, fewer side effects and relatively low cost. Despite the widespread use of complementary and alternative medicine (CAM), there is a lack of scientific evidence on the efficacy and safety of some of these herbal drugs. However, we are not aware of a satisfactory remedy for serious liver diseases and search for effective and safe drugs for liver disorders continues to be an area of interest. It is therefore very important to make continuous effort to develop more effective therapeutic strategies or prophylactic modalities to eradicate or stem liver disease.

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efficacy and safety of some of these herbal drugs. However, we are not aware of a satisfactory remedy for serious liver diseases and search for effective and safe drugs for liver disorders continues to be an area of interest. It is therefore very important to make continuous effort to develop more effective therapeutic strategies or prophylactic modalities to eradicate or stem liver disease.

MATERIALS AND METHODS

Chemicals and Biochemical. Some biochemical parameters (ALP, ALT, AST, LDH, GGT, albumin, creatinine, total protein, triglyceride and urea) were analyzed using kits from Randox Laboratories. The assays were performed according to manufacturer's instructions. D-galactosamine (Carbosynth, UK), LIV 52 syrup (Himalaya Drug company, India), Caspase 2, 3 and 9 assay kits (BioVision, USA) were used. Chromatography was performed on 10 x 10 and 20 x 10 cm Bioluminex HP-TLC silica gel 60 F_{254} plates. Silymarin and all other reagents were from Sigma Aldrich, USA otherwise specified.

Plants. Fresh leaves of *Gongronema latifolia, Ocimum gratissimum* and *Vernonia amygdalina* were purchased from Oyingbo market in Lagos metropolis, Nigeria. They were subsequently identified and authenticated by Mr Adeleke at the Department of Pharmacognosy, College of Medicine of the University of Lagos, Nigeria where voucher specimen (PCGH 444, PCGH 443 and PCGH 432 respectively) were deposited. The leaves were air dried at room temperature and finely ground using Corona® grinder.

Preparation of the 50 % Ethanolic Extract of *Gongronema latifolia* Benth, *Ocimum gratissimum Linn.* and *Vernonia amygdalina Del.* (GOV). 1 kg of each of the powdered leaves of *G. latifolia, O. gratissimum* and *V. amygdalina* was mixed and soaked in ten litres (10 L) of 50 % ethanol (v/v) for 24 hrs. It was filtered using three layers of cheese cloth. The solvent was removed by rotary evaporation under reduced pressure at temperatures below 45 °C while the water was removed by freeze-drying. The resultant extract is known as the triherbal formulation (GOV).

The percentage extract yield was estimated as (Parekh & Chanda, 2007):

$$\frac{\text{Dry weight X 100}}{\text{Dry material weight}}$$

High Performance Thin Layer Chromatography (HPTLC) Analysis. A HPTLC system comprising a CAMAG automatic TLC sampler 4 software, Optiquest monitor Q7 and CAMAG scanner 3 was used for this study. Ethyl acetate: formic acid: acetic acid: water (10:1.1:1.1: 2.6 v/v/v/v) was employed as mobile phase while anise aldehyde/ H_2SO_4 and natural products solutions were used as reagent sprays. Chromatography was performed on 10 x 10 and 20 x 10 cm Bioluminex HP-TLC silica gel 60 F_{254} plates. Methanol was developed off the top of HPTLC plate and dried at 100 ± 15 ^oC for 20 minutes.

Using a CAMAG automatic TLC sampler 4, methanolic solutions of samples and standard compounds of known concentrations were spotted on the plates. After spotting, the plate was placed in an oven at 100 ^oC for 2 minutes. The HPTLC plate was developed and dried in an oven at 40 ^oC for 20 minutes. The bands were visualized under Alpha Innotech Fluorchem® 8900 UV cabinet at 254 and 365 nm and the pictures were taken. The plate was sprayed with natural products and the bands were detected using movie files at 365nm and the 365 picture was taken. It was then sprayed with anise aldehyde and placed in an oven at 115 -

135 ^{o}C for 5 seconds. The bands were detected using movie files at 365nm and derivatized 365 pictures were taken. It was put back into the oven at 125 ^{o}C for 1 minute and the white light picture was taken. The identification of standard compounds was confirmed by matching the UV spectra of samples and standards within the same Rf window.

Animal Care. Studies were carried out using Swiss mice (20 - 30g) and Wistar albino rats (150 - 200 g) of either sex, obtained from the Laboratory animal centre of the College of Medicine, University of Lagos Nigeria. The animals were grouped and maintained under standard laboratory conditions with dark and light cycle (12/12 hr.). The animals were fed with standard rat chow supplied by Ladokun feeds, Ibadan, Nigeria and left for 14 days to acclimatize before commencement of experiments.

Acute Toxicity Test. This was performed according to the Organization of Economic Co-operation and Development (OECD) guidelines for testing Chemical, TG425 (OECD, 2001). 120 mice were randomly divided into 12 groups of 10 animals each. The animals were fasted overnight but allowed access to only water. Groups 1-5 were administered orally with GOV at different doses (1, 2, 4, 8 and 16 g kg^{-1}) by gastric intubation, while groups 7-11 were administered with different doses of GOV (0.5, 1, 1.5, 2 and 2.5 g kg^{-1}) intraperitoneally. Group 6 and 12 which are the control groups received the dosing vehicle i.e. distilled water (10 ml kg^{-1}). Signs of toxicity and mortality were observed after the administration of the extract at the first, second, fourth, sixth and twenty fourth hrs. Mortality in each group within 24 hrs was recorded. This study was conducted according to the rules and regulations of the University of Lagos Ethical Committee on the use of experimental animals.

The Effect of GOV on D-Galactosamine Induced Hepatotoxicity

Forty nine rats were randomized and divided into 7 groups of 7 rats each. Rats in Groups 1 and 2 served as normal and toxin control groups and received distilled water (10 ml kg^{-1} body weight p.o.) for 14 days. In groups 3, 4 and 5, the rats were treated with 2, 4 and 8 mg kg^{-1} body weight (p.o) of GOV respectively for 14 days while groups 6 and 7 were administered LIV 52 syrup and silymarin (300 mg kg^{-1} body weight p.o.) respectively for 14 days. D-GaIN (Sigma) in distilled water was administered to the rats in groups 2 to 7 in a single dose of 500 mg kg^{-1} body weight intraperitoneally (i.p.) 24 hours before sacrificing on the 14th day**.**

After the experimental period the animals were anaesthetized mildly with ether and blood was collected from the retro-orbital plexus. They were sacrificed and more blood samples were collected by cardiac puncture. The blood samples were used for biochemical and antioxidant studies. The liver and kidneys were also dissected out for assay of oxidative stress indicators or enzymes and histology.

Preparation of Homogenates. One gram (1 g) of the liver and kidneys were weighed, washed with 0.86 % ice-cold normal saline (to remove all the red blood cells) and homogenates (10 % w/v) were prepared in 0.4 M PBS using a Polytron® Ergonomic homogenizer. The homogenates were centrifuged at 1400–1600 rpm for 10 minutes at 4 ^{o} C, after removal of the cell debris, supernatant was used for the estimation of lipid peroxidative indices, enzymic and non-enzymic antioxidants.

Haematologic indices. To determine White blood cell (Leucocyte) count a Haemocytometer (improved Neubauer ruled chamber) and Turk's solution was used while for Leucocyte (White Cell) Differential Count, Wright stain solution was used employed (Houwen,

2001). The principle of Cyanmethemoglobin method was used to determine Haemoglobin (HB) level using Drabkin's neutral diluting fluid **(**Bain and Bates, 2001**)**. The method of Baker and Silverton (1976) was used to calculate the packed cell volume (PCV) using a microhaematocrit centrifuge. Red blood cell (RBC) count was ascertained using Gower's solution according to the method described by McPherson and Pincus (2007).

To calculate MCV, MCH, and MCHC, PCV and red blood cell counts, haemoglobin and red blood cell counts, and haemoglobin and PCV were employed, respectively.

Determination of Antioxidant Activity. Estimation of enzymic and nonenzymic antioxidants activities e.g. Catalase (Sinha 1972), Glutathione Peroxidase (Ellman, 1959), Reduced Glutathione (Ellman, 1959), Glutathione-S-Transferase (Habig & Jakoby, 1974), and Superoxide Dismutase (Kakkar *et al.,* 1984) and lipid peroxidation level (thiobarbituric acid reactive substances) (Niehaus & Samuelsson, 1968) were determined using liver and kidney homogenates and serum samples.

Assessment of Hepato- and Nephro-Protective Activity. Assessment of hepato- and nephro-protective activity was performed by determining the activities of some biochemical parameters e.g. ALP, ALT AST, GGT and LDH enzyme activity, while the chemical analytes were assessed by determining the albumin, creatinine, cholesterol, total protein, triglyceride and urea concentrations in serum. These assays were carried out using Randox® reagent kits and the procedures were followed as those described in the literature available with the kits.

Liver Histopathology (Mallory, 1961). A small chunk of liver and kidney were taken from the sacrificed experimental rats used for hepatotoxicity studies and were preserved in 10 % formal saline for histological studies. The tissues were processed and sectioned in paraffin. The paraffin sections of buffered formalin- fixed tissue samples (3 µm thick) were dewaxed and rinsed in alcohol and also water. It was stained with Harris' haematoxylin (Sigma) for 10 minutes, washed in running tap water for 1 minute, differentiated in acid alcohol for 10 seconds and washed again in running tap water for 5 minutes. The tissues were stained with eosin for 4 minutes and washed in running tap water for 10 seconds. It was dehydrated and mounted for photomicroscopic observations of the histological architecture of the different groups. The general structure of the livers and kidneys of the normal control group (group 1) was compared with those of the treated groups (groups 2-7).

Statistical Analysis. The results were expressed as mean $\pm$ SEM for seven rats. Statistical analysis of the data was performed using ANOVA statistical SPSS package (15.0) version. The significance of differences among all groups was determined by the Tukey HSD test. P – values less than 0.05 ($p \leq 0.05$) were considered to be statistically significant.

RESULTS AND DISCUSSIONS

The yield of the 50 % ethanol extract (GOV) obtained was 1.42 kg (15.69 %). The HPTLC chromatograms of GOV are shown in plates 1 - 4. The migration or Rf values of GOV were compared with reference compounds, sprayed with different reagents and then viewed at different wavelengths. The highest numbers of bioactive components were detected in the hexane: ethyl acetate solvent system while the natural product spray demonstrated the best colour separation compared with the other sprays.

HPTLC of the triherbal decoction (GOV) compared to standard reference compounds.

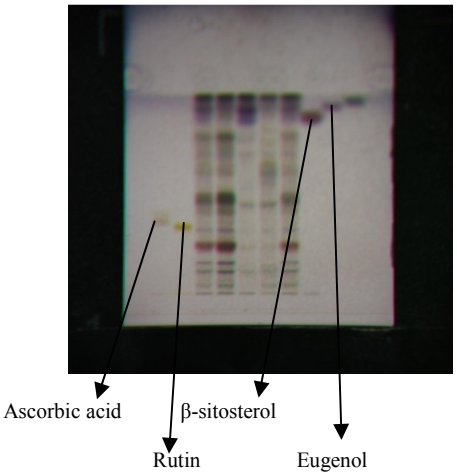

PLATE 1: chromatogram of GOV sprayed with natural product. Wth the aid of a scanner ascorbic acid, rutin, eugenol and β-sitosterol were detected (white light).

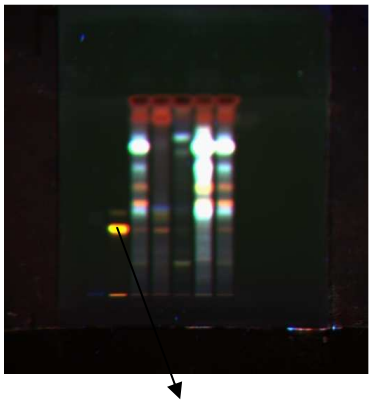

PLATE 2: chromatogram of aqueous and 50% ethanolic extract of GOV sprayed with anisaldehyde / H_2SO_4 solution. With the aid of a scanner, rutin was detected at 365 nm

Acute toxicity studies

The acute toxicity studies showed that GOV at 16000 mg kg^{-1} (p.o.) and 2500 mg kg^{-1} (i.p.) did not produce any mortality nor was there any significant change in the general behaviour of the mice.

Effect of GOV on the Haematologic Indices on D-GaIN Induced Hepatotoxicity on Rats. Tables 1 and 2 below shows the effect of GOV on hematologic indices in rats intoxicated with D-GaIN. Treatment, by gavage, with the triherbal decoction (GOV) produced various effects on blood parameters which included changes in hematological indices (Hb, PCV, RBC, WBC, platelet counts, MCV, MCH, MCHC, granulocytes, lymphocytes and monocytes) in albino rats intoxicated with D-GaIN. Administration of GOV significantly ($p< 0.05$) increased the PCV, RBC, Hb, WBC, platelet count, MCHC, granulocytes and lymphocytes while the MCV and monocytes were significantly ($p< 0.05$) depreciated in a dose dependent fashion compared to the toxin control group.

At 2 g kg^{-1}, GOV did not demonstrate any significant ($p\leq0.05$) difference in PCV, MCHC, MCH and granulocyte compared to the toxin control group. The administration of GOV at 4 g kg^{-1} and 8 g kg^{-1} increased the MCHC and MCH values and RBC and WBC values compared to all the groups respectively. Rats that received GOV at a dose of 4 g kg^{-1} exhibited increments in PCV, RBC, Hb, WBC and lymphocyte but the platelet numbers, MCV, granulocyte and monocytes depreciated compared to Liv 52 group. Compared to rats in the silymarin group, the rats in GOV (8 g kg^{-1}) group manifested an increment in PCV, RBC, WBC, MCV and monocytes while the Hb, platelet count, MCHC, MCH, granulocytes and

lymphocytes were decreased. Groups 5 and 7 demonstrated significant ($p \leq 0.05$) increase in RBC compared to group 3. In all the groups, there was no significant difference in MCH.

Effect of D-GaIN on Antioxidant Enzymes. Table 3 shows the effect of D-GaIN damage on serum antioxidant enzymes in rats pretreated with GOV. At 2 g kg^{-1}, GOV increased the CAT, GSH and GST compared to Liv 52 and silymarin while its GPx and SOD were increased compared to Liv 52. There was a significant ($p \leq 0.05$) increase in GSH at a dose of 2 g kg^{-1} compared to the 4 and 8 g kg^{-1} doses. The 2 g kg^{-1} attenuated the lipid peroxidation compared to Liv 52. GOV dose dependently increased the SOD activity compared to Liv 52.

TABLE 1: Effect of pretreatment with GOV on the hematologic indices in rats with D-GaIN induced hepatotoxicity.

Groups	Dose (g kg^{-1})	PCV (%)	RBC (10^6/µl)	Hb (g/dl)	WBC (10^3/µl)	Platelet (10^3/µl)
Control (Grp 1)		$43.77 \pm 2.27^{(b)}$	$6.36 \pm 0.16^{(b, c)}$	$11.86 \pm 0.67^{(b)}$	$12.84 \pm 0.44^{(b)}$	$44.99 \pm 4.45^{(b)}$
Toxin Control (Grp 2)	**0.5**	$31.29 \pm 0.94^{(a, d, e)}$	$3.84 \pm 0.2^{(a, c, d, e)}$	$6.31 \pm 0.66^{(a, c, d, e)}$	$2.28 \pm 0.35^{(a, c, d, e)}$	$21.3 \pm 0.82^{(a, c, d, e)}$
GOV + D-GaIN						
(Grp 3)	**2**	37.07 ± 1.9	$5.38 \pm 0.3^{(a, b, e)}$	$9.68 \pm 0.48^{(b)}$	$12.39 \pm 0.47^{(b)}$	$40.73 \pm 4^{(b)}$
(Grp 4)	**4**	$41.17 \pm 1^{(b)}$	$5.9 \pm 0.07^{(b)}$	$11.9 \pm 0.32^{(b)}$	$13.51 \pm 0.18^{(b)}$	$41.86 \pm 3.33^{(b)}$
(Grp 5)	**8**	$43.1 \pm 1.05^{(b)}$	$6.42 \pm 0.17^{(b, c)}$	$11.73 \pm 0.52^{(b)}$	$13.56 \pm 0.99^{(b)}$	$41.14 \pm 4.41^{(b)}$
LIV 52 + D-GaIN (Grp 6)	**0.3**	$40.39 \pm 1.03^{(b)}$	$5.88 \pm 0.2^{(b)}$	$11.47 \pm 0.41^{(b)}$	$11.8 \pm 0.11^{(b)}$	$49.43 \pm 5.52^{(b)}$
Silymarin + D-GaIN (Grp 7)	**0.3**	$42.6 \pm 2.11^{(b)}$	$6.36 \pm 0.25^{(b, c)}$	$12.14 \pm 0.56^{(b, c)}$	$12.48 \pm 0.43^{(b)}$	$48.7 \pm 6^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2 g kg-1) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4 g kg-1) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8 g kg-1) group. The significance of differences among all groups was determined by the Tukey HSD test.
Key: PCV =Packed Cell Volume, Hb = Haemoglobin, RBC = Red Cell Count, WBC = White Blood Cell.

Table 4 shows hepatic CAT, GPx, GSH, GST, MDA, SOD and total protein levels in rats fed GOV by intragastral gavage before administration of D-GaIN. The CAT, GPx, GSH, GST, SOD and total protein levels of liver homogenate in the toxin control group were significantly ($p \leq 0.05$) attenuated while the MDA level was significantly ($p \leq 0.05$) high compared to all the other experimental groups. The GPx, GSH, GST and SOD levels of experimental animals on pretreatment with GOV at 2 g kg^{-1} were higher compared to those of Liv 52 group while the CAT was higher than that of Liv 52 and silymarin. Silymarin group had the same GPx and GST values as 2 g kg^{-1} group while the 4 g kg^{-1} group has the same SOD

value as Liv 52 group. Control group was dose dependently and significantly ($p \leq 0.05$) different compared to GOV at the different doses.

Table 5 shows the levels of CAT, GPx, GSH, GST, MDA and total protein in kidney tissue homogenates obtained from all the experimental mice. MDA level (34.64 ± 4.79 nmol/g tissue) in kidney tissue homogenates of D-GaIN treated rats was found to be significantly ($p \leq 0.05$) higher than that level compared to normal control rats (7.43 ± 0.2 nmol/g tissue). Pre-treatment with GOV followed by toxin administration decreased the level significantly ($p \leq 0.05$). GOV significantly ($p \leq 0.05$) attenuated TBARS formation and elevated the activities of CAT, GPx, GSH, GST, SOD and total protein compared to D-GaIN intoxicated control group. GOV at 4 g kg^{-1} had higher CAT activity than Liv 52, increased GSH and GST compared to 2 and 8 g kg^{-1}, attenuated TBARS formation in D-GaIN induced kidney damaged rats compared to toxin control and GOV at 2 and 8 g kg^{-1}. The total protein concentration in GOV at 2 g kg^{-1} was almost equal to that of Liv 52. The GPx activity in the 8 g kg^{-1} group is significantly ($p \leq 0.05$) low compared to 4 g kg^{-1}, Liv 52 and silymarin groups while the total protein concentration is significantly ($p \leq 0.05$) low compared to silymarin.

TABLE 2: Effect of pretreatment with GOV on the hematologic indices in rats with D-GaIN induced hepatotoxicity.

Groups	Dose (g kg^{-1})	MCHC (%)	MCV (fl)	MCH (pg)	Granulocytes (%)	Lymphocyte (%)	Monocyte (%)
Control (Grp 1)		25.61 ± 2.05	68.72± 2.65$^{(b)}$	18.6 ± 0.8	8.4 ± 0.6	86.01 ± 1.18$^{(b)}$	5.59 ± 0.83$^{(b)}$
Toxin Control (Grp 2)	**0.5**	20.25 ± 2.12$^{(d)}$	82.48± 4.03$^{(a, c, d, e)}$	16.59 ± 1.82	4.63 ± 0.5$^{(d)}$	66.33 ± 1.92$^{(a, c, d, e)}$	29.04 ± 2.07$^{(a, c, d, e)}$
GOV + D-GaIN							
(Grp 3)	**2**	26.22 ± 0. 8	69.37± 3.07$^{(b)}$	18.23 ± 1.06	9.04 ± 0.8	84.8 ± 1.47$^{(b)}$	6.16 ± 1.05$^{(b)}$
(Grp 4)	**4**	28.98 ± 0.76$^{(b)}$	69.92± 2.06 $^{(b)}$	20.23 ± 0.68	10.53 ± 1.43$^{(b)}$	83.91 ± 2.14$^{(b)}$	5.41 ± 0.98$^{(b)}$
(Grp 5)	**8**	23.71 ± 2.84	67.63± 2.68$^{(b)}$	18.32 ± 0.82	9.64 ± 1.13	84.79 ± 1.62$^{(b)}$	5.57 ± 0.73$^{(b)}$
LIV 52 + D-GaIN (Grp 6)	**0.3**	28.43 ± 0.7$^{(b)}$	69.08± 2.87$^{(b)}$	19.58 ± 0.67	10.87 ± 0.89$^{(b)}$	83.34 ± 0.84$^{(b)}$	5.79 ± 0.57$^{(b)}$
Silymarin + D-GaIN (Grp 7)	**0.3**	28.65 ± 0.58$^{(b)}$	66.9 ± 2.17$^{(b)}$	19.1 ± 0.37	10.43 ± 2.1$^{(b)}$	85.66 ± 2.54$^{(b)}$	4.49 ± 1$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8 g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test. Key: MCV = Mean Cell Volume, MCH = Mean Cell Haemoglobin, MCHC = Mean Cell Haemoglobin Concentration,

TABLE 3: The effect of D-GaIN damage on serum antioxidant enzymes in rats pretreated with GOV

Groups	Dose (g kg^{-1})	CAT (µmol/min/ mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	Total protein (g/L)
Control (Grp 1)		92.21 ± 5.16$^{(b, d)}$	6.28 ± 0.1$^{(b cd, e)}$	37.57 ± 1.15$^{(b, d, e)}$	80.73 ± 2.33$^{(b cd, e)}$	36.65 ± 0.58$^{(b, d, e)}$	74.61 ± 1.63$^{(bc d e)}$	91.33 ± 3.61
Toxin Control (Grp 2)	**0.5**	46.19 ± 1.93$^{(a c d, e)}$	2.86 ± 0.1$^{(ac d e)}$	19.66 ± 0.28$^{(a c de)}$	38.60 ± 0.82$^{(a c d e)}$	81.69 ± 0.27$^{(a c d e)}$	22.43 ± 1.6$^{(a, c, d e)}$	50.34 ± 2.49$^{(a c de)}$
GOV + D-GaIN								
(Grp 3)	**2**	86.40 ± 1.8$^{(b)}$	5.18 ± 0.07$^{(a, b)}$	35.77 ± 1.19$^{(b, d, e)}$	70.10 ± 2.24$^{(a, b)}$	40.96 ± 1.1$^{(b)}$	48.66 ± 2.9$^{(a, b)}$	78.6± 4.31$^{(b)}$
(Grp 4)	**4**	79.45 ± 1.7$^{(a, b)}$	5.11 ± 0.08$^{(a, b)}$	31.25 ± 0.5$^{(a, b, c)}$	65.52 ± 1.42$^{(a, b)}$	45.55 ± 0.93$^{(a, b)}$	49.09 ± 3.06$^{(a, b)}$	76.25 ± 2.49$^{(a, b)}$
(Grp 5)	**8**	82.82 ± 1.94$^{(b)}$	4.93 ± 0.11$^{(a, b)}$	29.77 ± 0.53$^{(a, b, c)}$	68.60 ± 0.82$^{(a, b)}$	46.82 ± 0.9$^{(a, b)}$	48.83 ± 3.59$^{(a, b)}$	74.05 ± 3.34$^{(a, b)}$
LIV 52 + D-GaIN (Grp 6)	**0.3**	81.04 ± 2.73$^{(b)}$	5.09 ±.05$^{(a, b)}$	31.58 ± 0.38$^{(a, b, c)}$	68.37 ± 1.08$^{(a, b)}$	42.39 ± 3.01$^{(b)}$	45.63 ± 2.18$^{(a, b)}$	79.11 ± 2.58$^{(b)}$
Silymarin + D-GaIN (Grp 7)	**0.3**	80.52 ± 2.52$^{(b)}$	5.22 ± 0.06$^{(a, b)}$	35.5 ± 0.85$^{(b, d, e)}$	67.75 ± 0.96$^{(a, b)}$	39.83 ± 1.9$^{(b, e)}$	55.36 ± 1.18$^{(a, b)}$	80.73 ± 2.7$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.. KEY: CAT = Catalase, Gpx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

Effect of GOV on Serum Hepatic Enzymes and Chemical Analytes on D-GaIN intoxicated rats. Tables 6 and 7 below shows serum marker enzyme activities and levels of biochemical analytes in rats fed GOV before administration of D-GaIN. The increases in these enzyme activities were significantly ($p \leq 0.05$) declined at all doses of GOV tested, although the magnitude of the effect varied. At 2 g kg^{-1}, GOV attenuated the ALP activity compared to all the other experimental groups, had an ALT value lower than Liv 52 and almost equal to the control group and exhibited almost the same level of LDH activity compared to Liv 52 and silymarin.

TABLE 4: The effect of D-GaIN damage on liver antioxidant enzymes in rats pretreated with GOV

Groups	Dose (g kg^{-1})	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	Total protein (g/L)
Control (Grp 1)		393.52 ± 24.16$^{(b, c, d, e)}$	34.88 ± 4$^{(b, d, e)}$	75.97 ± 2.66$^{(b, c, d, e)}$	408.58 ± 17.46$^{(b, c, d, e)}$	7.6 ± 0.35$^{(b, c, d, e)}$	313.95 ± 17.95$^{(b, c, d, e)}$	102.85 ± 4.24$^{(b, d, e)}$
Toxin Control (Grp 2)	**0.5**	89.34 ± 3.37$^{(a, c, d, e)}$	9.9 ± 0.64 $^{(a, c, d, e)}$	35.07 ± 1.85$^{(a, c, d, e)}$	94.38 ± 6.08$^{(a, c, d, e)}$	22.54 ±1.04$^{(a, c, d, e)}$	77.74 ± 5.07$^{(a, c, d, e)}$	57.14 ± 1.92$^{(a, c, d, e)}$
GOV + D-GaIN (Grp 3)	**2**	308.23 ± 32.8$^{(a, b)}$	29.46 ± 2.24 $^{(b)}$	64.52 ± 1.45$^{(a, b)}$	302.1 ± 28.88$^{(a, b)}$	11.46 ± 0.79$^{(a, b)}$	178.22 ± 13.08$^{(a, b)}$	92.48 ± 2.24$^{(b)}$
(Grp 4)	**4**	287.04 ± 14.43$^{(a, b)}$	24.76 ± 1.37 $^{(a, b)}$	63.03 ± 0.49$^{(a, b)}$	230.95 ± 32.98$^{(a, b)}$	13.45 ± 0.63$^{(a, b)}$	171.52 ±15.52$^{(a, b)}$	83.22 ± 1.93$^{(a, b)}$
(Grp 5)	**8**	267.59 ± 15. 8$^{(a, b)}$	22.75 ± 1.31 $^{(a, b)}$	59.71 ± 1.65$^{(a, b)}$	288.26 ± 8.16$^{(a, b)}$	12.95 ± 0.44$^{(a, b)}$	174.89 ± 11.44$^{(a, b)}$	83.46 ±4.09$^{(a, b)}$
LIV 52 + D-GaIN (Grp 6)	**0.3**	289.16 ± 8.7$^{(a, b)}$	28.35 ± 0.94 $^{(b)}$	60.89 ± 0.58$^{(a, b)}$	282.69 ± 13.2$^{(a, b)}$	11.45 ± 0.58$^{(a, b)}$	171.4 ± 13.92$^{(a, b)}$	96.72 ± 3.36
Silymarin +D - GaIN (Grp 7)	**0.3**	298.93 ± 7.79$^{(a, b)}$	29.85 ± 0.63 $^{(b)}$	67.26 ± 3.38$^{(a, b)}$	302.24 ± 26.46$^{(a, b)}$	10.99 ± 0.77$^{(a, b)}$	190.34 ± 4.77$^{(a, b)}$	97.35 ± 3.89$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.
Key: CAT = Catalase, Gpx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

Pretreatment with GOV significantly ($p \leq 0.05$) attenuated the increased cholesterol, creatinine, triglyceride and BUN concentrations and elevated the reduced albumin and total protein concentrations induced by D-GaIN. At 2 g kg^{-1}, GOV increased albumin and total protein concentrations compared to 2 and 8 g kg^{-1} while the cholesterol concentration is low compared to Liv 52. In the 4 g kg^{-1} group, GOV reduced creatinine more than Liv 52 and silymarin, exhibited a lower triglyceride activity than Liv 52 but it is the same as silymarin. At 4 g kg^{-1}, BUN was reduced compared to 2 g kg^{-1} and 8 g kg^{-1}. At 2 and 8 g kg^{-1}, GOV has the same cholesterol value as Liv 52.

Effect of D-GaIN on Caspase Activities. Pretreatment with GOV significantly ($p \leq 0.05$) attenuated the increased cholesterol, creatinine, triglyceride and BUN concentrations and elevated the reduced albumin and total protein concentrations induced by D-GaIN. At 2 g kg^{-1}, GOV increased albumin and total protein concentrations compared to 2 and 8 g kg^{-1} while the cholesterol concentration is low compared to Liv 52. In the 4 g kg^{-1} group, GOV reduced creatinine more than Liv 52 and silymarin, exhibited a lower triglyceride activity than Liv 52

but it is the same as silymarin. At 4 g kg^{-1}, BUN was reduced compared to 2 g kg^{-1} and 8 g kg^{-1}. At 2 and 8 g kg^{-1}, GOV has the same cholesterol value as Liv 52.

Table 8 shows the fold increase in caspase 2, 3 and 9 activities in the white blood cell of rats pretreated with GOV before D-GaIN damage. At 2 and 4 g kg^{-1}, GOV lowered the fold increase in the caspase 3 activities compared to toxin control, GOV (8 g kg^{-1}), Liv 52 and silymarin groups. GOV significantly ($p \leq 0.05$) decrease the fold increase in caspase 2, 3 and 9 activities compared to the toxin control group.

TABLE 5: The effect of D-GaIN damage on kidney antioxidant enzymes in rats pretreated with GOV

Groups	Dose (g kg^{-1})	CAT (µmol/min/mg protein)	GPx (µmol/ml)	GSH (µmol/ml)	GST (µmol/ml)	MDA (nmol/ml)	SOD (µmol/ml)	Total protein (g/L)
Control (Grp 1)		457.5 ± 15.9$^{(b)}$	6.28 ± 0.1$^{(b,c,e)}$	4.19 ± 0.25$^{(bc,e)}$	345.71± 35.69$^{(b,e)}$	7.43 ± 0.2$^{(b)}$	236.73± 15$^{(bce)}$	71.75 ± 1.35$^{(bcde)}$
Toxin Control (Grp 2)	**0.5**	112.93 ± 14.08$^{(a)}$	2.7 ± 0.1$^{(acde)}$	1.43 ± 0.11$^{(acde)}$	108.52± 4.48$^{(acde)}$	34.64 ± 4.79$^{(acde)}$	63.08 ± 2.55$^{(acde)}$	45.79 ± 2.63$^{(acd)}$
GOV + D-GaIN								
(Grp 3)	**2**	277.14 ± 11.09$^{(a, b)}$	5.25 ± 0.23$^{(a, b)}$	2.82 ± 0.3$^{(a, b)}$	264.17± 33.05$^{(b)}$	9.79 ± 0.87$^{(b)}$	165.38± 9.27$^{(a b)}$	60.62 ± 2$^{(a, b)}$
(Grp 4)	**4**	286.52 ± 11.63$^{(a, b)}$	5.32 ± 0.28$^{(b,e)}$	3.37 ± 0.18$^{(a, b)}$	272.19± 16.23$^{(b)}$	9.33 ± 0.52$^{(b)}$	191.11± 23.02$^{(b)}$	59.9 ± 2.36$^{(a, b)}$
(Grp 5)	**8**	241.07 ± 7.52$^{(a, b)}$	4.35 ± 0.25$^{(a, b,d)}$	2.83 ± 0.28$^{(b)}$	222.19± 13.92$^{(a,b)}$	11.3 ± 0.87$^{(b)}$	153.88± 9.33$^{(ab)}$	54.54 ± 1.69$^{(a)}$
LIV 52 + D-GaIN (Grp 6)	**0.3**	285.18 ± 26.7$^{(a, b)}$	5.34 ± 0.33$^{(b,e)}$	3.44 ± 0.16$^{(b)}$	285.95 ± 7.71$^{(b)}$	8.87 ± 0.29$^{(b)}$	191.76 ± 9.44$^{(b)}$	61.62 ± 2.8$^{(a, b)}$
Silymarin + D-GaIN (Grp 7)	**0.3**	294.77 ± 29.88$^{(a, b)}$	6.07 ± 0.15$^{(b,e)}$	3.48 ± 0.12$^{(b)}$	285.38± 32.64$^{(b)}$	8.51 ± 0.54$^{(b)}$	196.9 ± 7.42$^{(b)}$	65.39 ± 1.4$^{(b,e)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test.

Key: CAT = Catalase, Gpx = Glutathione Peroxidase, GSH = Reduced Glutathione, GST = Glutathione-S-Transferase, MDA = Malondialdehyde, SOD = Superoxide Dismutase

TABLE 6: Serum levels of ALP, ALT, AST, GGT and LDH in rats pretreated with GOV before D-GaIN damage.

Groups	Dose (g kg^{-1})	LIVER FUNCTION ENZYMES				
		ALP (U/L)	ALT (U/L)	AST (U/L)	GGT (U/L)	LDH (U/L)
Control (Grp 1)		239.59 ± 4.84$^{(b)}$	14.06 ± 0.34$^{(b)}$	15.15 ± 0.73$^{(b)}$	1151.96 ± 62.01$^{(b, e)}$	14.04 ± 1.37$^{(b, e)}$
Toxin Control (Grp 2)	**0.5**	474.19 ± 45.63$^{(a, c, d, e)}$	68.18 ± 6.72$^{(a, c, d, e)}$	75.75 ± 7.51$^{(a, c, d, e)}$	3722.26 ± 80.33$^{(a, c, d, e)}$	67.99 ± 1.89$^{(a, c, d, e)}$
GOV + D-GaIN						
(Grp 3)	**2**	212.45 ± 18.62$^{(b)}$	14.49 ± 0.53$^{(b)}$	16.79 ± 2.6$^{(b)}$	1317.06 ± 72.5$^{(b)}$	15.78 ± 0.94$^{(b)}$
(Grp 4)	**4**	254.75 ± 10.76$^{(b)}$	16.45 ± 0.87$^{(b)}$	19.79 ± 0.9$^{(b)}$	1465.86 ± 29.33$^{(b)}$	17.59 ± 0.78$^{(b)}$
(Grp 5)	**8**	267.07 ± 8.47$^{(b)}$	17.83 ± 0.58$^{(b)}$	18.9 ± 0.72$^{(b)}$	1498.38 ± 131.08$^{(a, b)}$	20.47 ± 1.62$^{(a, b)}$
LIV 52 + D-GaIN (Grp 6)	**0.3**	241.23 ± 8.23$^{(b)}$	15.68± 0.63$^{(b)}$	15.61± 0.73$^{(b)}$	1285.52± 82.92	15.54± 0.95
Silymarin + D-GaIN (Grp 7)	**0.3**	240.03 ± 15.31$^{(b)}$	13.13 ± 0.38$^{(b)}$	15.02 ± 0.71$^{(b)}$	1184.02 ± 56.78$^{(b)}$	15.58 ± 1.51$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8 g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test. Key: ALP = Alkaline phosphatase, ALT = Alanine aminotransferase, AST = aspartate aminotransferase, GGT = L-γ-glutamyltransferase and LDH = Lactate dehydrogenase

Histopathology of rats pretreated with GOV before D-GaIN damage

The photo micrographs of the histopathologic studies on rat liver damaged with D-GaIN are shown in photo micrographs 1-6. The control group showed normal hepatocytes arranged in roughly rod or pillar-shaped pattern while the Toxin control group showed extensive hepatocellular necrosis, leukocyte infiltration, steatosis, centrizonal necrosis, hepatocellular swelling and vacuolation. Pretreatment with GOV 2 g kg^{-1} before intoxication using D-GaIN showed no remarkable changes while at 4 g kg^{-1} it showed mild steatosis. Pretreatment with Liv 52 and silymarin before damage with D-GaIN showed no remarkable changes.

Preliminary phytochemical screening in our laboratory revealed the presence of different phytoconstituents like flavonoids, triterpenoids, saponins, terpenoids and phenolics and alkaloids and these compounds are known to possess antioxidant and hepatoprotective activity.

TABLE 7: Serum levels of albumin, cholesterol, creatinine, total protein, triglyceride and BUN in rats pretreated with GOV before D-GaIN damage.

Groups	Dose (g kg^{-1})	BIOCHEMICAL PARAMETERS					
		TP (g/L)	ALB (g/L)	BUN (mmol/L)	CREA (mmol/L)	TG (mmol/L)	CHO (mmol/L)
Control (Grp 1)		91.33 ± 3.61$^{(b, d, e)}$	50.05 ± 3.45$^{(bcde)}$	7.08 ± 0.24$^{(b, e)}$	58.2 ± 3.3$^{(b)}$	0.99 ± 0.03$^{(b)}$	1.21 ± 0.08$^{(b)}$
Toxin Control (Grp 2)	**0.5**	50.34 ± 2.49$^{(a, c, d, e)}$	22.3 ± 0.97$^{(a, c)}$	14.89 ± 0.75$^{(a, c, d, e)}$	100.12 ± 1.68$^{(a, c, d, e)}$	3.01 ± 0.11$^{(a, c, d, e)}$	2.81± 0.29$^{(a, c, d, e)}$
GOV + D-GaIN							
(Grp 3)	**2**	78.6 ± 4.31$^{(b)}$	37.06 ± 0.76$^{(a, b)}$	9.22 ± 0.69$^{(b)}$	64.82 ± 4.89$^{(b)}$	1.13 ± 0.07$^{(b)}$	1.74 ± 0.15$^{(b)}$
(Grp 4)	**4**	76.25 ± 2.49$^{(a, b)}$	34.15 ± 0.85$^{(a)}$	8.47± 0.55$^{(b)}$	60.92 ± 4.25$^{(b)}$	1.08 ± 0.02$^{(b)}$	1.8 ± 0.11$^{(b)}$
(Grp 5)	**8**	74.05 ± 3.34$^{(a, b)}$	32.68 ± 1.5$^{(a)}$	10.61± 1.33$^{(a, b)}$	66.8 ± 2.65$^{(b)}$	1.13 ± 0.13$^{(b)}$	1.75 ± 0.18$^{(b)}$
LIV 52 + D-GaIN (Grp 6)	**0.3**	79.11 ± 2.58$^{(b)}$	40.62 ± 5.76$^{(b)}$	7.68 ± 0.29$^{(b)}$	63.79 ± 2.34$^{(b)}$	1.11± 0.06$^{(b)}$	1.75 ± 0.12$^{(b)}$
Silymarin + D-GaIN (Grp 7)	**0.3**	80.73 ± 2.7$^{(b)}$	40.29 ± 1.51$^{(b)}$	7.57 ± 0.34$^{(b, e)}$	63.1± 4.63$^{(b)}$	1.08 ± 0.1$^{(b)}$	1.41± 0.16$^{(b)}$

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to D-GaIN control group. (c) = $p \leq 0.05$ as compared with the GOV + D-GaIN (2 g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + D-GaIN (4 g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + D-GaIN (8 g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test. Key: TP = Total Protein, ALB = Albumin, BUN = Blood Urea Nitrogen, CREA = Creatinine, TG = Triglyceride, CHO = Cholesterol

Thin layer chromatographic (TLC) analysis of GOV, the ethanolic and water extracts of *G. latifolia, O. gratissimum* and *V. amygdalina* showed that water is not the best solvent for extracting polyphenolic compounds. This is in concordance with the findings reported by Marwah *et al*. (2007) that aqueous alcohols are the best solvents for extracting polyphenolic compounds from plant materials. Stigmasterol which is one of the phytoconstituents identified has been reported to possess anti-osteoarthritic (Gabay *et al*., 2010), antioxidant, hypoglycemic and thyroid inhibiting properties (Panda *et al*., 2009) while β-sitosterol exhibits anti-inflammatory activity in human aortic endothelial cells (Loizou *et al,* 2010) and is used in Europe for the treatment of breast cancer (Awad *et al,* 2000). Rutin is an antioxidant as reported by Jaganath *et al*. (2009), shows antinociceptive (Lapa *et al,* 2009) and anti-inflammatory activity in some animal and *in vitro* models (Chan *et al*., 2007). Hyperoside could have a protective antioxidant effect on cultured PC12 cells (a cell line derived from a pheochromocytoma of the rat adrenal medulla) (Zhiyong *et al*., 2005) and also in lung fibroblast cells (Piao *et al*., 2008). Eugenol shows protective activity against oxidized LDL-

induced cytotoxicity and adhesion molecule expression in endothelial cells (Ou *et al,* 2006) and exhibited antioxidant, anti-inflammatory and DNA-protective properties in thioacetamide-induced liver injury in rats (Yogalakshmi *et al*., 2010). Ascorbic acid selectively kills cancer cells and acts as a pro-drug to deliver hydrogen peroxide to tissues (Chen *et al*., 2005) while linoleic acid demonstrates antiapoptotic activities (Miner *et al*., 2001). Borneol exhibits antihypertensive and antioxidant activities (Kumar *et al*., 2010). The bioactivities of GOV could be adduced to the presence of some of these compounds identified from our phytoscreening.

TABLE 8: Colorimetric assay of caspase - 2, 3 and 9 activities in the white blood cell of rats treated with D-GaIN

Groups	Dose (g kg-^{1})	Caspase 2 (Units/mg of total protein).	Caspase 3 (Units/mg of total protein)	Caspase 9 (Units/mg of total protein)
Control (Grp 1)		$0.97 \pm 0.07^{(b)}$	$0.72 \pm 0.07^{(b)}$	$0.95 \pm 0.02^{(b)}$
Toxin control (Grp 2)	**0.5**	$2.27 \pm 0.15^{(a, c, d, e)}$	$6.28 \pm 0.33^{(a, c, d, e)}$	$4.66 \pm 0.72^{(a, c, d, e)}$
		(1.34)	(7.72)	(3.91)
GOV + D-GaIN				
(Grp 3)	**2**	$1.09 \pm 0.03^{(b)}$	$1.01 \pm 0.1^{(b)}$	$1.14 \pm 0.07^{(b)}$
		(0.12)	(0.4)	(0.2)
(Grp 4)	**4**	$1.2 \pm 0.07^{(b)}$	$1 \pm 0.07^{(b)}$	$1.22 \pm 0.04^{(b)}$
		(0.24)	(0.39)	(0.28)
(Grp 5)	**8**	$1.15 \pm 0.04^{(b)}$	$1.12 \pm 0.05^{(b)}$	$1.14 \pm 0.02^{(b)}$
		(0.19)	(0.56)	(0.2)
Liv 52 + D-GaIN (Grp 6)	**0.3**	$1.01 \pm 0.05^{(b)}$	$1.06 \pm 0.09^{(b)}$	$1.13 \pm 0.08^{(b)}$
		(0.04)	(0.47)	(0.19)
Silymarin + D-GaIN (Grp 7)	**0.3**	$1.00 \pm 0.06^{(b)}$	$1.03 \pm 0.01^{(b)}$	$1.11 \pm 0.06^{(b)}$
		(0.03)	(0.43)	(0.17)

Values are expressed as Mean ± SEM for seven rats. The Mean difference is significant at the .05 level. (a) = $p \leq 0.05$ as compared with the normal control group. (b) = $p \leq 0.05$ as compared to Alcohol control group. (c) = $p \leq 0.05$ as compared with the GOV + Alcohol (2g kg^{-1}) group. (d) = $p \leq 0.05$ as compared with the GOV + Alcohol (4g kg^{-1}) group. (e) = $p \leq 0.05$ as compared with the GOV + Alcohol (8g kg^{-1}) group. The significance of differences among all groups was determined by the Tukey HSD test. The figures in parenthesis indicates the fold-increase in caspase activity compared to the uninduced control.

D-GaIN produces diffuse type of liver injury simulating viral hepatitis (Srinath *et al*., 2010). Its toxicity increases cell membrane permeability leading to enzyme leakage which eventually causes cell death. Pretreatment with GOV reduced the increased enzyme activities of liver marker enzymes and malondialdehyde induced by D-GaIN damage. D-GaIN causes cholestasis and this can be attributed to its damaging effects on bile ducts. The bile duct obstruction caused pronounced elevation in ALP activity as ALP is mainly produced in the bile duct and its release is enhanced by cholestasis. The significant reduction in ALP on pretreatment with GOV can be attributed to its attenuation of the damaging effect on the bile ducts thereby preventing cholestasis. According to Drotman and Lawhan (1978), elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Rajkapoor *et al,* 2008). Serum ALP, bilirubin and total protein levels on other hand are related to the function of hepatic cell.

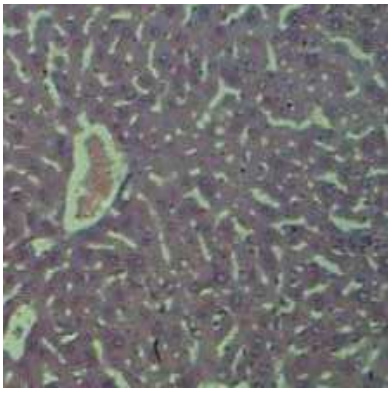

Photo micrograph 1: Control group

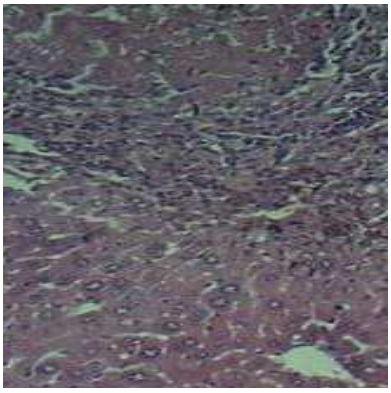

Photo micrograph 2: Toxin control group

Photo micrograph 3: GOV 2 g kg^{-1} + D-GaIN

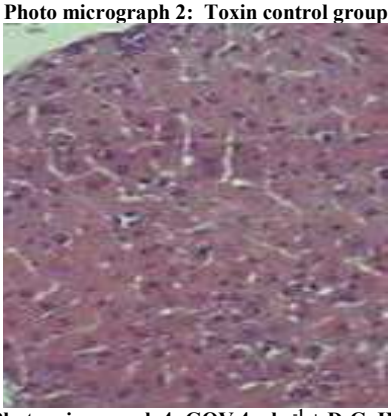

Photo micrograph 4: GOV 4 g kg^{-1} + D-GaIN

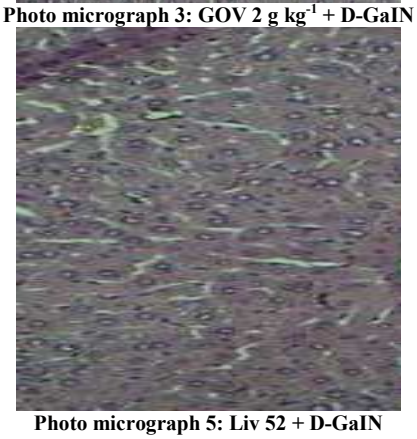

Photo micrograph 5: Liv 52 + D-GaIN

Photo micrograph 6: silymarin + D-GaIN

It has been shown that the lowering of serum albumin level is attributed to the reduction of albumin mRNA expression. The reduction in serum albumin concentration in D-GaIN treated rats after GOV treatment could be attributed to its ability to increase the blood flow and

irrigation to liver hence contribute to liver vitality. Serum cholesterol and triglyceride are elevated in cholestasis because metabolic degradation and excretion are impaired. Pretreatment with GOV significantly decreased serum cholesterol and triglyceride concentrations indicating its ability to enhance metabolic degradation and excretion. As D-GalN induced renal failure seems to occur at the end stage of liver cirrhosis, the protective role of GOV against D-GalN induced renal damages is likely to be an indirect effect probably coming into play via the protection against hepatic disorders. Decline in protein synthesis may have arisen due to disruption in synthesis of essential uridylate nucleotides which causes organelle injury and finally cell death, since depletion of essential uridylate nucleotides impede the normal synthesis of RNA. GOV attenuated the decrease in the total serum, kidney and liver proteins demonstrating that it participates in improving the conditions of liver and kidney. It shows that GOV may support optimum metabolic conditions for the high rate of energy dependent recovery processes required for repairing the tissues damaged by D-GaIN intoxication.

Histopathology of the liver of rats pretreated with GOV before D-GaIN damage

The restoration by GOV of the decreased serum, hepatic and renal levels of glutathione as well as decreased activities of glutathione S-transferase and glutathione peroxidase by GaIN towards normalization is suggestive of its hepatoprotective activity. This hepatoprotectivity may consist of maintaining adequate levels of hepatic glutathione for xenobiotics removal and increased blood flow to liver thus increasing its antioxidant capacity. The protective role of GOV against D-GaIN induced renal damages is likely to be an indirect effect, since GOV possesses hepatoprotective activity, it may first ameliorate liver damage and subsequently the renal disorders are reduced.

Caspases are known to mediate the apoptotic pathway (Kikuchi *et al*., 2010). The results obtained from this experiment indicated that D-GaIN induced cell death occurs through activation of caspases-2, 3 and 9. Zhivotovsky and Orrenius (2005), reported that genotoxic stress causes activation of caspase-2 upstream of mitochondria and that this caspase is the apical caspase which is required for apoptosis. In rats, it has been shown that D-GaIN causes apoptosis in the liver by activating caspase-3, which is released to the plasma by secondary necrosis, as indicated by the concomitant AST increase (Sun *et al*., 2003). The reduction in caspase 3 activity by GOV supports the attenuation of AST level earlier observed. Chan *et al*. (2006) and Kang and Reynolds (2009) reported that cytotoxic stress either from DNA damage or mitochondrial impairment leads to apoptosis via the intrinsic pathway. The intrinsic pathway involves the release of proapoptotic proteins including cytochrome C from the inner membrane of mitochondria to the cytosol leading to activation of caspase-9 (Riedl & Salvesen, 2007). It is likely that GOV may have decreased the extent of cytotoxic stress induced by these toxins by lowering the extent of release of proapoptotic proteins including cytochrome C and subsequent decrease in caspase activity. Thus it is suggested that GOV may inhibit apoptosis by down-regulating caspase-2, 3 and 9 activities.

CONCLUSIONS

This study complements the on-going activities of evaluation of different uses of medicinal plants and the development of new improved traditional medicine in Nigeria. The presence of rutin, borneo, stigmasterol, beta sitosterol, eugenol, hyperoside, ascorbic acid and

other antioxidants in GOV may be the contributing factor towards its hepatoprotective activity and justifies the folkloric use of the plant in treatment of liver diseases. The hepatoprotective properties of GOV may be attributed to the individual or combined action of these bioactive constituents.

It can be said that this triherbal formulation (GOV) has demonstrated liver protective effect against D- galactosamine-induced hepatotoxicity. It exhibited antioxidant activities in a dose dependent manner and demonstrated significant protection to the liver thus justifying its use as a hepatoprotective agent. The present findings provide scientific evidence to the ethnomedicinal use of this triherbal formulation in Eastern Nigeria in treating liver diseases.

REFERENCES

- Akande A. A., Olaosebikan O. F., Jimoh A. K., Abdulazeez S., Olawumi H. O. 2007. Lavudine therapy and hepatotoxicity as seen in a Nigerian tertiary antiretroviral treatment centre. *African Journal of Biochemistry Research*. 1 (6) 090-094.
- Awad A. B., Chan K.C., Downie A.C., Fink C.S. 2000. Peanuts as a source of β-sitosterol, a sterol with anticancer properties. *Nutrition and Cancer*. 36:238-241.
- Bain B., Bates I. 2001. *Basic haematological techniques*. *Dacie and Lewis practical haematology*. London: Church, Livingstone. pp. 20–22.
- Baker F., Silverton R. 1976. *Haematological estimation in Introduction to Medical Laboratory Technology.*5th ed., pp. 549-619, Butter Worths press, Boston, London.
- Chan H.J., Ji Y.L., Chul H.C., Chang J.K. 2007. Anti-asthmatic action of quercetin and rutin in conscious guinea-pigs challenged with aerosolized ovalbumin. *Archives of Pharmaceutical Research*. 30 (12): 1599–1607.
- Chan K.M., Rajab N.F., Ishak M.H., Ali A.M., Yusoff K., Din L.B., Inayat-Hussain S.H. 2006. Goniothalamin induces apoptosis in vascular smooth muscle cells. *Chemico-Biological Interactions*. 159:129–140.
- Chen Q., Espey M.G., Krishna M.C., Mitchell J. B., Corpe C.P., Buettner G.R., Shacter E., Levine M. 2005. Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues. *Proceedings of the National Academy of Sciences of the United States of America*; 102(38):13604-13609.
- Drotman R.B., Lawhorn G.T. 1978. Serum enzymes as indicators of chemically induced liver damage. *Drug and Chemical Toxicol*ogy. 1:163-71
- Ellman G.L. 1959. Tissue sulphydryl groups. *Archives of Biochemistry and Biophysics*. 82: 70-77.
- Gabay O., Sanchez C., Salvat C., Chevy F., Breton M., Nourissat G., Wolf C., Jacques, C., Berenbaum F. 2010. Stigmasterol: a phytosterol with potential anti-osteoarthritic properties. *Osteoarthritis Cartilage*. 18(1):106-116
- Habig W.H., Pabst M.J., Jakoby W.B. 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *The Journal of Biological Chemistry*. 249 :7130-7139
- Houwen B. 2001. The differential cell count. *Laboratory Hematology*. 7:89-100.
- Jaganath I.B., Mullen W., Lean M.E., Edwards C.A., Crozier A. 2009. *In vitro* catabolism of rutin by human faecal bacteria and the antioxidant capacity of its catabolites. *Free Radical Biology and Medicine*. 47 (8): 1180-1189
- Kakkar P., Das B., Viswanathan P.N. 1984. A modified spectrophotometric assay of superoxide dismutase (SOD). *Indian Journal of Biochemistry and Biophysics*. 21: 130-132.
- Kang M.H., Reynolds C.P. 2009. Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. *Clinical Cancer Research*. 15:1126–1132.
- Kikuchi T., Nihei M., Nagai H., Fukushi H., Tabata K., Suzuki T., Akihisa T. 2010. Albanol A from the root bark of *Morus alba* L. induces apoptotic cell death in HL60 human leukemia cell line. *Chemical and Pharmaceutical Bulletin.* 58 (4) 568—571.
- Kumar M. S., Kumar S., Raja B. 2010. Antihypertensive and antioxidant potential of borneol-a natural terpene in L-NAME – induced hypertensive rats. *International Journal of Pharmaceutical and Biological Archives*. 1(3): 271 – 279.
- Lapa F. R., Gadotti V.M., Missau F.C., Pizzolatti M.G., Marques M.C., Dafré A.L., Farina M., Rodrigues A.L., Santos A.R. 2009. Antinociceptive properties of the hydroalcoholic extract and the flavonoid rutin obtained from *Polygala paniculata* L. in mice. *Basic and Clinical Pharmacology and Toxicology*. 104(4):306-315.

- Loizou S., Lekakis I., Chrousos G. P., Moutsatsou P. 2010. Beta-sitosterol exhibits anti-inflammatory activity in human aortic endothelial cells. *Molecular Nutrition and Food Research*. 54 (4) 551-558
- Mallory F.B. 1961. *Pathological Technique*, Hafner Publishing., New York, c., pp. 152
- Marwah R.G., Fatope M.O., Mahrooqi R.A., Varma G.B., Abadi H.A., Al-Burtamani S.K.S. 2007. Antioxidant capacity of some edible and wound healing plants in Oman. *Food Chemistry.* 101(2):465–470.
- McPherson R.A., Pincus M.R. 2007. *Henry's Clinical Diagnosis and Management by Laboratory Methods.* 21st ed. Philadelphia, Pa: WB Saunders; 461-462.
- Miner J.L., Cederberg C.A., Nielsen M.K., Chen X., Baile C.A. 2001. Conjugated linoleic acid (CLA), body fat and apoptosis. *Obesity Research*. 9:129-134.
- Mumoli N., Cei M., Cosimi A. 2006. Drug-related hepatotoxicity. *The New England Journal of Medicine.* 354 (20): 2191–2193
- Niehaus W.G., Samuelsson B. 1968. Formation of malondialdehyde from phospholipid arachidonate during microsomal lipid peroxidation. *European Journal of Biochemistry.* 6: 126-130.
- OECD. 2001. The Organisation for Economic Co-operation and Development (OECD) 425 Guidelines for testing of chemicals acute oral toxicity up and down procedure. 26:1-26.
- Ou H.C., Chou F.P., Lin T.M., Yang C.H., Sheu W.H.2006. Protective effects of eugenol against oxidized LDL-induced cytotoxicity and adhesion molecule expression in endothelial cells. *Food and Chemical Toxicology*. 44(9):1485-1495.
- Panda S., Jafri M., Kar A., Meheta B.K. 2009. Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from *Butea monosperma*. *Fitoterapia*. 80(2):123-126.
- Parekh J., Chanda S. 2007. *In vitro* antimicrobial activity of *Trapa natans* L. fruit rind extracted in different solvents. *African Journal of Biotechnology*, 6(16): 1905-1909.
- Piao M. J., Kang K. A., Zhang R., Ko D. O., Wang Z. H., You H. J., Kim H. S., Kim J. S., Kang S. S., Hyun J. W. 2008. Hyperoside prevents oxidative damage induced by hydrogen peroxide in lung fibroblast cells via an antioxidant effect. *Biochimica et Biophysica Acta:* 1780 (12) 1448-1457.
- Rajkapoor B., Venugopal Y., Anbu J., Harikrishnan N., Gobinath M., Ravichandran V. 2008. Protective effect of *Phyllanthus polyphyllus* on acetaminophen induced hepatotoxicity in rats. *Journal of Pharmaceutical Sciences*. 21 (1)57-62
- Riedl S.J., Salvesen G.S. 2007. The apoptosome: signalling platform of cell death. *Nature Reviews Molecular Cell Biology*. 8:405–413.
- Russmann S., Kullak-Ublick G. A., Grattagliano I. 2009. Current concepts of mechanisms in drug-induced hepatotoxicity. *Current Medicinal Chemistry*. 16(23): 3041–3053.
- Shakya R., Rao B.S., Shrestha B. 2006. Evaluation of risk factors for antituberculosis drugs-induced hepatotoxicity in Nepalese population. K*athmandu University Journal of Science, Engineering and Technology*. 2(1): 1-8.
- Sinha K.A. 1972. Colorimetric assay of catalase. *Analytical Biochemistry.* 47: 389-394.
- Srinath A., Jyothi V., Asha- jyothi V. 2010. Hepatoprotective activity – a review. *International Journal of Pharmacy and Technology*. 2 (3): 354-366
- Sun F., Hamagawa E., Tsutsui C., Sakaguchi N., Kakuta Y., Tokumaru S., Kojo S. 2003. Evaluation of oxidative stress during apoptosis and necrosis caused by D-Galactosamine in rat liver. *Biochemical Pharmacology*. 65: 101–107
- Yogalakshmi B., Viswanathan P., Anuradha C.V. 2010. Investigation of antioxidant, anti-inflammatory and DNA-protective properties of eugenol in thioacetamide-induced liver injury in rats. *Toxicology*. 268(3):204-212.
- Zhivotovsky B., Orrenius S. 2005. Caspase-2 function in response to DNA damage. *Biochemical and Biophysical Research Communications*. 331: 859-857.
- Zhiyong L., Xinyi T., Chongwei Z., Yanhua L., Dongzhi W. 2005. Protective effects of hyperoside (quercetin-3-*o*-galactoside) to PC12 cells against cytotoxicity induced by hydrogen peroxide and *tert*-butyl hydroperoxide. *Biomedicine and Pharmacotherapy.* 59(9) : 481-490