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Serum lipids and oxidized low density lipoprotein levels in sickle cell disease: Assessment and pathobiological significance
Diatta Alassane, Cissé Fatou, Guèye Tall Fatou, Diallo Fatou, Touré Fall Awa Oumar, Sarr Gaston Ndéné, Lopez Sall Philomène, Sall Niama Diop and Touré Méïssa

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Okpala, J. C., Sani, I., Abdullahi, R., Ifedilichukwu, H. N. and Igwe, J. C.
Serum lipids and oxidized low density lipoprotein levels in sickle cell disease: Assessment and pathobiological significance

Diatta Alassane¹*, Cissé Fatou¹, Guéye Tall Fatou², Diallo Fatou¹, Touré Fall Awa Oumar³, Sarr Gaston Ndéné¹, Lopez Sall Philomène², Sall Niama Diop¹ and Touré Méissa¹

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One hundred and eighteen (118) subjects aged 15 to 36 years divided into control subjects (AA n = 42), heterozygous sickle cell patients (AS n = 33) and homozygous sickle cell patients (SS n = 43) were investigated for a lipid profile including the measurement of oxidized low density lipoprotein (LDL) to assess the risk of early atherosclerosis in sickle cell disease. The results show that total, high density lipoprotein (HDL) and LDL plasma cholesterol levels are significantly lower in the sickle cell patients than in control group (p < 0.05). In contrast, the triglyceride levels, the ratio of triglycerides to HDL-cholesterol and the oxidized LDL fraction are higher in patients (p < 0.05). These lipid abnormalities could represent a cardiovascular risk for sickle cell disease patients.

Key words: Atherosclerosis, dyslipidemia, oxidized low density lipoprotein (LDL), sickle cell disease.

INTRODUCTION

Sickle cell disease (SCD) is a genetic disorder caused by a single substitution (GTG for GAG) at the beta globin gene on chromosome 11. This gene defect codes for the sickle beta-hemoglobin characterized by the substitution of valine for glutamic acid at the sixth position of the beta-chain (Pauling et al., 1949; Ingram, 1957). This inherited affection also called Sickle cell anemia (SCA) is a condition characterized by defect in plasma and erythrocyte lipids associated with a chronic oxidative stress (Oztaz et al., 2011). These two morbid processes disturb lipid homeostasis (Rice et al., 1986; Diatta et al., 2002) and induce lipidperoxidation which promotes the accumulation of malondialdehyde, lysophosphatides and oxidizing agents (Diatta et al., 1999; Hebbel et al., 1982). These products, which are found at higher levels in the erythrocytes and plasma of SCA patients, act as powerful catalysts for LDL oxidation. The generated hydroperoxides and oxidized low density lipoproteins (oxLDL) induce an atherogenic process and serial deleterious effects (Peluso et al., 2012; Itabe, 2009). They promote a defect in lipid metabolism and abnormalities in lipids homeostasis by compromising the Lecithin cholesterol acyltransferase and the paroxonase activity (Bielicki and Forte, 1999; Aviram et al., 1999). These two enzymes have been shown to play a key role in esterification of cholesterol on high density lipoprotein (Santamarina-Fojo et al., 2000) and in the lipid oxidation (Hine et al., 2012).

Consistent with these data involved in atherogenesis, the aim of this study is to evaluate plasma lipids and oxidized LDL concentrations in SCA patients in order to verify the presence of an atherogenic lipid profile.

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MATERIALS AND METHODS

The subjects of this study were randomly selected from Aristide Le Dantec hospital and from two monitoring centers for SCA patients, namely the CNTS (Centre National de Transfusion Sanguine) and Albert Royer Children's Hospital of Fann.

These health facilities are located in Dakar in Senegal. Eligibility criteria of this study population included absence of traditional cardiovascular risk factors (obesity, diabetes mellitus, hypertension, tabagism, alcoholism) and absence of any therapy or affection underlying dyslipidemia. Informed consent from each subject was obtained for participation in the study. Then, 5 ml-blood was collected, by venipuncture in the antecubital fossa, in a tube containing lithium heparin and 5 ml in a dry tube. The serum obtained from the dry tubes was divided into aliquots and frozen at -80°C for the lipid profile and quantification of oxidized LDL. The heparin tubes were used for Emmel test and hemoglobin electrophoresis.

Plasma cholesterol and triglycerides were determined by enzymatic methods according to the manufacturer instructions on COBAS INTEGRA 400 analyzer (Roche Diagnostic®, Germany). HDL and LDL cholesterol were determined on the same instrument by direct methods. Oxidized LDL was quantified by ELISA method using kits marketed by Mercodia (Mercodia®, Sweden). Mercodia oxidized LDL ELISA is an enzyme immunoassay based on a direct sandwich technique utilizing anti-oxLDL antibodies as first antibody. Before the ELISA test, serum was diluted in order to obtain the same concentration of total LDL (0.5 g/L). This normalization concerns both patients and control subjects.

Statistical analysis

The results were expressed as mean ± standard deviation. Statistical analysis was carried out using EPI INFO 6. Student's t-test was used for comparing the different concentrations obtained. A difference to the value of p < 0.05 was considered significant.

RESULTS

This study population is composed of 118 individuals aged 15 to 36 years divided into three groups according to the electrophoretic patterns of hemoglobin and the Emmel test: 43 homozygous sickle cell patients (SS), 33 heterozygous sickle cell patients (AS) and 42 control subjects (AA). The individuals in the various groups were matched for age and sex. The lipid plasma concentrations (total, HDL and LDL cholesterol and triglycerides) of the three groups are shown in Table 1. Total, LDL and HDL cholesterol are significantly lower in homozygous SCA patients compared to the control groups (p < 0.001); triglycerides are significantly higher in SCA patients than in control group (Table 1). The decrease in LDL cholesterol is associated to an increase in LDL particle oxidized fraction (Table 1). Really, the oxidized LDL level per Gramm of total LDL is significantly higher in SCA patients than in the control groups (p < 0.05). Table 1 shows also the results of lipid indexes. The LDL/HDL ratio is not significantly different from one group to another among the study participants. In contrast, the ratio of triglycerides to HDL-c (TG/HDL- ratio) is significantly higher in SCA patients than in controls.

Table 2 presents the lipid data of the AA and SS groups according to the sex: apparently the differences between control subjects and SCA patients are more pronounced in males for all parameters but HDL- cholesterol and triglyceride levels show a more pronounced difference in females.

DISCUSSION

The hypocholesterolemia (total and LDL) observed in this study SCA patients confirms the results of a number of studies (El-hazim et al., 1987; Oztaz et al., 2011; Rahimi et al., 2006; Shores et al., 2003; Vanderjagt et al., 2001; Mokondjimob et al., 2012); the mean cholesterol concentration in this study patients is however particularly low (1.18 g/L).

The more pronounced difference observed in males has also been already reported by Shores et al. (Shores et al., 2003). The decrease of both LDL and HDL status attenuates the variation of the LDL-c/HDL-c ratio. In this case, the lipid index losses in this study the clinical utility reported by Fernandez and Webb (2008).

Hypocholesterolemia may be caused by several mechanisms. Plasma cholesterol is essential to the renewal of erythrocyte membranes and it is mobilized from plasma.
for this function. This turnover is exacerbated in SCA as a result of the alteration of erythrocyte components by intense oxidative stress (Diatta et al., 1999, 2002; Rice et al., 1986; El-hazmi et al., 1987; Ngogang et al., 1989). Hypocholesterolemia may also result from hemodilution caused by the decreased size of red blood cells resulting in an increased plasma volume and having thus a dilution effect on plasma constituents (Shores et al., 2003).

The concomitant decrease in HDL cholesterol is consistent with data reported by several authors (Vanderjagt et al., 2001; Mokondjimobe et al., 2012; Seixas et al., 2010; Nnodi et al., 2012). Other reports describe either no difference (Shores et al., 2003) or an increase in SCA patients compared to controls (Rahimi et al., 2006).

It is well known that HDL levels are associated with age. In women, HDL-Cholesterol levels increase progressively to the fifth decade and then decrease with menopause (Kim et al., 2000). This mechanism may influence the data on HDL Cholesterol concentrations reported by Rahimi et al. (2006): this study concerns women (with SS or AS phenotypes) who are older than the control subjects (23.7, 34.8 and 20.6 years respectively). This is probably the reason for the increase in HDL cholesterol in SCA patients observed by Rahimi et al. (2006). Another finding of this study is the higher triglyceride concentration observed in SCA patients compared to controls (Rahimi et al., 2006).

The values are expressed as mean ± standard deviation. *significantly different from the control group (AA) (p < 0.05).

### Table 2. Sex-related Lipid variations among Sickled hemoglobin carriers compared to healthy controls.

<table>
<thead>
<tr>
<th>Biological parameter</th>
<th>Males (n=44)</th>
<th>Females (n=74)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>SS</td>
</tr>
<tr>
<td>Chol (mg/ml)</td>
<td>1.60 ± 0.37</td>
<td>1.30 ± 0.21*</td>
</tr>
<tr>
<td>HDL-c (mg/ml)</td>
<td>0.42 ± 0.10</td>
<td>0.38 ± 0.09*</td>
</tr>
<tr>
<td>LDL-c (mg/ml)</td>
<td>1.00 ± 0.33</td>
<td>0.76 ± 0.20*</td>
</tr>
<tr>
<td>Triglyceride (mg/ml)</td>
<td>0.80 ± 0.20</td>
<td>0.85 ± 0.25*</td>
</tr>
<tr>
<td>Triglycerides/HDL</td>
<td>1.58 ± 0.66</td>
<td>2.83 ± 1.15</td>
</tr>
</tbody>
</table>

Indeed the role of oxidized LDL or Ox LDL in atherogenesis is well known (Belcher et al., 1999; Huang et al., 2008; Itabe, 2009). Their internalization mediated by macrophage receptors leads to the formation of foam cells (Nagy et al., 1998). The release of the contents of these cells induces inflammation which, in synergy with cytokines, causes the formation of atherosclerotic plaque (Daugherty and Roselaar, 1995; Berliner et al. 1995; Chilson et al., 1999). The outgrowth obtained then increases to the point of obstructing the lumen. In parallel, protein fibrous cap overlying the atheroma is degraded by the metalloproteinases released by macrophages and smooth muscle cells. This erosion promotes thrombus formation. Thus, the large fraction of oxidized LDL in this sickle cell population appears to be a significant risk factor for atherosclerosis. The presence of an atherogenic phenotype in SCA patients has already been reported (Seixas et al., 2010).

The combined effects of decrease in HDL-cholesterol, increased oxidized LDL status, raised triglyceridemia and TG/HDL-c ratio in SCA patients, despite the global hypocholesterolemia, could present a not negligible cardiovascular risk.

### Conclusion

The exploration of classical lipid parameters and oxidized LDL in the sickle cell disease shows that in addition to the qualitative abnormality of hemoglobin, an abnormal lipid profile which includes a decrease in HDL cholesterol level, an increase in triglycerides, in TG/HDL-c ratio and a high rate of oxidized LDL is observed in these patients. These lipid disorders by their powerful atherogenic potential can possibly carry an early cardiovascular risk. Without care, the atherogenic risk could be an obstacle to the improvement of the survival of patients with sickle cell disease.

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Full Length Research Paper

Treatment of lead-poisoned rats through oral administration of palm oil extracts

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Accepted 4 February, 2014

The palm fruit (*Elaeis guineensis*) is the source of palmitic-oleic rich semi-solid fat and fat-soluble minor components, made up of vitamin E (tocopherols and tocotrienols), carotenoids and phytosterols. This study was in two parts. Firstly, it examined the effects of palm oil fractionation methods on recovery and concentration of carotenoids, tocopherols and tocotrienols which are known to have nutritional and medicinal properties. Secondly, the study also investigated lead (Pb) poisoning effects on blood of albino rats and the efficacy of treatment with crude palm oil and palm oil extracts. The extraction methods employed in the study were adsorption chromatography, saponification and bleaching. The palm fruits used were of the *Tenera* variety, obtained from a plantation located at Goaso in Ghana. Examination of the palm oil extracts revealed that the unsaponifiable fraction contained 19,570 ppm carotene, 39,290 ppm vitamin E (tocols), 2.7% of carotenes, and 4.6% of free fatty acids (FFA). The adsorption chromatographic isolated fraction contained 16,310 ppm of carotenes, 19,870 ppm of tocols and 3.3% of FFA. The bleached fraction had a reduced level of carotenes and tocols with values of 209 ppm and 640 ppm respectively. A high FFA of 13.1% was recorded. The crude palm oil contained 530 ppm of carotenes, 1,040 ppm of tocols and 4.6% of FFA. Oral administration of 2 g/L lead acetate significantly decreased red blood cell count, haemoglobin level, haematocrit value and platelet count 40 days after treatment in rats, compared with the control group (that is, rats administered with deionized water). Crude palm oil and palm oil extracts administered (0.5 ml/kg body weight/day) significantly restored the normal blood conditions of the lead-poisoned rats. However, the bleached fraction had no significant effect on the rats. The unsaponifiable matter and the adsorption chromatographic fractions reversed the poisoning effect of lead in the rats to near normal levels. The efficiency of these extracts in treating lead toxicity depended on the contents of carotenoids and tocols, which are biological antioxidants. Considering oxidative stress as the major mechanism of lead toxicity, and this study has shown that the high level of antioxidants in palm oil extracts makes it effective natural product for the treatment of lead poisoning.

**Key words:** Antioxidant, carotene, lead poisoning, palm oil, tocopherols (tocols).

INTRODUCTION

Oil palm (*Elaeis guineensis*) is believed to have originated from West Africa. The commercial value of the crop lies mainly in its oil. Oil palm is unique in that it produces two types of oil. The fleshy mesocarp produces palm oil and the kernel produces palm kernel oil. Both are edible oils but with very different chemical composition, physical properties and applications (Poku, 2002).

There are three varieties of oil palm. The *Dura*, the main variety found in the groves, has been the main source of palm oil in Africa. The *Dura* has a large nut with...
a thick shell and thin mesocarp. The other variety is *Pisifera*. The *Pisifera* has a small fruit with no shell. Breeding works involving crossing the *Dura* and a shell-less *Pisifera*, have led to the development of a hybrid variety called *Tenera* which has much thicker mesocarp containing much more oil than either of its parents. The *Tenera* palm kernel is smaller than the *Dura* kernel although the *Tenera* bunch is much larger than *Dura*. In all, the *Tenera* is a much better variety for industrial and economic purposes.

Palm oil is mainly made up of palmitic and oleic fatty acids which tend to be semi-solid fat at room temperature (Sundram et al., 2001). Crude palm oil possesses 1% minor components; amongst them are carotenoids (α and β carotene), vitamin E (in the form of tocotrienols and tocopherol). Crude palm oil is considered to be the richest natural source of carotenoids (about 15 times more than in carrots). The human body uses carotenoids as Vitamin A (Choo et al., 1997). Although present in small quantities, these minor constituents, to a certain extent are responsible for the healing or medicinal properties of palm oil.

Carotenoids and tocols serve as biological antioxidants necessary for protection of cells and tissues from oxidative stress (Packer et al., 2001). These natural antioxidants act as buffers against free radicals and are believed to play a protective role in cellular ageing, atherosclerosis, cancer, arthritis, and Alzheimer’s disease (Goh et al., 2012; Parker, 1991). Aside the nutritional properties, the healing properties of palm oil have been recognized for generations (Sundram, 2011). Until modern medicine arrived, red palm oil was the remedy of choice for nearly every illness in most parts of Africa (Chandrasekharan et al., 2000). When someone got sick, drinking a cupful of palm oil was a remedy of choice. Even today, many traditional healers rely upon the age-old treatment technique (Kritchevsky, 2000). Today, scientists are recognizing the value of red palm oil in the treatment and prevention of diseases. There have been a number of scientific studies that support the medicinal use of palm oil. Hornstra, (1988) in the Netherlands first demonstrated that palm oil has an anti-clotting effect, and is as antithrombotic as the highly unsaturated sunflower seed oil. A human study showed that tocotrienols (from palm oil) supplementation can reduce re-stenosis of patients with carotid atherosclerosis (Goh et al., 2012). Tocopherol and its relative, tocotrienol in palm oil, inhibit human platelets from “sticking” to each other. A study conducted by Rand et al., (1988) showed that a palm oil diet either increases the production of prostacyclin which inhibits blood-clotting or decreases the formation of thromboxane which induces blood-clotting.

Chelating agents have long been used in the pharmacological treatment of lead poisoning and other heavy metal poisoning, the management of which is still a problem, particularly in developing countries. These drugs produce significant and frequent side effects, depending on their mechanisms of action which may lead to minor complications such as headache, nausea and vomiting, rashes, anaemia, nasal congestions (Tell et al., 2010), and severe complications including renal toxicity progressing to nephrotic syndrome, have been described (Tell et al., 2010). Economically, they are expensive and cannot be easily afforded by the rural poor who are mostly vulnerable to metallic poisoning. Natural products used for treatments have proven to have minimal side effects and are also cost-effective.

This study therefore aimed at producing and using various palm oil fractions for the treatment of lead poisoning in rat models.

**MATERIALS AND METHODS**

**Sample collection**

Ripened palm fruits were obtained from an oil palm plantation located at Gaaso in the Brong Ahafo Region of Ghana. The *Tenera* variety was selected for this study.

**Extraction of palm oil**

A 10 kg freshly harvested ripe oil palm fruits were boiled in a 20 L pressure cooker for four hours. A pulp mass was produced by pounding the boiled fruits using wooden pestle and mortar. The pulp was immersed in 10 L water and stirred thoroughly. The fibres and the seeds were filtered out using a basket as a sieve. The filtrate or infusion was poured into an aluminium cooking pot and boiled for four hours. The heated mixture was allowed to cool and 2 L of cold water was added to the surface by a sprinkler. The palm oil which set on top of the aqueous portion of the boiled filtrate was scooped into a fresh container. The collected oil was heated gently for ten minutes to remove traces of water.

**Fractionation of crude palm oil**

Three palm oil fractionation methods were employed in this study:

**Preparation of unsaponifiable extract**

This method was as described by Meloan, (2009) with some modifications. A 10 ml sample of crude palm oil was placed in a 250 ml conical flask and 15 ml of 20% sodium hydroxide added and stirred with the aid of a magnetic stirrer. The flask was corked and connected to a vacuum pump. The mixture was boiled on a hot plate until the solution thickened. The flask was removed from the hot plate and disconnected from the vacuum pump. Stirring continued as the solution cooled slowly. The cooled solution was transferred into a 500 ml separatory funnel and 200 ml of hexane was added. It was shaken and mounted on a retort stand to settle. The tap was opened to elute the bottom layer (soap). The upper layer was washed several times with distilled water until the waste was drained away completely. This upper layer was labelled as the unsaponifiable extract of palm oil. This was then stored in a cold dry place.

**Preparation of silica gel palm oil extract**

The method was based on the one described by Ahmad et al. (2008). A chromatographic glass tube, 3.0 cm in diameter, was mounted on a retort stand. A small glass wool was plugged at the
bottom of this tube. An adsorbent, silica gel (70 to 230 mesh), was used to fill the column up to a height of 5.0 cm. The sides of the column were tapped four times to remove air spaces. A glass rod was used to press down the adsorbent and flattened the surface. The eluting solvent consisted of 100 ml n-hexane. 10 ml of crude palm oil was dissolved in 30 ml of n-hexane and introduced into the column. The eluent was collected into a fresh flask until the adsorbent in the column became colourless. The collected fraction was stored in a cool, dry place to serve as the silica gel palm oil extract.

**Preparation of bleached palm oil extract**

Bleaching was carried out based on a method described by Patterson (1992). A mass ratio of 1 silica gel to 11 crude palm oil (CPO) was used. An amount of 110 g of CPO was poured into a beaker and heated on a hot plate. With the aid of a magnetic stirrer, CPO was stirred continuously until it reached 100°C. Thereafter, 6 drops of phosphoric acid was added to the CPO, followed by the addition of 10 g silica gel. The adsorbent was added slowly to ensure a uniform mixture with the CPO and also to prevent bubble formation. The mixture was heated again for an hour until it reached 150°C to complete bleaching process. After bleaching was achieved, the mixture was allowed to cool. The spent adsorbent was separated from the bleached palm oil by filtration using Whatman’s No. 1 filter paper. Filtration was carried out in the oven at 80°C, for a period of 22 h.

**Quantitation of carotene**

The carotene content of the crude palm oil (CPO) was determined according to the method described by Coursey (2000). 3 g of CPO was dissolved in 20 ml hexane in a 50 ml volumetric flask. A Spectrophotometer (V-1100, J.P.Selectra, Spain) was used to measure the absorbance at 446 nm. Hexane was used as the blank solution. Using the absorbances obtained, the concentration (ppm) of carotene in the sample was calculated, using the formula below;

\[
\text{[Carotene]} = \frac{K_D \times \text{Absorbance (446 nm) \times Volume (ml)}/100 \times \text{Sample weight (g)}}{K_{diff}}
\]

Where, Volume = volume of hexane mixed with palm oil, K_D (diffusion coefficient) = 383. The above procedure was repeated with other palm oil extracts.

**Quantitation of tocots as Vitamin E**

0.5 ml Crude palm oil (CPO) was dissolved in hexane in a 1.0 ml vial. The prepared CPO-hexane mix was then injected into a high performance liquid chromatography (HPLC) system (SMI3000SE, Hitachi, Germany) with a fluorescence detector (excitation at 295 nm and emission at 325 nm) and an analytical silica column (25 cm × 4.6 mm ID, stainless steel, 5 μm) was used to analyse vitamin E content, according to the method described by Ng and Tan (1999). The mobile phase used was hexane: tetrahydrofuran: isopropanol (100:6:4 v/v) at a flow rate of 1.0 ml/min. The HPLC system was calibrated using the standard vitamin E (obtained from Merck, Germany) and the concentration (ppm) of vitamin E in CPO was determined. The procedure was repeated with other palm oil extracts. Using the results obtained the concentrations of vitamin E in the samples were calculated, using the following formula:

\[
\text{[Vitamin E]} = \frac{A \times \text{STD}}{A \times \text{STD} \times \text{Wt sample}} \times \text{V hex (ml)} / \text{A sample} \times \text{STD} \times \text{Wt sample (g)}
\]

Where, [STD] = concentration of standard, A sample = area of sample, A STD = area of standard, V hex = volume of hexane, Wt sample = weight of sample.

**Determination of free fatty acid in the palm oil extracts**

Free fatty acid (FFA) contents in the palm oil extracts were determined by titration according to the PORM test method (1990). The palm oil samples for the analysis were melted at 60-70°C and 10 g each weighed into separate 50 ml Erlemeyer flasks. 20 ml ethanol and three drops of phenolphthalein were added and placed on the hot plate. The temperature was maintained at 40°C. The flask was swirled gently while titrating with 0.1 M NaOH to the first permanent pink colour. The volume of NaOH used was recorded. The percentage FFA in the sample was calculated, using the formula:

\[
\text{FFA % as palmitic acid} = \frac{25.6 \times M(\text{NaOH}) \times V}{W}
\]

Where, \( M = \text{Molarity of NaOH solution} \), \( V = \text{volume of NaOH solution used in ml} \), \( W = \text{weight of sample} \).

**In vivo investigation of lead detoxification by palm oil extracts in rat models**

Design and dosing of experimental animals were based on the method of Ashour (2002) with some modifications. Pre-adult male albino rats, weighing 100-120 g, obtained from the Breeding Unit of the Faculty of Pharmacy, KNUST in Kumasi were used in the study. The rats acclimatized for one week in ventilated plastic cages with wire mesh covers. The cages contained sawdust with high urine absorbing capacity. Each cage contained six rats at ambient temperature and humidity with an alternating 12 h light/dark cycle. Diet and water were supplied ad libitum throughout the experimental period. The feed consisted of milled dried maize and fish (5:1 kg). A sublethal dose of 2 g/L lead acetate was used. The dosage of lead acetate and palm oil were based on other studies (Flora et al., 2010; Wang and Quinn, 1999). The entire experimental treatment lasted for 40 days and it involved eight (8) treatments. The Control Group (A) was orally administrated with deionized water daily for 40 days. The first experimental Group (B) was orally administrated with lead acetate solution (2 g/L) daily for 40 days. In the second experimental Group (C) of animals, lead acetate treatment lasted for 35 days followed by five (5) days of deionized water. Rats in the third experimental Group (D) were given lead acetate and crude palm oil simultaneously for the entire 40 days. The Group D was used to show the prophylactic properties of palm oil against lead poisoning. The fourth experimental Group (E) was divided into four sub-groups E1, E2, E3 and E4. Each sub-group contained six rats. Sub-group E1 was administered the lead acetate for 35 days followed by oral administration of 0.5 ml crude palm oil per kg body weight per day for five days. Sub-group E2 was administered with lead acetate (2 g/L) for 35 days followed by 0.5 ml silica gel palm oil extract per kg body weight per day for the remaining five days. Finally, the sub-group E4 rats pre-treated with lead acetate for 35 days were orally treated with 0.5 ml unsaponifiable palm oil extract per kg body weight per day for the remaining five days (Table 1). Lead acetate was administered through drinking water while the palm oil extracts through oral gavaging.

**Blood sampling and haematological analysis**

On the 40th day, animals were anesthetized and blood was taken through cardiac puncture, into two separate 10 ml centrifuge tubes. The first tube contained EDTA and was used for complete blood count (CBC) analysis. Blood sample in the other tube without EDTA was allowed to clot. Serum was subsequently produced by centrifugation at 3000 rpm for 20 min and then kept in the refrigerator at 4°C for analysis. Haematological parameters including red blood cells, haemoglobin, haematocrit and platelet were determined using...
Table 1. Experimental design for the administration of lead acetate, crude palm oil and palm oil extracts in male albino rats.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Oral administration of lead acetate (2g/L)</th>
<th>Oral administration of palm oil and its extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Negative control)</td>
<td>- (Day 0-40)</td>
<td>- -</td>
</tr>
<tr>
<td>B (Positive control 1)</td>
<td>+ (Day 0-40)</td>
<td>- -</td>
</tr>
<tr>
<td>C (Positive control 2)</td>
<td>+ (Day 0-35)</td>
<td>- -</td>
</tr>
<tr>
<td>D</td>
<td>+ (Day 0-40)</td>
<td>+ (Day 0-40) - -</td>
</tr>
<tr>
<td>E1</td>
<td>+ (Day 0-35)</td>
<td>+ (Day 36-40) - -</td>
</tr>
<tr>
<td>E2</td>
<td>+ (Day 0-35)</td>
<td>- + (Day 36-40) - -</td>
</tr>
<tr>
<td>E3</td>
<td>+ (Day 0-35)</td>
<td>- - + (Day 36-40) -</td>
</tr>
<tr>
<td>E4</td>
<td>+ (Day 0-35)</td>
<td>- - - + (Day 36-40)</td>
</tr>
</tbody>
</table>

The animals in each group were treated for 40 days. The symbol (+) indicates presence of the factor and (-) represents absence of the factor under consideration.

Table 2. Concentration of carotenes and tocols in crude palm oil and palm oil extracts.

<table>
<thead>
<tr>
<th>Phytonutrient</th>
<th>Concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude palm oil</td>
</tr>
<tr>
<td>Carotenes</td>
<td>530</td>
</tr>
<tr>
<td>Tocols</td>
<td>1040</td>
</tr>
</tbody>
</table>

Table 3. Free fatty acid (FFA) content of crude palm oil and palm oil extracts.

<table>
<thead>
<tr>
<th>Crude palm oil and fraction</th>
<th>Free fatty acid content (%) v/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude palm oil</td>
<td>4.6</td>
</tr>
<tr>
<td>Silica gel extract of palm oil</td>
<td>3.3</td>
</tr>
<tr>
<td>Bleached palm oil</td>
<td>13.8</td>
</tr>
<tr>
<td>Unsaponifiable matter of palm oil</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Sysmex analyzer ((Lincolnshire, USA).

Statistical analysis

Data collected were expressed as mean values ±SE (n = 6). Means of samples were compared by one-way analysis of variance (ANOVA). Significant differences between means were determined by Bonferroni’s multiple comparison test (P<0.05) and results presented graphically. The software used was GraphPad Prism version 4.0 (San Diego California, USA).

RESULTS

Quantitation of carotene and tocol contents of palm oil and its extracts

The unsaponifiable palm oil recorded the highest concentration of carotenes (19570 ppm) and tocols (39290 ppm). This was closely followed by silica gel extract which recorded 16310 ppm of carotenes and 19870 ppm of tocols. The bleached palm oil had the least amount of both carotenes and tocols (Table 2).

The bleached palm oil was found to contain the highest amount of free fatty acids (13.8% v/v) than the other palm oil extracts (Table 3). The crude palm oil was second highest in terms of free fatty acid content and about three times lower than bleached oil. Silica gel extract contained 3.3% (v/v) FFA, about four times as in the bleached oil extract. The unsaponifiable matter had the least level of FFA (Table 3).

Haematological analysis

Oral administration of lead acetate solution (2 g/L) for a period of 40 days caused very significant decrease (P<0.01) in RBC count. When lead acetate was administered for 35 days, RBC count still exhibited a highly significant decrease (Figure 1). In animals orally gavaged with palm oil (0.5 ml/kg body weight) alongside lead acetate, a significant decrease was observed as compared to the control. The oral gavaging of crude palm oil and palm oil fractions to the 35-day long lead-poisoned rats (Groups E1, E2, E3 and E4) showed various degrees of increments in their RBC counts. The unsaponifiable palm oil extract exhibited the highest effect in restoring normal RBC count and the bleached palm oil with the least effect
Figure 1. Effects of orally administered lead acetate (2 g/L), crude palm oil and palm oil extracts on red blood cells count of albino rats. A (negative control), B (rats given lead acetate for 40 days), C (rats given lead acetate for 35 days), D (rats given lead acetate and crude palm oil), E1 (rats treated with crude palm oil after 35 days of lead poisoning), E2 (rats treated with silica gel extract after 35 days of lead poisoning), E3 (rats treated with bleached palm oil after 35 days of lead poisoning) and E4 (rats treated with unsaponifiable matter after 35 days of lead poisoning). Values are means ± SE (n=6). Bars with different letters show significant differences (p<0.05).

The groups administered lead acetate (2 g/L) for 35 days showed a significant decrease in haemoglobin content, compared to the control (Figure 2). The effect was similar in the group treated with lead acetate over the 40 day period. The groups given palm oil or palm oil fractions with the exception of those given bleached palm oil had higher haemoglobin levels. Silica gel palm oil extract and unsaponifiable palm oil extract were the most effective in restoring normal levels of haemoglobin. Bleached palm oil fraction (Group E3) produced the least effect on haemoglobin. The rats that were given lead acetate alongside palm oil (Group D) showed increase in haemoglobin content when compared with the groups that were given lead acetate only (Figure 2).

There was significant reduction in haematocrit (Hct) value when animals were given lead acetate (Figure 3). The rats in the Group C in which lead acetate was discontinued showed significant decrease in Hct value relative to the negative control Group A). Administration of palm oil and palm oil extracts to 35-day long lead-poisoned animals significantly increased Hct value over the untreated rats. The treatment with crude palm oil maintained the Hct values close to the normal level (Figure 3).

The mean values of platelet count of lead-treated rats showed significant decrease against the negative control (P<0.05). The group administered with crude palm oil and lead acetate simultaneously showed significant decrease in the platelet count compared to the negative control (Group A). The rats given crude palm oil and palm oil extracts recorded platelet counts close to the normal levels. With the exception of bleached palm oil extract, all the palm oil fractions significantly restored the platelet count in the lead-poisoned rats.

DISCUSSION

The present study has shown that saponification and adsorption chromatography are good techniques for concentrating tocols and carotene contents of palm oil. The palm oil fractionation techniques employed in the study successfully produced palm oil extracts containing nearly ten times (10x) tocols and carotene as found in the crude palm oil. The amounts of phytonutrient in palm oil were studied by Choo et al. (1997). Earlier studies have established crude palm oil to contain nearly 1% minor components made up of phytonutrients among which include carotenoids, tocopherols and tocotrienols and sterols. Thermal bleaching on the contrary, led to significant reductions in the levels of the phytonutrients in palm oil (Table 2). This could be due to instability of tocols and carotene to heat (Borner et al., 2009).

The free fatty acid (FFA) analysis of the crude palm oil and purified extracts showed direct correlation between temperature and FFA during the thermal bleaching process. FFA liberation from the parent structure, triglyceride,
Figure 2. Effects of orally administered lead acetate (2 g/L), crude palm oil and palm oil extracts on haemoglobin content of albino rats. A (negative control), B (rats given lead acetate for 40 days), C (rats given lead acetate for 35 days), D (rats given lead acetate and crude palm oil), E1 (rats treated with crude palm oil after 35 days of lead poisoning), E2 (rats treated with silica gel extract after 35 days of lead poisoning), E3 (rats treated with bleached palm oil after 35 days of lead poisoning) and E4 (rats treated with unsaponifiable matter after 35 days of lead poisoning). Values are means ± SE (n=6). Bars with different letters show significant differences (p<0.05).

Figure 3. Effects of orally administered lead acetate (2 g/L), crude palm oil and palm oil extracts on blood haematocrit value of albino rats. A (negative control), B (rats given lead acetate for 40 days), C (rats given lead acetate for 35 days), D (rats given lead acetate and crude palm oil), E1 (rats treated with crude palm oil after 35 days of lead poisoning), E2 (rats treated with silica gel extract after 35 days of lead poisoning), E3 (rats treated with bleached palm oil after 35 days of lead poisoning) and E4 (rats treated with unsaponifiable matter after 35 days of lead poisoning). Values are means ± SE (n=6). Bars with different letters show significant differences (p<0.05).
occurs in a process characterized by high temperature and other hydrolytic solvents (Siew, 2011). The bleached palm oil contained 13.8% (v/v) FFA, an amount nearly 300% of what is found in the crude palm oil. Conversely, thermal bleaching of palm oil reduces carotene and tocol contents. Fractionation using saponification and adsorption chromatography produced extracts with reduced levels of FFA. Saponification and adsorption chromatography produced opposite effect - thus high concentrations of carotene and tocols, and reduced FFA content.

When 2 g/L of lead acetate was orally administered for 40 days, there was a highly significant increase in blood lead levels (BLL). Earlier studies have shown time-and-dose dependency of blood lead levels (Flora et al., 2010). In the second part of the present study, the effects of oral administration of lead acetate (2 g/L) on haematology of male rats weighing 100-120 g were determined. The current study shows oral administration of 2 g/L lead acetate to male albino rats for a period of 35-40 days to be effective in inducing haematological impairments or insults. The lead dose administered is comparable to amounts found in common sources of lead in domestic and industrial settings such as paint, crayon, pencils, chalks and some clay.

The lead-poisoned rats showed significant decreases in their RBC count (Figure 1). Patrick (2006) earlier reported in a study that erythrocytes have high affinity for lead, binding 99% of the lead in the bloodstream. Lead destabilizes the plasma membranes resulting in the decrease in RBC fluidity and subsequently increased erythrocytes haemolysis (Philip and Gerson, 2012).

The results establish RBC count (Figure 1), haemoglobin content (Figure 2), haemtocrit value (Figure 3) and platelet count (Figure 4) to be significantly decreased in rats exposed to lead acetate. Lead interferes with haemoglobin synthesis. Specifically, lead inhibits amino-levulinic acid dehydratase (ALAD) enzyme (Ellenhorn, 2012) which is critical enzyme in the biosynthetic pathway of haem (Farrant and Wigfield, 1998). The decrease in haematocrit and platelets counts result from lead-induced anaemia through haem biosynthesis and reduced life span of the RBC (Gurer-Orhan, 2008).

Treatment of the lead-poisoned rats by oral administration of various doses of crude palm oil and the three palm oil extracts showed significant recovery indicated by RBC count, haemoglobin concentration, haematocrit and platelet count (Figures 1-4). The prophylactic effect of palm oil on lead-poisoned rats, characterized by the improvement of haematological indices has been reliably shown by this study. The two palm oil extracts, unsaponifiable matter fraction and silica gel extract, contained high amounts of phytonutrients composed mainly of carotenes, tocopherols and tocotrienols. These properties made the two extracts more effective in alleviating the lead toxicity in the animals.

The two palm oil extracts independently reversed haematological effects of lead poisoning to near normal levels. Crude palm oil, though not as effective as the unsa-
ponifiable and silica gel palm oil extracts, was also effective in detoxifying lead poisoning in the rats. Antioxidant properties of tocots and carotenens have long been established (Packer et al., 2001; Brian, 1991). Thus the high efficacy of the silica gel and unsaponifiable extracts and to a lesser extent the crude palm oil might be due to anti-oxidation capacity of carotenens and tocots. The bleached palm oil, with trace amounts of carotenens and tocots had no significant effect on the haematological indices of lead-poisoned rats (Figures 1-4).

Oxidative stress generated by reactive oxygen species (ROS) and the depletion of antioxidant reserves is the dominant and logical mechanism for the lead toxicity (Ercal et al., 2001). Palm oil has been shown to elevate blood levels of antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (Ian et al., 2005). It can therefore be suggested that antioxidant action of crude palm oil and palm oil extracts might play a role in the treatment of lead poisoning. Other mechanisms of action such as chelation of the lead ion (Pb²⁺) and enhanced excretion of the metal ion caused by carotenens and tocots could be important in the body’s detoxification of lead (Hsu, 2011).

CONCLUSIONS

Palm oil extracts derived from saponification and silica gel chromatography contained high amounts of tocots and carotenens. The levels ranged from 19 to 38 times that of the crude palm oil. The thermal bleached palm oil extract contained trace amounts of tocots and carotenens than that in the crude palm oil. Oral administration of various doses of unsaponifiable matter and silica gel extract of palm oil at different treatment times significantly reversed abnormal haematological indices in lead-poisoned rats. The bleached palm oil extract, on the other hand, was ineffective in treating lead poisoning in the affected rats.

ACKNOWLEDGEMENTS

We acknowledge the immense contribution of the following laboratory staff of the Kwame Nkrumah University of Science and Technology: Mr. Thomas Ansah, a technician of the Department of Pharmacy, Faculty of Pharmacy; Mr. Frank Bonsu Ayegman and Mr. William Appau, technicians, and Mr. Benjamin Afful, a research assistant of the Clinical Analysis Laboratory (CAmLab) and Aflatoxin Laboratory of the Department of Biochemistry and Biotechnology.

REFERENCES


Extraction and comparison of fibrolytic enzyme additives from gut of 11 ungulates

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Accepted 16 January, 2014

Microbial populations in herbivores gut attack, degrade and ferment structural carbohydrates in forage cell walls, producing volatile fatty acids and microbial proteins for the host. Exocellulases, endocellulases and cellobiases are the major cellulolytic enzymes while hemicellulase (xylanase) exposes cellulose for fermentation. This study aimed to isolate proteins that hydrolyse fibre or assist fibrolysis in any way from 11 herbivores gut microbial ecosystems (cow, sheep, horse, camel, elephant, zebra, llama, wildebeest, giraffe, impala and buffalo), optimize their working conditions and compare fibrolytic activities as potential feed additives. Exocellulase, endocellulase, xylanase and cellobiase in rumen and fecal crude enzyme extracts were assayed and compared. A broad in vitro pH range (4.5 to 8.0) of endocellulase activity was observed for all ecosystems. Enzymes from horse, zebra, impala, wildebeest and elephant showed the highest potential for degrading fibre encouraging further investigation as feed additives.

Key words: Crude cellulase extraction, hemicellulase, rumen and faecal inoculum, ungulates.

INTRODUCTION

Cellulolytic microbes possess three types of enzymes which enable partial or complete solubilisation of plant cell walls. These enzymes are exocellulases (EC 3.2.1.91), endocellulases (EC3.2.1.4) and cellobiases (EC 3.2.1.21). These complex enzyme consortia function synergistically to hydrolyse plant cell walls. Exocellulases remove cellobiose units from the non-reducing ends of the cellulose chain, endocellulases act in a random fashion on the region of low crystallinity on the cellulose chain and β-glucosidase produces glucose units from cellobiose (Lynd et al., 2002). Although glucosidase is not strictly necessary for cell wall solubilisation, its presence enhances cellulose hydrolysis because these three enzymes function synergistically and inhibition of endocellulase and exocellulase by cellobiose is avoided. Solubilisation of plant cell walls does not solely depend on cellulases because of the complexity of its association with other macromolecules (Lynd et al., 2002). Apart from celluloses, hemicelluloses are the second most abundant plant fraction available in nature with xylan being the most abundant of all the hemicelluloses (Petzold et al., 2008). Lignin, pectin and tannins are examples of macromolecules that are often found in close association with cellulose (Mussatto et al., 2008). Therefore for solubilisation of cellulose in roughages to be successful the enzymes hemicellulase, ligninase, tannase and pectinase are required (Fon and Nsahlai, 2012).

Not all the microbes (bacteria, fungi and protozoa) that are found in both the fore- and hindguts have fibrolytic or hemicellulolytic properties but can assist in the fermentation process either directly or indirectly. Fermentation yields volatile fatty acids which can reduce the pH of the rumen fluid to less than 6, thereby halting the activity of most rumen microbes which function optimally at pH 6.2 and above (Russell and Dombrowski 1980). The presence of

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non-fibrolytic microbes that utilise acetic acid as a source of energy reduces its concentration hence maintaining the optimal rumen pH.

Different studies have shown that adding exogenous enzymes to herbivore diet increases milk production due to an increase in feed digestion (Schingoethe et al., 1999; Kung et al., 2000; Yang et al., 2000). In a similar study on fibre rich forages, Feng et al. (1996), Krause et al. (1998), Yang et al. (1999) and Beauchemin et al. (2000) observed only small increments in fibre digestion. In vitro increases in dry matter (DM) digestibility have also been reported by Nakashima et al. (1988), Feng et al. (1996), and Yang et al. (1999).

It is possible that microbial enzymes from herbivorous species consuming different food items in the pasture can vary in plant cell wall hydrolysis (Antonio et al., 2011). The major limitations in investigating the fibrolytic potential of the different microbial ecosystems especially that from the wild includes; animals being expensive to kill for rumen fluid, expensive extraction and purification techniques. Several methods have been applied to isolate proteins from the rumen fluid or faecal samples including ammonium sulfate precipitation, three phase partitioning precipitation, gel filtration and acetone precipitation. Ammonium sulphate is one of the most used methods in precipitating crude enzymes because of its simplicity, availability and cost effectiveness (Henry et al., 1974). The main objective of this study was to identify potential fibrolytic enzyme additives secreted by microbes from gut of 11 ungulates.

MATERIALS AND METHODS

Experimental animals and nutrition

Animals were chosen with no preference to sex or species. Bostaurus (fistulated Jersey cows), Equus caballus (horse) and Ovis aries (fistulated sheep) were from Ukulinga Research farm, University of KwaZulu Natal (UKZN), Pietermaritzburg. Llama glama (llama), Loxodonta africana (elephant) and Camelus dromedarius (camel) were provided by Brian Boswell Circus, Pietermaritzburg. Equeus quagga (zebra), Connochaetes taurinus (wildebeest), Aepyceros melampus (impala), Syncerus caffer (buffalo) and Giraffa camelopardalis (giraffe) were provided by the Taia Game Reserve, Umbumbulu, KwaZulu-Natal (SA).

This study was conducted in winter (April – August). Camel, elephant and llama were kept in an enclosed area and fed with grass hay (Eragrostis dominant) on a regular basis. Game animals such as the impala, wildebeest, zebra and buffalo were grazing on a dry land in an open field where Pennisetum clandestinum (Kikuyu grass) dominated, with other grasses. The giraffe browsed on a variety of tree leaves (various acacia species). At the Ukulinga research farm, horse, sheep and cow were fed entirely on grass hay (Eragrostis dominant).

Faecal and rumen fluid collection

A modified procedure previously described by Smith et al. (1974) was employed when collecting rumen samples from fistulated cows and sheep. Rumen digesta (200 ml) was collected through a fistula, strained through four layers of cheese cloth, and treated immediately with 150 µl of phenylmethylsulfonyl fluoride (0.1 mmol/L PMSF) to inhibit proteases from lysing enzymes of interest (Owolabi et al., 1888; Vercoe et al., 2003). On the other hand, faeces were collected in situ from the horse, giraffe, buffalo, impala, wildebeest, zebra, elephant, llama and and camel and transported in an airtight insulated flask maintained at 38°C. All experimental animals sample collection was governed by the UKZN ethical roles (083/10/animal).

Protein exclusion and concentration measurements

Protein isolation was done using a modified procedure described by Henry et al. (1974). Both rumen fluid (100 ml) and faeces were used for protein isolation. In the case of faeces, 20 g was dissolved in 80 ml of a homogenization buffer (50 mmol/L sodium-acetate buffer, 0.02% (m/v) NaNO₃, 0.1 mmol/L PMSF and 0.1 mmol/L EDTA at pH 5.0) before topping to 100 ml. Sample solutions in sealed centrifuge tubes were placed on a shaker for 30 min to facilitate bacteria detachment from fibres before centrifuging (7500 xg, 30min at 4°C) to sediment particulate matter (Figure 1). The supernatant was centrifuged (30 000 xg, 15 min at 4°C) to sediment bacteria cells. The sedimented particles and bacteria cells were dissolved in 10 ml and 5 ml of homogenisation buffer, respectively, sonicated for 3 min at 1 min intervals using a O125 Ultrasonic Processor (to lyse bacteria cells) and centrifuged (30 000 xg, 15 min at 4°C). The supernatants of the three different steps were pooled and centrifuged (30 000 xg, 15 min at 4°C) to sediment any unlysed cells. Ammonium sulfate (60% (m/v) (NH₄)₂SO₄) was dissolved in the sample solution to facilitate protein precipitation before centrifuging (7000 xg, 15 min at 4°C). The precipitate was dissolved in 10 ml of storage buffer (20 mmol/L sodium acetate, 0.02 % (m/v) NaNO₃, and 0.1 mmol/L EDTA at pH 5.0) before dialyzing (12 h in storage buffer). Dialyzed sample solutions were concentrated using polyethylene glycol 20 000.

Bradford (1976) dye-binding assay was used to determine crude enzyme concentrations. A standard curve for a micro assay was prepared with bovine serum albumin concentrations of 0, 5, 10, 20, 30 and 40 µg/100 µl of reaction buffer (20 mmol/L sodium acetate, 0.02% (m/v) NaNO₃ and 0.1 mmol/L EDTA at pH 5.0; Goeser et al., 2009). Bradford reagent (900 µl of 600 mg Coomassie Brilliant Blue G-250 dissolved in 1 L of 2% perchloric acid and filtered through Whatman number 1 filter paper) was pipetted onto the standard solution (100 µl) and absorbance read at 595 nm after 3 min. For the unknown protein sample solutions, 5 µl was diluted into 100 µl of the reaction buffer before adding Bradford reagent (900 µl) and allowed to stand for 3 min. Each assay was performed in triplicates and absorbance was measured at 595 nm. Unknown protein concentrations were determined from the standard curve.

Activities of crude enzyme samples and pH optimization

The method described by Seyis and Aksoz (2005) for the optimisation of xylanase activity from Trichoderma1073 D3 was modified slightly and used in this assay. Enzyme stability within a pH range of 4.0 to 8.0 was determined by pre-incubating the crude enzyme extracts in 500 µl sodium-acetate buffer (400 µg/500 µl 20 mmol/L sodium-acetate buffer) at different pH values for 30 min before adding 500 µl of carboxymethyl cellulose (1% (m/v) CMC in 20 mmol/L sodium acetate, 0.02% (m/v) NaNO₃ and 0.1 mmol/L EDTA) and incubating for 2 h at 39°C. The substrate concentrations as well as the crude enzyme concentration were constant throughout the experiment. Enzyme reactions were stopped by heating at 100°C. Incubated samples were centrifuged at maximum speed (12875 xg) on a desktop centrifuge for 5 min and 400 µl of the sample solution was used for reducing sugar analysis.

Quantification of reducing sugars

Dinitrosalicylic (DNS) method (Miller, 1959) was chosen for analysis because of its sensitivity, simplicity and availability. A standard curve...
of micrograms of reducing sugars (glucose or xylose) against absorbencies at 540 nm was plotted as described by Wood and Bhat (1988). DNS reagent, 600 µl (0.001 M sodium metabisulfite, 0.708 M potassium sodium tartrate, 0.25 M sodium hydroxide, and 0.021 M phenol) was pipetted into 400 µl of the sample solution and boiled for 5 min. The reaction mixture was then cooled under running water and the absorbance measured at 540 nm. The absorbance values were translated into concentration of reducing sugars using the standard curve.

**Enzyme assays**

Exocellulase activity was assayed following a modified version of the method described by Gerrit et al. (1985). Exocellulase activity was measured by pipetting 0.5 ml of 1% (m/v) crystalline cellulose in the reaction buffer into 0.5 ml of crude enzyme solution obtained from different herbivores and incubating for 48 h at 39°C and pH 5.5 (standard assay condition). The enzyme reaction was stopped by boiling at 100°C, and the reaction mixture was centrifuged (6000 x g for 5 min), following which p-nitrophenol (pNP) liberated was analysed by reading the absorbance at 407 nm. The molar absorption coefficient (ε) of pNP at 407 nm was taken to be 18300 mol⁻¹ cm⁻¹ from similar calculations done by Frutos et al. (2002). The number of moles of pNP released was assumed to be directly proportional to the amount of glucose.

Xylanase hydrolyses xylan into xylose and oligosaccharides (Chivero et al., 2001). A modified procedure described by Khanna (1993) was used to assay xylanase activity. The assay was carried out by pipetting 0.6 ml of 0.1% (m/v) xylan solution in the reaction buffer (pH 5.0) into 0.4 ml of crude enzyme solution and incubating at 39°C for 1 h. The reaction was stopped with 100 µl of 200 mmol/L NaHCO₃, centrifuged (at 6000 x g for 5 min) and analysed for reducing sugars using the DNS method. Each ecosystem was represented by three samples each of which was analysed in triplicate. Specific activities of the above enzymes were defined as µg of xylose/mg crude enzyme.

**Statistical analysis**

The results from exocellulase, endocellulase, cellobiase and hemicellulase specific activities as well as total protein, purification fold and yields were subjected to analysis of variance (ANOVA) using the general linear model of SAS. The model was: Yᵢ = µ + Tᵢ + eᵢ, where Yᵢ is the individual observation, µ is the overall mean, Tᵢ
Table 1. Purification of crude enzyme extracts from herbivores using ammonium sulphate.

<table>
<thead>
<tr>
<th>Sample source</th>
<th>Sample fraction</th>
<th>Total protein (mg)</th>
<th>Total activity (nmol glucose)</th>
<th>Specific activity[^1]</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>Supernatant</td>
<td>310[^ab]</td>
<td>2928[^d]</td>
<td>9.4[^i]</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Sheep</td>
<td>Supernatant</td>
<td>313[^ab]</td>
<td>2783[^f]</td>
<td>8.9[^g]</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>(NH$_4$)$_2$SO$_4$ precipitate</td>
<td>138[^b]</td>
<td>1378[^f]</td>
<td>10.0[^i]</td>
<td>1.125</td>
<td>43.9</td>
</tr>
<tr>
<td>Horse</td>
<td>Supernatant</td>
<td>588[^a]</td>
<td>5553[^c]</td>
<td>9.4[^i]</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>(NH$_4$)$_2$SO$_4$ precipitate</td>
<td>233[^b]</td>
<td>8263[^a]</td>
<td>35.0[^a]</td>
<td>3.765</td>
<td>39.6</td>
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<tr>
<td>Camel</td>
<td>Supernatant</td>
<td>396[^ab]</td>
<td>3300[^e]</td>
<td>8.3[^g]</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>(NH$_4$)$_2$SO$_4$ precipitate</td>
<td>333[^ab]</td>
<td>2960[^f]</td>
<td>8.9[^g]</td>
<td>1.067</td>
<td>84.1</td>
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<td>Elephant</td>
<td>Supernatant</td>
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<td>5390[^f]</td>
<td>11.7[^d]</td>
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<td>100</td>
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<td>146[^b]</td>
<td>4536[^f]</td>
<td>31.1[^a]</td>
<td>2.667</td>
<td>31.6</td>
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<tr>
<td>Zebra</td>
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<td>876[^a]</td>
<td>7773[^f]</td>
<td>8.9[^g]</td>
<td>1</td>
<td>100</td>
</tr>
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<td></td>
<td>(NH$_4$)$_2$SO$_4$ precipitate</td>
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<td>5231[^f]</td>
<td>33[^a]</td>
<td>3.688</td>
<td>18.2</td>
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<tr>
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<td>Supernatant</td>
<td>518[^ab]</td>
<td>4317[^f]</td>
<td>8.3[^g]</td>
<td>1</td>
<td>100</td>
</tr>
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<td></td>
<td>(NH$_4$)$_2$SO$_4$ precipitate</td>
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<td>2010[^g]</td>
<td>10.0[^i]</td>
<td>1.2</td>
<td>38.8</td>
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<td>938[^ab]</td>
<td>8337[^f]</td>
<td>8.9[^g]</td>
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<td>100</td>
</tr>
<tr>
<td>Giraffe</td>
<td>Supernatant</td>
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<td>2800[^f]</td>
<td>8.3[^g]</td>
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<td>100</td>
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<tr>
<td></td>
<td>(NH$_4$)$_2$SO$_4$ precipitate</td>
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<td>747[^f]</td>
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<td>1.067</td>
<td>25</td>
</tr>
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<td>Impala</td>
<td>Supernatant</td>
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<td>375[^f]</td>
<td>8.3[^g]</td>
<td>1</td>
<td>100</td>
</tr>
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<td>(NH$_4$)$_2$SO$_4$ precipitate</td>
<td>135[^b]</td>
<td>2025[^g]</td>
<td>15.0[^c]</td>
<td>1.8</td>
<td>29.9</td>
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<td>Buffalo</td>
<td>Supernatant</td>
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<td>4320[^f]</td>
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<td>1</td>
<td>100</td>
</tr>
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<td>(NH$_4$)$_2$SO$_4$ precipitate</td>
<td>170[^b]</td>
<td>2742[^f]</td>
<td>16.1[^b]</td>
<td>1.813</td>
<td>35.0</td>
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<tr>
<td>SEM</td>
<td></td>
<td>52.1</td>
<td>290.1</td>
<td>1.51</td>
<td>0.13</td>
<td>6.81</td>
</tr>
</tbody>
</table>

[^1]: nmol glucose/mg/min.

is the effect of the treatment (enzyme sources, pH or crude enzyme concentrations) and $e_{ij}$ is the random variation.

**RESULTS**

Crude enzymes (endocellulases) activities

The colour of the protein precipitates consisted of variable shades of brown and green. The horse, wildebeest, zebra, buffalo and impala were characterized by a light reddish brown precipitate while those from the elephant, giraffe, llama and camel were characterised by a whitish-brown precipitate. Precipitates from the cow and sheep were characterized by a greenish colour. The crude enzyme extracts were active to the extent that their enzyme specific activities on carboxymethyl cellulose increased (P<0.05) as protein concentration decreased following purification (Table 1). The activity recovered (purification fold) was highest (P<0.05) in the horse and zebras, intermediate in the wildebeest, impala and buffalo, and lowest in the camel, sheep and giraffe. It was interesting to note that the camel with the highest percentage yield (84.1%) had the lowest recovery (1.06 fold).

**pH optimization**

Endocellulase had a broad (P<0.05) pH range of activity for all the experimental microbial ecosystems (Figure 2). Despite the broad pH range, some distinct (P<0.05) peaks of endocellulase activities were observed within the pH range 4.5–5.0 for the elephant, at pH 5 for the horse and the zebra, within pH 5.0–6.0 for the sheep, and within pH 5.0–7.0 for the wildebeest. Endocellulase activity for the rest of the animals (cow, llama, giraffe and camel) responded minimally within pH 4.0 to 8.0 range.

**Optimization of crude enzyme concentrations for enzyme assay**

Generally, product formation increased (P < 0.05) with increasing crude enzyme concentration from the sampled herbivore microbial ecosystems. Glucose liberated from crystalline cellulose, CMC and pNP-G following incubation with crude extracts, increased (P < 0.05) non-linearly (Figure 3). Xylose liberated from xylan by xylanase from crude enzyme extracts also increased (P < 0.05) non-
Figure 2. The effect of pH on endocellulase activity. Product formation was monitored for samples from the reaction mixture of cow (■), sheep (□), horse (●), camel (○), elephant (▲), zebra (Δ), llama (◊), wildebeest (*), giraffe (+), impala (−) and buffalo (▪).

DISCUSSION

Collecting fresh samples with limited exposure to oxygen maximised the presence of anaerobic cellulolytic and hemicellulolytic microbes as well as their enzymes. Limited exposure to oxygen prevents cell death, which was vital for the release of periplasmic proteins during sonication (Dunn et al., 2000). Ammonium sulfate precipitation reduced the amount of unwanted proteins in the samples. This was confirmed by the increase in specific activity of crude enzyme samples with a decrease in protein concentration (Groleau and Forsberg 1983). The reddish-brown protein precipitate observed in horse, wildebeest, impala and zebra was due to high cellobiase content while the greenish colour in cow and sheep precipitates was probably due to the plant pigment chlorophyll. Specific activity and enzyme stability were both affected by pH and temperature. The rate of enzyme reactions might be compromised if these factors were neglected. pH optimisation was vital as enzymes were isolated from 11 different ecosystems which might vary greatly. The pH profile showed that activity occurs with a wide range as opposed to the narrow in vivo pH range (5.6 to 8) described by many authors (Russell and Dombrowski, 1980). This disparity can be ascribed to the presence of enzymes only in in vitro systems unlike in vivo where very low or high pH might compromise microbial growth and survival. However, the peaks observed in the horse (pH 5.5) and elephant (pH 5) demonstrated that these ecosystems were harbouring microbes that were active in slightly acidic conditions. Variations can also be attributed to the genetic differences among strains of microbes in various ecosystems in the expression of these enzymes.

Other factors also affecting activity include enzyme crystalline cellulose, CMC and pNP-G following incubation x substrate concentrations can be observed in the work linearly (Figure 3D). In Figure 3A, the highest increase was obtained in the zebra, horse and wildebeest while the elephant and impala showed moderate increases. High (P < 0.05) levels of product formation were also observed in horse, impala and wildebeest when endocellulase concentrations were varied (Figure 3C). A similar situation was also observed when cellobiase was incubated with pNP-G (Figure 3B). Product formation from the reaction mixtures of the horse, zebra, impala, wildebeest and sheep were dominant while the rest were crowded together at low product formation. The result was not very different when hemicellulases were incubated with xylan (Figure 3D). The product formed in the sheep was as high as those observed from the horse and zebra, while the cow, camel and giraffe activities tended to be high. An enzyme concentration of 340 µg/ml for optimal activity was established as suitable for exocellulase and endocellulase.
The effect of enzyme concentrations on product formation. Product formation from crystalline cellulose (3A), carboxymethylcellulose (3B), p-nitrophenyl β-1,4-glucopyranoside (3C) or xylan (3D) were monitored from the reaction mixture of cow (■), sheep (□), horse (●), camel (○), elephant (▲), zebra (Δ), wildebeest (★), giraffe (+), impala (×) and buffalo (▫). The positive controls (—·—) were commercial cellulase from Aspergillus niger (3A (exocellulase) and 3B (endocellulase)) cellobiase (3C) and xylanase from Thermomyces lanuginosus (3D). The negative control (...) was BSA for all experiments. Exocellulase = crystalline cellulase, Endocellulase = carboxymethyl cellulase.

of Henry et al. (1974) but should be optimised for every sample as enzyme concentration and activity vary relatively to its purification method (Jahir et al., 2011). The results arising from incubating different crude enzyme concentrations with the different substrates (crystalline cellulose, CMC, xylan and cellobiose) established that 340 µg was the ideal concentration to apply in enzyme assays. It was also noted that as the crude enzyme concentration increased the rate also increased linearly but linearity was lost at high crude enzyme concentrations. This was expected because at a high crude enzyme concentration, substrate concentration would become rate limiting. This was similar to the results obtained by Groleau and Forsburg (1983) in studies on cellobiase and endocellulase.

In an attempt to optimise extract concentrations for enzyme assays with the different substrates, it was noted that the rate at which protein samples from the different ecosystems hydrolyse specific substrates was not the same. Crude enzyme samples from the horse, zebra and wildebeest were the most active when incubated with all
four substrates but for the elephant which was also very active when incubated with CMC. Interestingly, the hindgut fermenters contained the most active protein samples. Although, many studies have stated that there are no major differences among the microbes in both fore- and hindgut fermenters (Smith and Mackie, 2004; Smith and Mackie, 2004, Alexander, 1993), the results obtained from this study clearly indicate that there might be some factors influencing activity in these chambers. If time spent by digesta in the hindgut is a major factor (Mould et al., 2005; Schingoethe, 1993) then it is imperative to postulate that microbes in the hindgut will be more efficient in order to extract sufficient nutrients from the food before elimination as faeces. However, not all the foregut fermenters exhibited low activities as the wildebeest; the impala and cow showed intermediate activities with all four substrates. Variation in these activities might have been influenced by differences in ecological niches or composition of microbial species. For herbivores within the same geographical region, the differences in enzyme activity could be attributed to differential genetic evolution of microbes which might have resulted in variable fibrolytic efficiencies.

Conclusion

An enzyme concentration of 340 µg/ml for optimal activity was established as suitable for exocellulase and endocellulase. Crude enzyme samples from the zebra, horse and wildebeest were the most active when incubated with all four substrates while those from the elephant, impala, cow and giraffe were intermediate. Future studies to confirm high activity among these ecosystems would include determining the affinity of enzyme to substrate, maximum velocity as well as their catalytic efficiencies. These results can assist in establishing cellulosomes from microbial population of zebra, horse, impala and wildebeest as feed additives.

ACKNOWLEDGEMENTS

We would like to acknowledge National Research Foundation for funding this project and the University of Zululand and University KwaZulu-Natal for facilitating the project.

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applied to forages at the time of feeding. J. Dairy Sci. 82:996-1003.


Effects of n-butanol fraction of *Gongronema latifolium* leave extract on some biochemical parameters in CCl₄-induced oxidative damage in Wistar albino rats

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Accepted 16 January, 2014

Effects of n-butanol fraction of *Gongronema latifolium* leave extract on some biochemical parameters in carbon tetrachloride-induced oxidative damage in Wistar albino rats were assessed. Fifty-four (54) Wistar albino rats were divided into treatment group and LD₅₀ groups. Group A (normal control) was given feed and water, Group B (vehicle control) was injected with olive oil intraperitoneally, while the rest groups (C, D, E, F and G) were injected intraperitoneally with a single dose of CCl₄ (148 mg/kg) as a 1:1 (v/v) solution in olive oil and all the animals were fasted for 36 h. This was repeated once every week for a period of four (4) weeks. At the end of 28 days of treatment, liver marker enzymes studies showed that there was significant (*p*<0.05) increase in the serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin concentrations in CCl₄-induced control group when compared with the normal control and induced treated groups but there was no significant (*p*>0.05) difference of these liver marker enzymes and bilirubin levels between the normal control and induced treated groups. Kidney function studies showed that there was significant (*p*<0.05) increase in creatinine and urea concentrations of CCl₄-induced control group when compared with the normal control and induced treated groups but there was no significant (*p*>0.05) difference of these liver marker enzymes and bilirubin levels between the normal control and induced treated groups. Also, the kidney and liver antioxidant study revealed significant (*p*<0.05) decrease in superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activities as well as an increase in MDA concentration in CCl₄-induced control rats when compared with the normal control rats. Histological section of the organs shows that the levels of hepatic and renal damage were higher in CCl₄-induction control rats when compared with the normal control and induced treated groups. These findings suggest that n-butanol fraction of methanolic leaves extract of *G. latifolium* may have anti-hepatotoxic, anti-nephrotoxic and antioxidative effects against CCl₄-induced liver and kidney damage in albino rats.

**Key words:** *Gongronema latifolium*, antioxidant, CCl₄, liver, kidney.

**INTRODUCTION**

*Gongronema latifolium* (Asclepiadaceae), is a perennial climber forest leafy vegetable with woody hollow glabrous stems below and characterized by greenish yellow flowers (Okafor, 1989). It is widespread in tropical Africa such as Senegal, Chad and DR Congo as well as grows in the forest of south eastern and western Nigeria where it is widely used...
for medicinal and nutritional purposes (Ugochukwu et al., 2003). G. latifolium occurs in rainforest, deciduous and secondary forests, and also in mangrove and disturbed roadside forest, from sea-level up to 900 m altitude. In Nigeria, information available from the indigenous traditional healers claimed that a decoction of the chopped (Ajibola and Satake, 1992) leaves of G. latifolium has been used in the production of several herbal products which are taken orally (Okafor, 1989) for the treatment of stomach upsets and pains, dysentery, malaria, typhoid fever, worm and cough (Akpan, 2004). Asthma patients chew fresh leaves to relieve wheezing (Okafor, 1989) and a decoction of the roots, combined with other plant species, is taken to treat sickle cell anaemia. A maceration of the leaves in alcohol is taken to treat bilharzia, viral hepatitis and as a general antimicrobial agent (Okigbo et al., 2009). It is also taken as a tonic to avert loss of appetite (Akpan, 2004). Previous studies have revealed that other plants with polyphenols exhibit clear anti-hepatotoxic and anti-nephrotoxic properties (Okafor, 1989) and that flavonoids could protect the liver against oxidative injury induced by CCl₄ in vivo (Akpan, 2004). Although many other plants have been reported to possess anti-hepatotoxic and anti-nephrotoxic properties, the scientific authentication of most of them such as G. latifolium which is used traditionally to treat several diseases is unavailable (Ajibola and Satake, 1992).

The aim of this work was to provide some scientific support for the health benefit of G. latifolium. To achieve this, studies were carried out to investigate the phytochemical constituents of G. latifolium and to evaluate the anti-hepatotoxic and anti-nephrotoxic activities of n-butanol fraction of methanolic leaves extract of G. latifolium against oxidative damage induced by CCl₄ in Wistar albino rats.

MATERIALS AND METHODS

Chemicals/reagents

All assays kits were from Randox Laboratories Ltd. Ardmore, Co. Antrim UK. Chemicals and reagents used were purchased from Sigma Chemical Company St. Louis U.S.A and chemicals used were of analytical grade. Folin ciocaltelu phenol reagent, gallic acid, carbon tetrachloride (Sigma-Aldrich), distilled water and normal saline were used.

Plant material and extraction

Fresh leaves (blend) of G. latifolium were obtained from a homestead garden at Isuofia, Aguata L.G.A., Anambra State, Nigeria in the month of February 2013 and authenticated at the herbarium unit by Gallah U. J. in the Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria where a voucher specimen was deposited. The collected plants were rinsed in clean water and air dried at room temperature for two weeks. The dried leaves were pulverized into powder using Thomas-Wiley laboratory mill (model 4) manufactured by Arthur H. Thomas Company, Philadelphia, PA., U.S.A. before being extracted.

A portion of five hundred grams (500 g) of the pulverized plant leaves was suspended in 2.5 L of methanol for 48 h in large amber bottles with intermittent shaking. At the end of the extraction, the crude methanol extract was filtered using Whatmann No. 1 filter paper (1 mm mesh size) and then concentrated in a water bath maintained at 45°C until greenish black residues were obtained. Certain gram of the crude extract was then subjected to phytochemical analysis using standard procedures (Sofowora, 1993). Also, 51 g of the crude extract was reconstituted with 250 ml of methanol for further fractionation and the fractions were kept in sealed containers and refrigerated at 2-4°C for further use. The percentage yield of both the crude methanol leaves extract and fractions were determined as a percentage of the weight (g) of the extract to the original weight (g) of the dried sample used.

Fractionation of crude extract

The crude extract of G. latifolium was subjected to liquid-liquid partition separation to separate the extract into different fractions. 250 ml of the reconstituted extract was placed in a separator funnel and 250 ml of n-hexane, ethylacetate and n-butanol solvents were added sequentially as a 1:1 (v/v) solution and rocked vigorously (Abbot and Andrews, 1970). The sample was left standing for 30 min for each solvent on the separator funnel until a fine separation line appear clearly indicating the supernatant from the sediment before it was eluted sequentially. The process was repeated thrice in order to get adequate quantity for each fraction. The n-hexane, ethylacetate, n-butanol as well as the aqueous residue fractions were evaporated to dryness in a water bath to afford four fractions (grams) respectively.

Preliminary phytochemical screening

Test for glycosides, saponin, flavonoids, anthaquinones and tannins was carried out according to the method of Trease and Evans (1983). Test for Alkaloids was carried out according to the method of (Sofowora, 1982).

Quantitative analysis of phytochemicals

Determination of saponin and glycosides was carried out according to the gravimetric method of (AOAC, 1984). Determination of total flavonoids was done using the method of Boham and Kocipal-Abyazan (1974). Determination of tannin was done using the standard method described by AOAC (1980). Determination of total phenolic contents (TPC) using the Folin-Ciocalteu method adopted by Amin et al. (2004) was used. Ascorbic acid contents were determined using the method described by Barros et al. (2007). Determination of alkaloids was carried out using the procedure described by Harborne (1973) with slight modification by Edeoga et al. (2005).

Animals

A total of 54 apparently healthy Wistar albino rats of both sexes weighing between 100-150 g were obtained from the Animal House, Department of Pharmacology, Ahmadu Bello University, Zaria, Kaduna State after ethical approval was granted by the departmental ethical committee. The animals were separated into male and female in well aerated laboratory cages in the Animal House, Department of Pharmacology, Ahmadu Bello University, Zaria, Kaduna State and were allowed to acclimatize to the laboratory environment for a period of two weeks before the com-
mencement of the experiment. They were fed daily with grower mash from Vital Feeds Company and water *ad libitum* during the stabilization period.

**Acute toxicity study**

The median lethal dose (LD₅₀) of *n*-butanol fraction was conducted in order to select a suitable dose for the evaluation of the effects of *n*-butanol fraction. This was done using the method described by Lorke (1983). In the initial phase, rats were divided into 3 groups of 3 rats each and were treated with 10, 100 and 1000 mg of *n*-butanol fraction per kg body weight orally. They were observed for 24 h for signs of toxicity, including death. In the final phase, 3 rats were divided into 3 groups of one rat each, and were treated with *n*-butanol fraction based on the findings in the first phase. The LD₅₀ was calculated from the results of the final phase as the square root of the product of the lowest lethal dose and the highest non-lethal dose, that is, the geometric mean of the consecutive doses with 0 and 100% survival rates were recorded.

**Animal grouping**

A total of 54 Wistar albino rats were used. The rats were divided into carbon tetrachloride-induced liver and kidney damage group of 6 rats each and LD₅₀ group. Carbon tetrachloride induced group. Group A: Normal control rats were given feed and water only. This served as the normal control group (NC). Group B: Rats were treated with olive oil and served as vehicle control group (VC). Group C: Rats were treated with 148 mg/kg b.wt. carbon tetrachloride (CCl₄) in olive oil. This serves as the CCl₄-induced liver and kidney damage group (IC). Group D: Rats were treated with 148 mg/kg b.wt. CCl₄ in olive oil + 100 mg/kg b.wt. Silymarin as standard drug (CCl₄ + Std). Group E: Rats were treated with 148 mg/kg b.wt. CCl₄ in olive oil + 100 mg/kg b.wt. *n*-butanol fraction (CCl₄ + BF). Group F: Rats were treated with 148 mg/kg b.wt. CCl₄ in olive oil + 150 mg/kg b.wt. *n*-butanol fraction (CCl₄ + BF). Group G: Rats were treated with 148 mg/kg b.wt. CCl₄ in olive oil + 200 mg/kg b.wt. *n*-butanol fraction (CCl₄ + BF).

**Induction of liver and kidney damage**

The liver and kidney damage was induced by the administration of carbon tetrachloride (CCl₄). Rats were injected intraperitoneally with a single dose of CCl₄ (148 mg/kg body weight) as a 1:1 (v/v) solution in olive oil and were fasted for 36 h before the administration of *n*-butanol fraction (Manoj and Aqued, 2003). This was done once a week for a period of four weeks. The administration of *n*-butanol fraction was done daily by oral intubation for the period of 28 days.

**Collection and preparation of sera samples**

At the end of 28 days of treatment, the animals were sacrificed by decapitation using chloroform anaesthesia and blood samples were collected from the head wound in plain bottles (for biochemical parameters). The blood samples collected in plain tubes were allowed to clot and the serum separated by centrifugation using Labofuge 300 centrifuge (Heraeus) at 3000 rpm for 10 minutes and the supernatant (serum) collected was subjected to biochemical screening.

**Collection of liver and kidney**

Immediately after the blood was collected, the liver and kidneys were quickly excised, trimmed of connective tissues, rinsed with saline to eliminate blood contamination, dried by blotting with filter paper and weighed (so as to calculate the relative weight) and kept on ice. Certain gram of the liver and kidney were crushed in 50 mM potassium phosphate buffer (pH 7.4) using mortar and pestle (homogenization) while the rest of the organs were placed in freshly prepared 10% formalin for histopathological studies. The homogenized organs were then centrifuged at 4000 rpm (2700 xg) for 15 min and the supernatant was collected using Pasteur pipette for further endogenous antioxidant activity assay. The percentage change in organ weight of each of the animals was calculated as follows:

\[
\text{Change in weight (\%)} = \frac{\text{organ weight}}{\text{animal weight}} \times 100
\]

**Haematological assay determination of packed cell volume (PCV)**

The PCV is the volume of red blood cells (RBC) expressed as a fraction of the total volume of the blood. The microhaematocrit method was used (Cheesbrough, 2000). Principle: The red blood cells are heavier than plasma with specific gravity of 1090 and 1030, respectively. When blood is placed in a capillary tube and centrifuge, they settle and are packed because of the centrifugal force acting on them. The volume occupied by the cells is measured with a heamatocrit reader relative to the volume of the whole blood.

**Biochemical studies**

Assessment of aspartate aminotransferase (AST) activity and assessment of alanine aminotransferase (ALT) activity were determined by the method described by Amador and Wacker (1962). Assessment of alkaline phosphatase (ALP) activity: Serum activity of ALP was determined by the method described by Haussament (1977). Determination of serum bilirubin concentration: The serum total and direct bilirubin was determined by the method of Jendrassik and Gröf (1938). Determination of total protein level: Total protein was determined colorimetrically according to the method described by Fine (1935). Determination of albumin level: The serum albumin was determined by the method of Doumas et al. (1971). Determination of serum urea concentration: This was assessed using the method described by Fawcett and Scout (1960). Determination of serum creatinine concentration: The colorimetric method was used to determined serum creatinine concentration according to Bartels and Bohmer (1973). Estimation of superoxide dismutase (SOD) activity: Superoxide dismutase activity was measured using the method described by Martin et al. (1987). Estimation of catalase activity: Catalase activity was determined using the method described by Aebi and Bergmeyer (1983). Estimation of glutathione peroxidase: Glutathione peroxidase assay is an adaptation of the method of Paglia and Valentine (1967). Estimation of thiobarbituric acid reactive substance (TBARS): TBARS in the tissues was estimated in the form of MDA using the method described by Fraga et al. (1988).

**Histopathological studies**

A portion of the liver and kidneys of the animals were cut into two to three pieces and fixed in 10% formalin (Lillie, 1965). The paraffin sections were prepared and stained with haematoxylin and eosin. The thin sections of liver and kidneys were made into permanent slides and examined under high (250x) resolution microscope with photographic facility and photomicrographs were taken.
Statistical analysis

The data were analyzed by the analysis of variance (ANOVA) using SPSS program (version 17.0 SPSS Inc., Chicago, IL, USA). The differences between the various animal groups were compared using the Duncan multiple range test. The results were expressed as mean ± standard error of mean (SEM). P value less than 0.05 was considered as significant (P<0.05).

RESULTS

The percentage yield of methanolic leave extract and fractions of G. latifolium

The percentage yield (w/w) of the crude extract is 10.24% and the various fractions have aqueous residue as the highest yield (45.80%), followed by n-butanol fraction (25.14%), ethylacetate fraction (10.70%) and n-hexane fraction has the lowest yield (6.66%).

Qualitative phytochemical screening of the methanolic leaves extract of G. Latifolium

The qualitative phytochemicals screening of the methanolic leaves extract of G. latifolium revealed the presence of glycosides, alkaloids, saponin, flavonoids, tannins and the absence of free anthraquinone.

Quantitative analysis of the phytochemical constituents (mg/g) of G. latifolium

The quantitative analysis of phytochemical constituents of G. latifolium leaves is presented in Table 1. The crude extract showed high tannin content followed by glycosides, alkaloids and saponin. The results in Table 2 also showed that the n-butanol fraction has higher flavonoids, polyphenols and ascorbic acid content than the ethylacetate fraction.

Lethal dosage (LD_{50}) determination for n-butanol fraction of G. latifolium

No death was recorded after the oral administration up to a dose of 5000 mg per kg body weight.

Effects of n-butanol fraction of G. latifolium on packed cell volume

The effects of sub-chronic oral administration of n-butanol fraction of G. latifolium methanolic leaves extract and silymarin (standard drug) at 100, 150 and 200 mg/kg b.wt. on packed cell volume in CCl_{4}-induced liver and kidney damage in albino rats for 28 days is shown in Table 3. The result showed that the PCV level of induced control group was significantly (P<0.05) lowered than the PCV level of normal control group, but there was no significant (P>0.05) difference between the PCV level of the normal control animals and all the induced treated animals.

Effects of n-butanol fraction of G. latifolium on body and organ weight change

Changes in body weight of rats induced liver and kidney damage treated with n-butanol fraction of G. latifolium methanolic leaves extract and silymarin (standard drug) for a period of 28 days is represented in Table 4. The results showed no significant (P>0.05) difference in the body weight change of all the induced treated groups when compared with the normal control group. However, the CCl_{4}-induced liver and kidney damage control group showed a significant (P<0.05) decrease in body weight when compared with the induced treated and normal control groups. Changes in organ weight of rats induced liver and kidney damage treated with n-butanol fraction of G. latifolium methanolic leaves extract and silymarin (standard drug) for a period of 28 days is represented in Table 5. The result showed that there was no significant (P>0.05) difference between the percentage change in liver and kidney weight of all the induced treated groups when compared with the normal control group.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Polyphenol (mg/g)</th>
<th>Flavonoid (mg/g)</th>
<th>Ascorbic acid (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n- Butanol</td>
<td>4.53</td>
<td>5.15</td>
<td>2.24</td>
</tr>
<tr>
<td>Ethylacetate</td>
<td>2.39</td>
<td>4.51</td>
<td>0.62</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Leave</th>
<th>Alkaloid (mg/g)</th>
<th>Saponin (mg/g)</th>
<th>Glycoside (mg/g)</th>
<th>Tannin (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>1.26</td>
<td>0.82</td>
<td>2.57</td>
<td>10.60</td>
</tr>
</tbody>
</table>
kidney weights of the entire induced treated group when compared with the normal control rats. However, the induced control rats present a significant (P<0.05) higher percentage change in kidney weights when compared with the normal control rats.

**Biochemical studies**

**Assessment of liver function indices**

Liver function indices of alanine aminotransferases (ALT), aspartate amino transferases (AST), alkaline phosphatases (ALP), total protein (TP), albumin (ALB) and bilirubin (DB and IB) concentrations in the serum of CCl₄-induced liver and kidney damage rats after the daily oral administration of n-butanol fraction of *G. latifolium* and silymarin for 28 days is represented in Tables 6 and 7.

There was significant (P<0.05) increase in activities of all these liver marker enzymes (ALT, AST and ALP) in the CCl₄-induced liver damage control group when compared with the normal control. The activities of ALT, AST and ALP in the induced treated groups were however significantly (P<0.05) reduced when compared with induced not treated group. The n-butanol fraction and silymarin significantly (P<0.05) increase the serum total protein levels of the induced treated groups when compared with the induced not treated group but there was no significant (P>0.05) difference between the serum total protein levels of all the induced treated groups and the normal control group. Also, serum albumin concentrations of the induced not treated group was significantly (P<0.05) lower than the normal control and the induced treated

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### Table 3. Mean changes in PCV values of CCl₄-induced liver and kidney damage rats treated daily with oral administration of n-butanol fraction of *G. latifolium* and silymarin (STD).

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>PCV (%) Week 1</th>
<th>PCV (%) Week 2</th>
<th>PCV (%) Week 3</th>
<th>PCV (%) Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>47.3 ± 2.57²</td>
<td>50.7 ± 0.72²</td>
<td>50.2 ± 1.87²</td>
<td>53.7 ± 0.42²</td>
</tr>
<tr>
<td>VC</td>
<td>45.8 ± 1.35²</td>
<td>51.8 ± 0.48²</td>
<td>53.0 ± 1.16²</td>
<td>52.3 ± 0.92²</td>
</tr>
<tr>
<td>IC</td>
<td>37.3 ± 1.78²</td>
<td>28.7 ± 1.86²</td>
<td>31.7 ± 2.26²</td>
<td>31.0 ± 2.48²</td>
</tr>
<tr>
<td>CCl₄ + BF</td>
<td>49.8 ± 1.92²</td>
<td>52.5 ± 0.76²</td>
<td>54.7 ± 0.72²</td>
<td>56.2 ± 1.01²</td>
</tr>
<tr>
<td>CCl₄ + BF</td>
<td>48.7 ± 2.09²</td>
<td>54.3 ± 0.80²</td>
<td>57.7 ± 0.62²</td>
<td>57.5 ± 0.76²</td>
</tr>
<tr>
<td>CCl₄ + BF</td>
<td>49.3 ± 2.35²</td>
<td>54.7 ± 1.31²</td>
<td>61.0 ± 0.58²</td>
<td>60.0 ± 0.58²</td>
</tr>
<tr>
<td>CCl₄ + Std</td>
<td>49.3 ± 1.82²</td>
<td>53.3 ± 0.49²</td>
<td>53.8 ± 1.54²</td>
<td>56.3 ± 0.62²</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Values with different superscript down the columns are significantly different (P<0.05). NC: Normal control rat, VC: Vehicle control rats, CCl₄: Carbon tetrachloride, IC: CCl₄ Induced Liver and Kidney damage control rats, CCl₄ + BF: CCl₄ Induced Liver and kidney damage rats+100mg/kg b.wt. of n-butanol fraction, CCl₄ + BF: CCl₄ Induced Liver and Kidney damage rats+150mg/kg b.wt. of n-butanol fraction, CCl₄ + BF: CCl₄ Induced Liver and Kidney damage rats+200mg/kg b.wt. of n-butanol fraction, CCl₄ + Std: CCl₄ Induced Liver and kidney damage rats+100 mg/kg b.wt. of standard drug (Silymarin).

### Table 4. Mean changes in body weights of CCl₄-induced liver and kidney damage rats treated daily with oral administration of n-butanol fraction of *G. latifolium* and silymarin (STD).

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>Weight change Week 1 (g)</th>
<th>Weight change Week 2 (g)</th>
<th>Weight change Week 3 (g)</th>
<th>Weight change Week 4 (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>1.50 ± 0.22²</td>
<td>2.83 ± 0.31³</td>
<td>6.67 ± 0.67³</td>
<td>10.3 ± 0.72²</td>
</tr>
<tr>
<td>VC</td>
<td>1.50 ± 0.22²</td>
<td>3.33 ± 0.21³</td>
<td>6.50 ± 0.22²</td>
<td>9.67 ± 0.56²</td>
</tr>
<tr>
<td>IC</td>
<td>0.17 ± 0.54³</td>
<td>0.83 ± 1.05³</td>
<td>2.67 ± 1.05³</td>
<td>4.00 ± 1.55³</td>
</tr>
<tr>
<td>CCl₄ + BF</td>
<td>1.83 ± 0.17³</td>
<td>3.50 ± 0.22³</td>
<td>6.83 ± 0.48³</td>
<td>10.5 ± 0.34³</td>
</tr>
<tr>
<td>CCl₄ + BF</td>
<td>2.00 ± 0.26³</td>
<td>3.67 ± 0.67³</td>
<td>7.67 ± 0.84³</td>
<td>11.5 ± 0.76³</td>
</tr>
<tr>
<td>CCl₄ + BF</td>
<td>1.67 ± 0.21³</td>
<td>3.00 ± 0.26³</td>
<td>6.67 ± 0.62³</td>
<td>10.2 ± 0.87³</td>
</tr>
<tr>
<td>CCl₄ + Std</td>
<td>1.67 ± 0.21³</td>
<td>3.50 ± 0.34³</td>
<td>7.00 ± 0.37³</td>
<td>10.5 ± 0.22³</td>
</tr>
</tbody>
</table>

Values are Means ± SEM. Values with different superscript down the columns are significantly different (P<0.05). NC: Normal control rat, VC: Vehicle control rats, CCl₄: Carbon tetrachloride, IC: CCl₄ Induced Liver and Kidney damage control rats, CCl₄ + BF: CCl₄ Induced Liver and Kidney damage rats+100mg/kg b.wt. of n-butanol fraction, CCl₄ + BF: CCl₄ Induced Liver and Kidney damage rats+150mg/kg b.wt. of n-butanol fraction, CCl₄ + BF: CCl₄ Induced Liver and Kidney damage rats+200mg/kg b.wt. of n-butanol fraction, CCl₄ + Std: CCl₄ Induced Liver and Kidney damage rats+100 mg/kg b.wt. of Standard Drug (Silymarin).
Table 5. Mean changes in organ weights of CCl₄-induced kidney damage rats treated daily with oral administration of Silymarin and n-Butanol fraction of *G. latifolium*.

<table>
<thead>
<tr>
<th>Groups (n=6)</th>
<th>Percentage change in kidney weight (g)</th>
<th>Percentage change in liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>0.52 ± 0.01a</td>
<td>4.31 ± 0.14a</td>
</tr>
<tr>
<td>VC</td>
<td>0.58 ± 0.02a</td>
<td>4.58 ± 0.32a</td>
</tr>
<tr>
<td>IC</td>
<td>0.85 ± 0.05b</td>
<td>6.94 ± 0.38b</td>
</tr>
<tr>
<td>CCl₄ BF</td>
<td>0.57 ± 0.03a</td>
<td>4.70 ± 0.17a</td>
</tr>
<tr>
<td>CCl₄ BF</td>
<td>0.52 ± 0.02a</td>
<td>4.21 ± 0.53a</td>
</tr>
<tr>
<td>CCl₄ BF</td>
<td>0.51 ± 0.01a</td>
<td>4.40 ± 0.14a</td>
</tr>
<tr>
<td>CCl₄ Std</td>
<td>0.55 ± 0.03a</td>
<td>4.64 ± 0.14a</td>
</tr>
</tbody>
</table>

Values are Means ± SEM. Values with different superscript down the columns are significantly different (P<0.05). NC: Normal control rat, VC: Vehicle control rats, CCl₄: Carbon tetrachloride, IC: CCl₄ Induced Liver and Kidney damage control rats, CCl₄ BF: CCl₄ Induced Liver and kidney damage rats+100mg/kg b.wt. of n-butanol fraction, CCl₄ BF: CCl₄ Induced Liver and Kidney damage rats+150 mg/kg b.wt. of n-butanol fraction, CCl₄ BF: CCl₄ Induced Liver and Kidney damage rats+200mg/kg b.wt. of n-butanol fraction, CCl₄ + Std: CCl₄ Induced Liver and Kidney damage rats+100 mg/kg b.wt. of Standard Drug (Silymarin).

Table 6. Effects of daily doses of n-butanol fraction of *G. latifolium* on serum liver function parameters of CCl₄-induced liver and kidney damage albino rats.

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>ALP (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>45.8 ± 2.85a</td>
<td>42.5 ± 1.63ab</td>
<td>60.2 ± 2.18a</td>
</tr>
<tr>
<td>VC</td>
<td>44.5 ± 3.24a</td>
<td>41.3 ± 1.89a</td>
<td>59.5 ± 1.77a</td>
</tr>
<tr>
<td>IC</td>
<td>60.3 ± 3.02b</td>
<td>56.8 ± 2.18c</td>
<td>76.0 ± 3.44b</td>
</tr>
<tr>
<td>CCl₄ BF</td>
<td>48.0 ± 2.15a</td>
<td>47.3 ± 1.54a</td>
<td>64.7 ± 2.33a</td>
</tr>
<tr>
<td>CCl₄ BF</td>
<td>47.3 ± 1.63a</td>
<td>43.8 ± 1.74ab</td>
<td>62.2 ± 1.11a</td>
</tr>
<tr>
<td>CCl₄ BF</td>
<td>45.5 ± 1.73a</td>
<td>44.5 ± 1.71ab</td>
<td>61.8 ± 1.78a</td>
</tr>
<tr>
<td>CCl₄ Std</td>
<td>48.3 ± 2.03a</td>
<td>46.7 ± 1.71ab</td>
<td>64.0 ± 1.81a</td>
</tr>
</tbody>
</table>

Values are Means ± SEM. Values with different superscript down the column are significantly different (P<0.05). NC: Normal Control rat, VC: Vehicle control rats, CCl₄: Carbon tetrachloride, IC: CCl₄ Induced Liver and Kidney damage control rats, CCl₄ BF: CCl₄ Induced Liver and kidney damage rats+100mg/kg b.wt. of n-butanol fraction, CCl₄ BF: CCl₄ Induced Liver and Kidney damage rats+150 mg/kg b.wt. of n-butanol fraction, CCl₄ BF: CCl₄ Induced Liver and Kidney damage rats+200mg/kg b.wt. of n-butanol fraction, CCl₄ + Std: CCl₄ Induced Liver and Kidney damage rats+100 mg/kg b.wt. of Standard Drug (Silymarin). ALT:Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase.

Table 7. Effects of daily doses of n-butanol fraction of *G. latifolium* on serum total protein, albumin, direct and indirect bilirubin, creatinine and urea levels of CCl₄-induced liver and kidney damage albino rats.

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>TP (g/dl)</th>
<th>ALB (g/dl)</th>
<th>DB (mg/dl)</th>
<th>IB (mg/dl)</th>
<th>CR (mg/dl)</th>
<th>Urea (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>61.2 ± 1.82cd</td>
<td>37.2 ± 2.09</td>
<td>6.68 ± 0.41a</td>
<td>5.30 ± 0.69a</td>
<td>54.3 ± 1.65a</td>
<td>3.28 ± 0.22a</td>
</tr>
<tr>
<td>VC</td>
<td>60.3 ± 1.94bcd</td>
<td>33.5 ± 1.52bc</td>
<td>7.13 ± 0.66a</td>
<td>5.10 ± 0.23a</td>
<td>52.8 ± 3.26c</td>
<td>3.55 ± 0.28bc</td>
</tr>
<tr>
<td>IC</td>
<td>44.8 ± 2.21a</td>
<td>24.8 ± 1.74a</td>
<td>10.5 ± 0.57b</td>
<td>6.82 ± 0.24b</td>
<td>63.8 ± 1.85b</td>
<td>5.45 ± 0.16b</td>
</tr>
<tr>
<td>CCl₄ BF</td>
<td>55.2 ± 2.02cde</td>
<td>32.2 ± 1.30c</td>
<td>7.70 ± 0.89a</td>
<td>5.78 ± 0.25ab</td>
<td>56.8 ± 1.35c</td>
<td>3.78 ± 0.15a</td>
</tr>
<tr>
<td>CCl₄ BF</td>
<td>59.5 ± 1.48c</td>
<td>29.5 ± 2.49ab</td>
<td>7.62 ± 0.95a</td>
<td>4.72 ± 0.24a</td>
<td>55.5 ± 2.32a</td>
<td>3.43 ± 0.14a</td>
</tr>
<tr>
<td>CCl₄ BF</td>
<td>63.3 ± 2.06d</td>
<td>33.3 ± 1.71c</td>
<td>6.83 ± 0.78a</td>
<td>5.55 ± 0.33a</td>
<td>55.5 ± 2.32d</td>
<td>3.38 ± 0.11a</td>
</tr>
<tr>
<td>CCl₄ Std</td>
<td>54.5 ± 2.45b</td>
<td>33.0 ± 1.93bc</td>
<td>8.23 ± 0.62a</td>
<td>4.83 ± 0.51a</td>
<td>58.0 ± 1.29ab</td>
<td>3.82 ± 0.15a</td>
</tr>
</tbody>
</table>

Values are Means ± SEM. Values with different superscript down the column are significantly different (P<0.05). NC: Normal control rat, VC: Vehicle control rats, CCl₄: Carbon tetrachloride, IC: CCl₄ Induced Liver and Kidney damage control rats, CCl₄ BF: CCl₄ Induced Liver and kidney damage rats+100mg/kg b.wt. of n-butanol fraction, CCl₄ BF: CCl₄ Induced Liver and Kidney damage rats+150 mg/kg b.wt. of n-butanol fraction, CCl₄ BF: CCl₄ Induced Liver and Kidney damage rats+200mg/kg b.wt. of n-butanol fraction, CCl₄ + Std: CCl₄ Induced Liver and Kidney damage rats+100 mg/kg b.wt. of Standard Drug (Silymarin). TP: Total protein, ALB: Albumin, DB: Direct bilirubin, IB: Indirect bilirubin, CR: Creatinine.
groups, but there was no significant (P>0.05) difference between the serum albumin levels of all the induced treated groups and the normal control group. Also, the levels of bilirubin in the induced treated groups were however significantly (P<0.05) reduced when compared with induced not treated group, but there was no significant (P>0.05) difference between the bilirubin levels of all the induced treated groups and the normal control group.

**Effects of n-butanol fraction of *G. latifolium* on kidney function parameters**

Creatinine concentrations in the serum of normal and CCl₄ induced liver and kidney damage rats after the oral administration of n-butanol fraction of *G. latifolium* and silymarin for 28 days is presented in Table 7. The results showed that the concentration of creatinine in the serum of CCl₄ induced not treated rats was significantly (P<0.05) higher when compared with the normal control rats. However, there was no significant (P>0.05) difference between the concentration of creatinine in all the induced treated groups when compared with the normal control group. Also, the urea concentrations in the serum of normal and CCl₄-induced liver and kidney damage rats after the oral administration of n-butanol fraction of *G. latifolium* and silymarin for 28 days (Table 7) showed that the concentration of urea in the serum of CCl₄-induced not treated rats was significantly (P<0.05) higher when compared with normal control rats and all the induced treated groups. However, there was no significant (P>0.05) difference between the concentration of urea in the serum of all the induced treated groups when compared with the normal control group.

**In vivo antioxidant studies**

**Effects of n-butanol fraction of *G. latifolium* on some endogenous antioxidant enzymes in the liver of CCl₄-induced liver and kidney damage albino rats**

The effects of daily oral administration of n-butanol fraction of *G. latifolium* and Silymarin for 28 days on the level of malondialdehyde (MDA) and some endogenous antioxidant enzymes (catalase, glutathione peroxidase and superoxide dismutase) of the liver of CCl₄-induced liver and kidney damage rats is represented in Table 8. There was a significant (P<0.05) increase in the level of MDA and a significant (P<0.05) decrease in the level of CAT, glutathione peroxidase (GPx) and superoxide dismutase (SOD) of the CCl₄-induced liver and kidney damage control rats when compared with the normal control. There was no significant (P>0.05) difference in the levels of MDA and endogenous antioxidant enzymes of all the induced treated groups when compared with the normal control group.

**Effects of n-butanol fraction of *G. latifolium* on some endogenous antioxidant enzymes in the kidney of CCl₄-induced liver and kidney damage albino rats**

The effects of daily oral administration of n-butanol fraction of *G. latifolium* and Silymarin for 28 days on the level of malondialdehyde (MDA) and some endogenous antioxidant enzymes (catalase, glutathione peroxidase and superoxide dismutase) of the liver of CCl₄-induced liver and kidney damage rats is presented in Table 9. The result showed that there was a significant (P<0.05) increase in the level of MDA of
the CCl4-induced control rats when compared with the normal control but, there was no significant (P>0.05) difference between the MDA levels of normal control and the induced treated groups. However, the GPx, SOD and CAT of CCl4 induced control group were significantly (P<0.05) lowered than the normal control group but there was no significant (P>0.05) difference in the levels of endogenous antioxidant enzymes of all the induced treated groups when compared with the normal control group.

Histopathological studies

Effects of n-butanol fraction of *G. latifolium* on liver and kidney

The histological section of the liver and kidneys of CCl4-induced oxidative damage rats treated with n-butanol fraction of *G. latifolium* methanolic leave extract and silymarin for 28 days is shown in Plates 1 and 2. The histopathological examinations of liver section of normal control group showed normal cellular architecture with distinct hepatic cells. CCl4-induced control group liver showed an intense hepatic necrosis with vascular congestion, vacuolation, lymphocyte hyperplasia and degeneration of normal hepatic cells. The induced treated groups showed almost normalisation of the hepatic cells after administration of n-butanol fraction of *G. latifolium*.

Also, the histopathological examinations of kidney section of normal control group showed normal glomerulus and tubules. However, oxidative damage using CCl4 resulted into intense glomerular and tubular necrosis. Daily oral administration of n-butanol fraction of *G. latifolium* methanolic leave extract and silymarin brought the kidneys back to moderate glomerular necrosis.

DISCUSSION

The preliminary phytochemical studies revealed the presence of glycosides, saponins, tannins, alkaloids and flavonoids in the crude methanolic leave extracts of *G. latifolium*. The presence of these phytochemicals in the plant, accounts for their usefulness as medicinal plant (Jayathilakan et al., 2007). The quantitative phytochemical analysis showed that tannins had the highest concentration in the crude extract (Table 1) whereas the n-butanol fraction had the highest concentration of flavonoids, ascorbic acid and polyphenols (Venkatalakshmi et al., 2012; Omonkhelin et al., 2007) when compared with the ethylacetate fraction (Table 2). Plant phenolics, flavonoids and ascorbic acid constitute major groups of phytochemicals acting as primary *in vitro* antioxidants or free radical terminators (El-Sayed et al., 2012). Therefore, it was reasonable to determine their concentration in the n-butanol and ethylacetate plant fractions with the aim of utilising the fraction with the highest concentration of *in vitro* antioxidant (Kumbhare et al., 2012; Makepeace et al., 1985). Polyphenols, flavonoids and ascorbic acid scavenging potentials and metal chelating ability is (Wang et al., 2008) dependent upon their unique structure, the number and position of the hydroxyl groups (Pazos et al., 2005; Smith and Eyzaguine, 2007; Kumar et al., 2009). The potential health benefits associated with these phytochemicals has generated great interest among scientists for the development of natural *in vitro* antioxidant compounds from plants (Rohman et al., 2010; Masoumeh et al., 2011).

Haematological investigation provides information on the general pathophysiology of the blood and reticuloendothelial system (Baker and Silverton, 1985; Mishra et al., 2009). Fairbarks (1967) showed that xenobiotics causes low PCV level which may be associated with the oxidation of sulphhydryl groups of the erythrocyte membrane thus, inflicting injury to the erythrocytes membrane. This is in agreement with the present study as packed cells volume (PCV) values in rats exposed to CCl4 gave low levels of PCV. The n-butanol fraction appeared to

Table 9. Effects of daily doses of n-butanol fraction of *G. latifolium* on some endogenous antioxidant enzymes in the kidney of CCl4-induced liver and kidney damage albino rats.

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>MDA (µM)</th>
<th>SOD (U/ml)</th>
<th>CAT (U/ml)</th>
<th>GPx (mU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>1.30 ± 0.06a</td>
<td>2.35 ± 0.08b</td>
<td>47.3 ± 1.41b</td>
<td>44.2 ± 1.20b</td>
</tr>
<tr>
<td>VC</td>
<td>1.32 ± 0.08a</td>
<td>2.28 ± 0.08b</td>
<td>47.0 ± 1.37b</td>
<td>44.7 ± 0.99b</td>
</tr>
<tr>
<td>IC</td>
<td>2.35 ± 0.14b</td>
<td>1.57 ± 0.09a</td>
<td>34.2 ± 1.30a</td>
<td>35.2 ± 1.20a</td>
</tr>
<tr>
<td>CCl4 + BF</td>
<td>1.58 ± 0.08a</td>
<td>2.15 ± 0.08b</td>
<td>45.8 ± 1.25b</td>
<td>44.8 ± 0.91b</td>
</tr>
<tr>
<td>CCl4 + BF</td>
<td>1.55 ± 0.08a</td>
<td>2.32 ± 0.10b</td>
<td>46.7 ± 1.36b</td>
<td>46.5 ± 1.09b</td>
</tr>
<tr>
<td>CCl4 + BF</td>
<td>1.45 ± 0.08a</td>
<td>2.33 ± 0.09b</td>
<td>46.7 ± 1.43b</td>
<td>46.3 ± 1.15b</td>
</tr>
<tr>
<td>CCl4 + Std</td>
<td>1.47 ± 0.09a</td>
<td>2.33 ± 0.09b</td>
<td>44.8 ± 0.95b</td>
<td>44.7 ± 1.45b</td>
</tr>
</tbody>
</table>

Values are Means ± SEM. Values with different superscript down the column are significantly different (P<0.05). NC: Normal Control rat, VC: Vehicle control rats, CCl4: Carbon tetrachloride, IC: CCl4 Induced Liver and Kidney damage control rats, CCl4 + BF: CCl4 Induced Liver and kidney damage rats+100mg/kg b.wt. of n-butanol fraction, CCl4 + BF: CCl4 Induced Liver and Kidney damage rats+150mg/kg b.wt. of n-butanol fraction, CCl4 + BF: CCl4 Induced Liver and Kidney damage rats+200mg/kg b.wt. of n-butanol fraction. CCl4 + Std: CCl4 Induced Liver and Kidney damage rats+100mg/kg b.wt. of Standard Drug (Silymarin). MDA: Malondialdehyde, SOD: Superoxide dismutase, CAT: Catalase, GPx: Glutathione peroxidase.
boost blood cells as the values of PCV approached the normal control (Table 3). This finding suggests that the administration of the n-butanol fraction of the methanolic leaves extract of *G. latifolium* to patient with remarkable low PCV level may increase their packed cell volume. It implies that the n-butanol fraction may possess constituents that would trigger the production of more blood cells (Patrick-Iwuanyanwu et al., 2007; Emeka and Obioa, 2009).

Changes in the body weight after CCl₄ dosing have been used as a valuable index of CCl₄-related organ damage by Bruckner et al. (1986) and Pradeep et al. (2005) and thus, will be applicable in this study in order to justify the effects of CCl₄ on the body and organ weights of these animals. The decrease in changes in body weight (Table 4) and consequent increase in liver and kidney weights (Table 5) seen in CCl₄-induced control group was considered to be as a result of direct toxicity of CCl₄ and/or indirect toxicity that lead to liver and kidney damage. This indicates that, CCl₄ may have induced hypertrophy of the cells of these organs as well as elicit remarkable tissue damage (Li et al., 2011) which may have lead to the observed effects on the body and organ weights of these animals. However, all the induced treated groups experienced a significant increase in body weight changes as well as reduced change in organ weights, suggesting the possible curative effects of the n-butanol fraction of *G. latifolium* against liver and kidney injury after CCl₄ induction.

Assessment of liver can be made by estimating the activities of serum ALT, AST and ALP which are enzymes originally present at higher concentration in cytoplasm (Reham et al., 2009). When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent of liver damage (Nkosi et al., 2005; Dominic et al., 2012). Administration of CCl₄ caused a significant (P<0.05) elevation of these liver marker enzyme levels and a consequent decrease in the level of serum proteins when compared with normal control group (Table 6). The elevated level of these liver marker enzymes with a corres-
Plate 2. The Representative Kidney Region of CCl₄-Induced Liver and Kidney damage rats treated with n-butanol fraction of *G. latifolium* and silymarin (H&E STAIN X250). Group A: Normal Control, Group B: Vehicle Control, Group C: CCl₄-Induced Control, Group D: CCl₄ + STD 100 mg/kg, Group E: CCl₄+ BF 100 mg/kg, Group F: CCl₄+ BF 150 mg/kg, Group G: CCl₄+ BF 200 mg/kg.

ponding decrease in serum protein levels observed in the CCl₄-induced not treated group corresponded to extensive liver damage induced by CCl₄ which may be as a result of an impaired protein turnover (Table 7). These results are in agreement with previous finding that the activity levels of serum ALT, ALP and AST were significantly elevated and there was a significant decrease in serum protein levels in rats after CCl₄ administration (Khan et al., 2012; Battu et al., 2012; Shahid et al., 2012; Etim et al., 2008).

Also, the significant (P<0.05) elevation of bilirubin levels in the CCl₄-induced not treated group when compared with the normal control and the induced treated groups (Table 7) may be as a result of haemolytic anemia that may be associated with oxidative damage to red blood cells thus, leading to elevated bilirubin level since bilirubin is an intermediate product in haemoglobin breakdown in the liver (Reham et al., 2009). Again, this elevated bilirubin level may also be associated with reduced hepatocyte uptake of bilirubin, impaired conjugation of bilirubin and reduced hepatocyte secretion of bilirubin (Table 7) (Nkosi et al., 2005; Dominic et al., 2012). Also, there are significant elevation of direct (conjugated) and indirect (unconjugated) bilirubin levels in the blood serum of CCl₄-induced not treated group which may be attributed to the inability of the hepatocyte to secrete conjugated bilirubin as envisioned in elevated direct bilirubin level or may also be due to obstruction in the flow of bile from the bile duct as a result of severe liver damage (Emeka and Obioa, 2009). Furthermore, elevated indirect bilirubin level may be as a result of liver necrosis which is capable of causing the liver not to
conjugate bilirubin and may also cause the hepatocytes to lose its ability to take up bilirubin (Khan et al., 2012; Battu et al., 2012; Shahid et al., 2012).

There was significant (P<0.05) restoration of these liver marker enzymes levels as well as bilirubin and serum proteins levels on administration of the n-butanol fraction and silymarin for 28 days at a dose of 100, 150 and 200 mg/kg b.wt. The reversal of these serum liver marker enzymes in CCl₄-induced treated groups towards a near normalcy by the n-butanol fraction observed in this study may be due to the prevention of the leakage of these intracellular enzymes as a result of the presence of polyphenols, flavonoids and ascorbic acid in the n-butanol fraction as well as their membrane stabilizing activity which may be attributed to their ability to mop up free radicals that attack cell membranes. Also, the repeated contact of these in vitro antioxidants with hepatocytes may lead to increased stability of the cell membrane (Muthu et al., 2008; Chavan et al., 2012). Again, the ability of the n-butanol fraction to reduce the bilirubin level to near normalcy may be as a result of its ability to assist in reducing oxidative damage to red blood cells which may lead to reduction in haemoglobin breakdown by the liver. This is in agreement with the commonly accepted view that serum levels of transaminases, bilirubin and serum proteins returns to normalcy with the healing of hepatic parenchyma cells as well as the regeneration of hepatocytes (Chavan et al., 2012; Ugwu and Eze, 2010). It is therefore, a clear manifestation of the hepatocurative effects of the n-butanol fraction of G. latifolium.

The kidney helps in maintaining homeostasis of the body by reabsorbing important material and excreting waste products. Creatinine is a break down waste product formed in the muscle by creatine phosphate metabolism. Creatine is synthesized in the liver, passes into the circulation and is taken up almost entirely by skeletal muscle for energy production. Creatinine retention in the blood is evidence of kidney impairment. Urea is the main end product of protein catabolism. Amino acid deamination takes place in the liver, which is also the site of urea cycle, where ammonia is converted into urea and excreted through urine. It represents 90% of the total urinary nitrogen excretion. Urea varies directly with protein intake and inversely with the rate of excretion. Renal diseases which diminish the glomerular filtration lead to urea retention. Administration of CCl₄ causes nephrotoxicity as indicated by significant (P<0.05) elevation in serum level of creatinine and urea (Table 7). These results are in agreement with earlier findings (Venkatannarayana et al., 2012; Yacout et al., 2012). From the present study, it is evident that elevation in plasma urea and creatinine levels can be attributed to the damage of nephron structural integrity (Khan and Siddique, 2012). The different doses of n-butanol fraction significantly (P<0.05) lowered urea and creatinine levels in the CCl₄-induced treated groups when compared with the CCl₄-induced not treated groups. This indicates that the n-butanol fraction of G. latifolium may improve renal function in kidney disease rats.

Antioxidant activity or scavenging activity of the generated free radicals is important in the curative effect of CCl₄-induced hepatotoxicity. The body has an effective defence mechanism to prevent and neutralize free radicals-induced damage. This is accomplished by a set of endogeneous antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase. Decrease in enzyme activity of SOD is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in liver injury (Mohajeri et al., 2011; Tamilarasi et al., 2012). The increased level of MDA in the liver tissue of the rats administered CCl₄ (Table 8) may be as a result of the enhanced membrane lipid peroxidation by free radicals generated and failure of antioxidant defence mechanisms to prevent formation of excessive free radicals (Liu et al., 2009; Kim et al., 2010; Khan et al., 2012). Also, the decreased activity of SOD, GPx and CAT in the liver tissues of CCl₄-induced rats may be due to high concentration of these free radicals generated by CCl₄ which may lead to decreased level or inactivation of these endogenous antioxidant enzymes (Showkat et al., 2010). Treatment with n-butanol fraction of G. latifolium significantly (P< 0.05) increased the levels of SOD, GPx and CAT activities and a consequent significant (P< 0.05) reduction in MDA. The effects of the n-butanol fraction were comparable to the standard drug (Silymarin). Thus, this result suggests that n-butanol fraction of G. latifolium contains free radical scavenging activity due to the presence of in vitro antioxidants, which could exert beneficial action against pathophysiological alterations caused by the presence of superoxide and hydroxide free radicals as well as hydrogen peroxide indicating the regeneration of damaged liver cells (Etim et al., 2008; Ugochuwku et al., 2003).

Furthermore, a non significant (P>0.05) difference was observed in the kidney level of MDA (Table 9) in the normal control and all the induced treated groups in contrast to a significant increase in the level of MDA in the CCl₄-induced control group. Also, there was significant decrease in the activities of SOD, GPX and CAT in the CCl₄-induced control group as compared to the normal control and induced treated groups that showed elevated activities of these endogenous antioxidant enzymes. This suggests that, the n-butanol fraction of G. latifolium could improve renal function in animals due to its in vitro antioxidant potentials that may have assisted the endogenous enzymatic antioxidants to mop up free radicals generated by CCl₄ or could be as a result of gradual restoration of the endogenous enzymatic antioxidant levels as less demand is placed on them thus, reversing the oxidative stress. This result is in agreement with the report of Ragip et al. (2008), Etim et al. (2008) and Ugochuwku et al. (2003).

The histopathological studies of the liver and kidneys in the CCl₄-induced control group showed that CCl₄ caused an intense vascular congestion, vacoulation, lymphocyte
hyperplasia and necrosis (Plates 1 and 2) indicating its hepatotoxicity and nephrotoxicity. This result is in agreement with that of Venkatanarayana et al. (2012). Following the administration of the n-butanol fraction of G. latifolium and silymarin, the hepatocytes showed close to normal cellular architecture while the glomerulus showed moderate necrosis which may be as a result of regeneration and repair of liver and kidney cells (Emeka and Obioa, 2009; Etim et al., 2008).

In line with these findings, it is obvious that histopathological examinations are in agreement with observed biochemical analysis. This result is in agreement with the report of Etim et al. (2008) and Ugochuwku et al. (2003). It was demonstrated that liver is not the only target organ of CCl₄; it also causes free radical generation in other organs, such as heart, lung, testis, brain and blood (Khan and Ahmed, 2009; Khan et al., 2009; Khan and Zehra, 2011).

Conclusions

The result of this study has scientifically justified the traditional use of G. latifolium in the management of human diseases. The result showed that the n-butanol fraction of methanolic leave extract of G. latifolium possess in vitro antioxidants which may have contributed to its significant anti-hepatotoxic properties. The histological examination showed that the n-butanol fraction of G. latifolium has curative effect on the liver in CCl₄ induced liver damage rats. The n-butanol fraction of G. latifolium is comparable to the standard drug (silymarin). This work provides the phytotherapeutic potential of n-butanol fraction of G. latifolium that may be useful to scientists and researchers in the nutraceutical industry.

RECOMMENDATIONS

1. There is need to carry out a bioactivity-guided fractionation, isolation and identification of the bioactive constituents of the n-butanol fraction which is responsible for the observed pharmacological activities.

2. There is need to carry out chronic toxicity studies of the n-butanol fraction of the plant so as to ascertain the safety of long term usage on animals.

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UPCOMING CONFERENCES

12th European Conference on Fungal Genetics (ECFG12), Seville, Spain, Mar 23 2014

International Conference on Molecular Biology, Biochemistry and Biotechnology, Tokyo, Japan, May 29 2014

ICMBBB 2014 : International Conference on Molecular Biology, Biochemistry and Biotechnology
February 2014
5th International Conference on Legal Medicine, Medical Negligence and Litigation in Medical Practice & 5th International Conference on Current Trends in Forensic Sciences, Forensic Medicine & Toxicology (IAMLE 2014), Goa, India, Feb 25 2014

March 2014
12th European Conference on Fungal Genetics (ECFG12), Seville, Spain, Mar 23 2014

American College of Medical Genetics and Genomics (ACMG) Annual Clinical Genetics Meeting, Nashville, USA, Mar 25 2014

April 2014
18th Annual International Conference on Research in Computational Molecular Biology, Pittsburgh, USA, Apr 2 2014

Toxicology and Risk Assessment Conference (TRAC), Cincinnati, USA, Apr 7 2014

International Conference on Biochemistry and Molecular Biology, Vienna, Austria, Apr 17 2014

May 2014
International Conference on Molecular Biology, Biochemistry and Biotechnology, Tokyo, Japan, May 29 2014