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Effects of Fractionated Methanolic Leaf Extract of *Gongronema latifoliumon* CCl_4-induced Wistar Albino Rats

Okpala Jude Chinedu[1]*, Sani Ibrahim[1], Nzelibe Henry Chukwuemeka[1] and Akpa Alex Uwadiegwu[2]

[1]*Department of Biochemistry, Ahmadu Bello University, Nigeria*

[2]*Department of Medical Biotechnology, National Biotechnology Development Agency, Nigeria*

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***Corresponding author:** Okpala, Jude Chinedu, Department of Biochemistry, Ahmadu Bello University, Nigeria, Email: judeokpch@yahoo.co.uk

Abstract

The general aim of this study is to assess the effects of fractionated methanolic leaf extract of *Gongronema latifoliumon* CCl4-induced wistar albino rats. Fifty-four (54) Wistar albino rats were divided into seven treatment groups. Group A was given feed and water, Group B was injected with olive oil intra peritoneally, while the rest of the groups (C, D, E, F and G) were injected intra peritoneally with a single dose of CCl_4 (148 mg/kg). After 36 hours of induction, group E, F and G were given 100 mg/kg, 150 mg/kg and 200 mg/kg body weight of n-butanol fraction of methanol leaf extract of *Gongronema latifolium* by oral gavage. Group D was given 100 mg/kg of silymarin (standard drug) whereas group C served as CCl_4-induced group. At the end of 28 days of treatment, there were significant (P<0.05) reduction in PCV, Hb concentration and serum protein levels as well as a significant (P<0.05) increase in percentage change in liver weights of CCl4-induced control rats when compared with the induced treated groups. Liver marker enzymes studies showed that there was significant (P<0.05) increase in the serum activities of ALT, AST, ALP and bilirubin concentrations in CCl4-induced control group when compared with the induced treated groups. Antioxidant assay on the liver homogenate showed that there was significant (P<0.05) decrease in SOD, CAT, GPx and a significant increase (P<0.05) in MDA of CCl_4-induced control rats when compared to the normal control rats. These findings suggested that n-butanol fraction of methanol leaf extract of *G. latifolium* may have anti-hepatotoxic and antioxidative effects against CCl_4-induced liver damage rats.

Keywords: *Gongronema latifolium*; CCl_4; Liver; Antioxidants; Bilirubin

Abbreviations: TBARS: ThioBarbituric Acid Reactive Substance; SOD: Superoxide Dismutase; GR: Glutathione Reductase; ANOVA: Analysis of Variance; PCV: Packed Cell Volume; RBC: Red Blood Cells; ALP: Alkaline Phosphatase; CCl_4: Carbon Tetrachloride; MDA: Malondialdehyde; ALT: Alanine Amino Transferases; AST: Aspartate Amino Transferases; TP: Total Protein; ALB: Albumin

Introduction

Gongronema latifolium (Asclepiadaceae) is a perennial climber forest leafy vegetable which has a woody hollow glaborous stems and is characterized by greenish yellow flowers [1]. It is widespread in tropical Africa as well as grows in the forest of south eastern and western Nigeria where it is widely used for medicinal and nutritional purposes [2]. G. latifolium can mostly be found 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 herbalist claimed that a decoction of the grounded [3] leaves of G. latifolium has been used in the production of several herbal products which are taken orally [1] for the treatment of stomach upsets and pains, dysentery, malaria, typhoid fever, worm and cough [4]. Asthma patients chew fresh leaves to relieve wheezing [1] 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 [5]. It is also taken as a tonic to treat loss of appetite [4]. Previous research studies have revealed that other plants with in vitro antioxidant properties such as polyphenols, flavonoids and ascorbic acid were able to ameliorate hepatotoxic effects [1] and thus, protect the liver against oxidative injury induced by CCl4 in vivo [1]. Although many plants have been reported to possess anti-hepatotoxic capabilities, the scientific authentication of most of them such as G. latifolium which is used traditionally to treat several diseases is unavailable [3]. The aim of this work is 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 properties of *n*-butanol fraction of methanolic leave 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. Antrm UK. Chemicals and reagents used were purchased from Sigma Chemical Company St. Louis U.S.A. and chemicals used were of analytical grade. Folinciocalteu phenol reagent, gallic acid, carbon tetrachloride (Sigma-Aldrich), distilled water and normal saline.

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 Mallam Gallah U.J. in the Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria where a voucher specimen with voucher number 1274 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) 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 hours 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 (1mm mesh size) and then concentrated in a water bath maintained at 45ºC until greenish black residue was obtained. This process was repeated thrice. The crude extract was then subjected to phyto chemical analysis using standard procedures [6]. Also, 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 ºC 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

Fractionation procedure was carried out according to the method described by [7]: The methanolic extract of *G. latifolium* was subjected to liquid-liquid partition separation to separate the extract into different fractions. 250 ml of the reconstituted extract (extract was reconstituted with methanol solvent) 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. The sample was left standing for 30 minutes for each solvent on the separator funnel until a fine separation line appear clearly indicating the supernatant from the sediment before it was eluted. The n-hexane, ethylacetate, n-butanol as well as the aqueous residue fractions were concentrated over a water bath maintained at 45 ºC. The concentrated fractions were kept in sealed containers and refrigerated at 2-4ºC for further use.

Preliminary phytochemical screening

Test for carbohydrates was carried out using the method of [8]

Molisch's Test: 2 ml of Molisch's reagent was added to 3 ml of the extract dissolved in distilled water in a test tube and shaken properly. 2 ml of concentrated sulphuric acid (H_2SO_4) was carefully allowed to run down the side of the test tube. A purple to violet ring colour at the interface indicates the presence of carbohydrate.

Fehlings Test: 2 ml of extract dissolved in distilled water was added to 5ml of a mixture of Fehling's solution A and B in the ratio of 1:1 and the mixture was boiled for few minutes. A brick red precipitate indicates the presence of free reducing sugar.

Cardiac glycosides was carried out using the method of [8]

KellaKilliani Test: Extract was dissolved in glacial acetic acid containing traces of ferric chloride. The test tube was held at an angle of 45 degree, 1ml of concentrated sulphuric acid was added down the side of the test tube, a purple ring colour at the interface indicate the presence of cardiac glycosides.

Kadde Test: One millimeter of 2% 3, 5-dinitrobenzoie acid in 95% alcohol was added to the extract, the solution was made alkaline with 5% sodium hydroxide. Appearance of purple-blue colour indicates the presence of cardenolides in the sample.

Anthraquinones was carried out using the method of [8].

Test for free anthraquinones (Borntrager's Test): 0.2 g of the extract was shaken with 10ml of benzene and filtered. 5ml of 10% ammonia solution was added to the filtrate and stirred. The production of a pink-red or violet colour indicated the presence of free anthraquinones.

Test for combined anthracene (Modified Borntrager's Test): Sample (extract) was boiled with 5ml of 10% hydrochloric acid for 3 mins. This hydrolyzed the glycosides to yield a glycones which are soluble in hot water only. The solution was filtered hot; the filtrate was cooled and extracted with 5ml of benzene. The benzene layer was pipetted off and shaken gently with half its volume of 10% ammonia solution. A rose-pink or cherry red colour indicates the presence of combined anthracene.

Saponins was carried out using the method of [8]

Frothing Test: 0.5 g of the extract was dissolved in 10ml of distilled water. This was then shaken vigorously for 30 seconds and was allowed to stand for 30 minutes. A honey comb froth that lasted for more than 15 minutes indicates the presence of saponin.

Flavonoids was carried out using the method of [8]

Shinoda Test: About 0.5g of extract was dissolved in 2ml of

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50% methanol in the heat. Metallic magnesium and four drops of concentrated HCI were added. A red or orange colour indicates the presence of flavonoid aglycones.

Sodium Hydroxide Test: 2 ml of aqueous NaOH were added to 5ml of extract (0.5 g of extract dissolved in distilled water) a yellow colouration shows the presence of flavonoid.

Tannins was carried out using the method of [8]

Lead Sub-Acetate Test: In a test tube containing about 5 ml of the extract solution (0.5 g dissolved in distilled water), 3 drops of 1% lead sub-acetate solution was added. A red precipitate indicates the presence of tannins.

Ferric chloride Test: About 0.5 g of extract was dissolved in 10ml of distilled water, and then filtered. Few drops of ferric chloride solution were added to the filterate. Formation of a blue-black precipitate indicates hydrolysable tannins and green precipitates indicate the presence of condensed tannin.

Test for alkaloids was carried out using the method of [9]

Sample quantity of the extract (4 mg) was stirred with 5 ml of 1% aqueous HCl on water bath and then filtered. Of the filtrate, 1 ml was taken individually into 2 test tubes.

Meyers Test: To the first portion, 1 ml of Mayer's reagent was added and buff-coloured precipitate indicates the presence of alkaloids.

Dragendoff's Test: To the second portion, 1 ml of Dragendorff's reagent was added and occurrence of rose-red precipitate indicates the presence of alkaloids.

Quantitative analysis of phytochemicals and *In vitro* antioxidant assay

Determination of saponin was carried out using the gravimetric method of [10]

Procedure: Five grams of G. latifolium sample was weighed into a thimble and put in a soxhlet extractor with a condenser fitted on top. Extraction was done with acetone in a 250 ml round bottom flask for 3 hrs, after which the other weighed 250 ml round bottom flask containing methanol was fitted to the same extractor and extracted for another 3 hrs. At the end of the second extraction, the methanol was recovered by distillation and the flask oven-dried to remove the remaining solvent in the flask. The flask was allowed to cool in a dessicator and weighed. This test was carried out in triplicates.

Calculation:

$$Saponin = \frac{A - B}{W}$$

Where A= weight of flask and extract (saponin)

B = weight of empty flask

W = weight of sample.

Glycosides were carried out using the gravimetric method of [10]

Procedure: Five grams of sample was placed in a 200 ml conical flask; 50 ml of distilled water was added. The content of the flask was stirred and allowed to stand for 4 hrs. The filtrate was steam distilled into 20 ml of 2.5% NaOH. Seventy five milliliters (75 ml) of the distillate was collected. This was titrated with 0.02 N AgNO3 (Silver nitrate) after the addition of 8 ml of 6 N ammonium hydroxide and 2 ml of 2% potassium iodide. Permanent turbidity indicated end-point. This test was carried out in triplicates. The cyanogenic glycosides was calculated by multiplying the volume of 0.02 N AgNO3 used (titre value) by 1.08 mg, using the equation below;

1ml 0.02 N $AgN0_3$= 1.08 mg HCN

Therefore cyanogenic glycosides content in the sample (mg) = titre value x 1.08g.

Determination of total flavonoids was carried out using the method of [11]

Principle: Aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. However, it forms acid labile coloured complexes with the orthodihydroxyl groups in the A or B-ring of flavonoids.

Procedure: The aluminium chloride method was used for the determination of the total flavonoid content of the test samples. 2 ml of each fraction (0.2 g) were made up to 3ml with methanol. Then 0.1ml $AlCl_3$ (10%) (10 g in 100 ml methanol), 0.1ml Na-K tartarate and 2.8 ml distilled water were added sequentially. The test solution was vigorously shaken. Absorbance at 415 nm was recorded after 30 minutes of incubation. A standard calibration plot was generated at 415 nm using define concentrations of quercetin. This test was carried out in triplicates. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent /g of sample.

Determination of tannin was carried out using the standard method described by [12]

Procedure: Two grams of the dried sample was boiled in 300 ml of distilled water for 30 minutes. The sample was cooled and transferred into a standard volumetric flask and filtered through a non-absorbent cotton wool to get a clear filtrate. A volume of 25 ml of the filtrate was measured into a 2 litre porcelain dish and titrated with 0.1N potassium permanganate (0.1N potassium permanganate was standardized against 0.1N Oxalic acid) until the blue solution changed to green, then few drops of 0.1N potassium permanganate were added drop wise until the solution turns golden yellow. This test was carried out in triplicates. The

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tannin content in the sample was calculated by multiplying the volume of 0.1 N potassium permanganate used (titre value) by 0.0066235 g. Using the equation below;

1ml of 0.1 N potassium permanganate (titre value) = 0.0066235 g Tannins

Therefore Tannins content in the sample (mg) = titre value x 0.0066235 g

Determination of total phenolic contents (TPC) was carried out using the Folin-Ciocalteu method adopted by [13]

Principle: The reaction is based on the reduction of phosphor-wolframate-phosphomolybdate complex by phenolics to a blue reaction product with a maximum absorption at 765nm which can be measured spectrophotometrically (Amin et al., 2004).

Procedure: 2.5 ml of 10-fold diluted Folin–Ciocalteu reagent was mixed with 2 ml of saturated sodium carbonate (75 g/litre) and 6050 µl (6.05 ml) of each sample (0.13 g fractions dissolved in methanol). The mixture was vigoriously shaken for 10 seconds and heated for 30 minutes at 45°C. The absorbance was measured at 765 nm after cooling to room temperature. This test was carried out in triplicates. The data obtained was calculated by comparison between a standard curve (µg Gallic acid/ml) and the absorbance of each sample. The data obtained were expressed as mg Gallic acid equivalent per gram of dry matter.

Ascorbic acid content determination was carried out according to the method described by [14]

Principle: Ascorbic acid reacts with 2, 6-dichlorophenolindophenol (DCPIP) (blue colour) in a 1:1 order changing the blue colour. The absorbance is read spectrophotometrically at 515 nm.

Procedure: One gram of each fraction was dissolved with 10 ml of 0.5% oxalic acid and the mixture was shaken and left for 20 minutes at room temperature and was filtered through Whatman No. 4 filter paper. Precisely 1 ml of the filtrate was mixed with 9 mL of 0.1mol L–1 of 2, 6-dichlorophenolindophenol reagent. A reagent blank using distilled H_2O instead of sample was prepared. The absorbance was read within 30 minutes at 515 nm against the prepared blank. This test was carried out in triplicates. The ascorbic acid content was calculated using the calibration curve, prepared from standard L-ascorbic acid (µg/ml). The data obtained were expressed as mg L-ascorbic acid equivalent per gram of dry matter.

Determination of alkaloids was done using the procedure described by [15] with slight modification by [16]

Procedure: Five grams of the sample was weighed into 250 ml beaker. 100 ml of 10% acetic acid in ethanol was then added. The mixture was shaken, covered and allowed to stand for 4 hours. It was then filtered using no. 4 filter paper and the filtrate concentrated on a water bath to ¼ of the original volume. Thereafter, concentrated ammonium hydroxide was added drop wise until precipitation was completed. The mixture was then allowed to settle and the precipitate collected, washed with diluted ammonium hydroxide and filtered. The filtrate was concentrated in a water bath maintained at 45°C. The dry weight of the residue is equivalent to the weight of alkaloids in mg/g of the dry sample weight. This was carried out in triplicates.

The DPPH radical scavenging activity of the plant fractions were assayed by the DPPH radical scavenging method of [17]

Principle:1,1-Diphenyl-2-picrylhydrazyl (DPPH) contains an odd electron in its structure. Its purple colour is reduced to yellow-coloured diphenyl picryhydrazine when it reacts with an antioxidant, which can donate a hydrogen atom or remove an electron from it. The change in colour can be measured spectrophotometrically at 520 nm using a UV/Visible light spectrophotometer.

Procedure: DPPH solution was prepared by dissolving 6 mg of DPPH in 100 ml of methanol. To 1ml of each fraction of various concentrations (0.02, 0.04, 0.06 and 0.08 mg/ml), 2 ml of DPPH solution (0.1mM) was added. An equal amount of methanol and DPPH served as control. The mixture was shaken vigorously and was left to stand in dark for 30 min. The resulting solution was measured spectrophotometrically at 520 nm and the absorbances were recorded. The experiments were performed in triplicate and the percentage scavenging activity of each fraction on DPPH radical was calculated using the following formula:

Scavenging activity (%) = {(1- Absorbance of the sample) / (Absorbance of the control)} ×100.DPPH radical scavenging activities of the fractions were expressed as IC50 values. IC50, the effective concentration of the fractions required for 50% scavenging of DPPH radical was calculated from the graph of scavenging activity plotted against sample concentration using Microsoft Excel software.

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. 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 commencement of the experiment in line with international best standard for animal use as stipulated and approved by the university departmental ethical board in charge of animal use protocols. The animals 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 (LD_{50}) of n-butanol fraction was

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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 [18]. In the initial phase, rats were divided into 3 groups of 3 rats each and were treated with 10 mg, 100 mg and 1000 mg of n-butanol fraction per kg body weight orally. They were observed for 24 hours 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 LD_{50} 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, i.e. 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 damage group of 6 rats each and LD_{50} 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 148mg/kg b.wt. carbon tetrachloride (CCl_4) in olive oil. This serves as the CCl_4-induced liver damage group (IC).

Group D: Rats were treated with 148 mg/kg b.wt. CCl_4 in olive oil + 100 mg/kg b.wt. Silymarin as standard drug (CCl_4+Std) [19].

Group E: Rats were treated with 148 mg/kg b.wt. CCl_4 in olive oil + 100 mg/kg b.wt. n-butanol fraction. (CCl_4+BF)

Group F: Rats were treated with 148 mg/kg b.wt. CCl_4 in olive oil + 150 mg/kg b.wt. n-butanol fraction. (CCl_4+BF)

Group G: Rats were treated with 148 mg/kg b.wt. CCl_4 in olive oil + 200 mg/kg b.wt. n-butanol fraction. (CCl_4+BF).

Induction of liver damage

The liver damage was induced by the administration of carbon tetrachloride (CCl_4). Rats were injected intraperitoneally with a single dose of CCl_4 (148 mg/kg body weight) as a 1:1 (v/v) solution in olive oil and were fasted for 36 hours before the administration of n-butanol fraction [20]. This was done once a week for a period of four weeks. The administration of n-butanol fraction was done daily by oral gavage 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

Immediately after the blood was collected, the liver was 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 was crushed in 50mM 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. It was then centrifuged at 4000 rpm (2700 xg) for 15 minutes. Then the supernatant was collected using Pasteur pipette. The percentage change in organ weight of each of the animals was calculated as follows;

$$\%\ Change\ in\ weight = \frac{Organ\ weight}{Animal\ weight} \times 100$$

Heamatological 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 micro haematocrit method was used to determine PCV on a weekly basis [21].

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 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.

Procedure: Weekly blood collected from the tail of the rats was allowed by capillary action to flow through the heparinized capillary tube after which one end of the tube was sealed by flaming. It was then centrifuged at a speed of 7000 rpm for 5 minutes. The PCV was estimated using a micro heamatocrit reader and expressed as percentage erythrocytes that the blood contain.

Determination of heamoglobin concentration (Hb)

Haemoglobin concentration (Hb) was determined on a weekly basis using the cyanmethaemoglobin method of [22].

Principle: Blood is mixed with Drabkin's solution, a solution that contains ferricyanide and cyanide. The ferricyanide oxidizes the iron in the hemoglobin, thereby changing heamoglobin to metheamoglobin. Metheamoglobin then unites with the cyanide to form cyanmetheamoglobin. Cyanmetheamoglobin produces a brownish-colored solution which is then measured in a spectrophotometer at 540 nm. The color relates to the concentration of heamoglobin in the blood.

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Procedure: Weekly blood collected from the tail of the rats was allowed by capillary action to flow through the heparinized capillary tube. Sample solutions and standard solutions were prepared as follows.

Random sample: 5000 µl of Drabkin reagent was mixed with 20 µl of distilled water

Standard: 5000 µl of Drabkin reagent was mixed with 20 µl of sample solution of haemoglobin

Target sample: 5000 µl of Drabkin reagent + 20 µl of blood

The concentration of haemoglobin was marked with Drabkin's method, with the use of a spectrophotometer. Once Drabkin reagent was mixed with the blood, the solution was incubated at room temperature for the duration of 5 mins and absorbance was measured at 540 nm against distilled water.

Measurement formula: Haemoglobin and its derivatives affected by Drabkin reagent transform into cyanmethemoglobin, whose optical density was measured photometrically.

The concentration of hemoglobin was calculated according to the following formula:

$$Hb\ concentration\ (g/dl) = \frac{Absorbance\ of\ tested\ sample}{Absorbance\ of\ \text{standard}} \times concentration\ of\ \text{standard}\ in\ g/dl$$

Biochemical studies

Assessment of aspartate aminotransferase (AST) activity

AST activity was determined by the method described by [23]

Principle: In this reaction L-Aspartate and α-Ketoglutarate react in the presence of AST in the sample to yield oxaloacetate and L-glutamate. The oxaloacetate is reduced by malate dehydrogenase to yield L-malate with the oxidation of NADH to NAD+. The reaction is monitored by measurement of the decrease in the absorbance of NADH at 340nm. The rate of reduction in absorbance is proportional to AST activity in the sample.

Procedure: 1 ml of reagent was added to all required test tubes followed by the addition of 0.05 ml of the sample to the sample test tube and none to the blank. It was incubated at room temperature for 20 min, mixed immediately and first absorbance of test was read exactly at 1 minute and thereafter at 30, 60, 90 and 120 seconds at 340 nm. The mean change in absorbance per minute was determined and the test results were calculated.

$$Serum\ AST\ activity\ (IU/L) = \frac{Change\ in\ A}{\min} \times 3376$$

Assessment of alanine aminotransferase (ALT) activity

ALT activity was determined by method described by [23].

Principle: In this reaction, L-Alanine and α-ketoglutarate react in the presence ALT in the sample to yield Pyruvate and L-glutamate. Pyruvate is reduced by lactate dehydrogenase to yield lactate with oxidation of NADH to NAD. The reaction is monitored by measurement of the decrease in absorbance at 340 nm. The rate of reduction is proportional to ALT activity in the sample.

Procedure: 1 ml of reagent was added to all required test tubes followed by the addition of 0.05 ml of the sample to the sample test tube and none to the blank. It was incubated at room temperature for 20 min, it was mixed immediately and first absorbance of test was read at exactly 1 minute and thereafter at 30, 60, 90 and 120 seconds at 340 nm. The mean change in absorbance per minute was determined and test results were calculated.

$$Serum\ ALT\ activity\ (IU/L) = \frac{\triangle A}{\min} \times 3376$$

Assessment of alkaline phosphatase (ALP) activity

Serum activity of alkaline phosphatase (ALP) was determined by the method described by [24].

Principle

$$\left(P\text{-}nitrophenylphosphate + H_2O \xrightarrow{ALP} phosphate + p\text{-}nitrophenol\ (405\ nm)\right)$$

Alkaline Phosphatase in a sample hydrolyses paranitrophenyl phosphate into paranitrophenol and phosphate, in the presence of magnesium ions. The rate of increase in absorbance of the reaction mixture at 405 nm and 37°C due to liberation of paranitrophenol is proportional to the alkaline phosphatase activity.

Procedure: Reagent (1 ml) containing diethanolamine buffer, magnesiu chloride and substrate (P-nitrophenylphosphate) was added into a clean test tube and incubated at 370C followed by the addition of 0.02 ml of sample. This was mixed thoroughly and immediately absorbance of test was read exactly at 30, 60, 90 and 120 seconds at 405nm against the reference blank (distilled water).The mean change in absorbance per minute was determined and the test results were calculated. Calculation:

The ALP activity was calculated using the following formulae:

Determination of serum bilirubin concentration

The serum total and direct bilirubin was determined by the method [25].

Principle: Bilirubin is estimated by reacting it with diazotised sulfanilic acid obtained from sodium nitrite and sulfanilic acid solutions. Bilirubin when reacted with diazotised sulfanilic acid forms a pink coloredazocompound that is measured at 546 nm. The unconjugated or free bilirubin takes longer time to react and requires caffeine as accelerator. The indirect bilirubin is calculated from the difference between the total and direct bilirubin.

Procedure for total bilirubin:

Sample (0.05 ml) was pipetted into both the standard and test tubes. Then to the standard tube, 0.1 ml of 2-bilirubin solution

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was added followed by the addition of 1.0 ml of 3-bilirubin solution. Also, to the test tube, 0.1 ml of working reagent was added followed by 1.0 ml of 3-bilirubin solution. The reaction was incubated at room temperature for 5 minutes and absorbance was read at 546 nm against sample blank.

Procedure for direct bilirubin:

Sample (0.05 ml) was pipette into both the standard and test tubes. Then to the standard tubes, 1.0 ml of normal saline was added and followed by the addition of 0.1 ml of 2-bilirubin solution. Also, 1.0 ml of normal saline was added to the test tubes and followed by the addition of 0.1 ml of working reagent. The reaction was incubated at room temperature for 3 minutes and absorbance was read at 546 nm against sample blank.

Calculation:

$$Serum\ bilirubin\ (mg/dl) = \left(Absorbance\ of\ sample - Absorbance\ of\ sample\ blank\right) \times 26.312$$

$$Total\ Bilirubin - Direct\ Bilirubin = Indirect\ bilirubin$$

Determination of total protein level

Total protein was determined colorimetrically according to the method described by [26].

Principle: In an alkaline medium, protein reacts with the copper in the biuret reagent causing an increase in absorbance. The increase in the absorbance, at 540nm due to formation of the coloured complex (alkaline-copper-protein) which is directly proportional to the concentration of protein.

Procedure: Biuret reagent (2.5 ml) was added to all the required test tubes (sample blank, standard and test sample). Also, 0.05 ml of the sample was added to the test sample tubes and 0.05 ml of the standard reagent was added to the standard test tube. It was mixed well and allowed to stand at room temperature for 10 minutes. The absorbance of the test sample and standard were read at 540 nm against sample blank. The concentration of the sample was calculated using the formula.

$$Total\ protein\ Conc.(g/dl) = \frac{Absorbance\ of\ test}{Absorbance\ of\ s\tan dard} \times Concentration\ of\ s\tan dard$$

Determination of albumin level

The serum albumin was determined by the method of [27].

Principle: In an acidic medium, albumin binds with bromocresol green (BCG) causing a shift in the absorption spectra of the yellow BCG dye. The blue green colour formed is directly proportional to the albumin present when measured at 630 nm.

Procedure: Bromocresol green reagent (2.5 ml) was added to three clean test tubes labelled test sample, standard and sample blank. Also, 0.01ml of the sample was added to the test sample and 0.01 ml of the standard reagent was added to the standard test tube respectively. The mixtures in each of the test tubes were mixed well and allowed to stand at room temperature for 10 minutes. The absorbance of the test sample and of the standard was read after 10 mins at 630 nm against the sample blank.

Calculation:

$$Album\text{in}\ concentration\ (mg/dl) = \frac{Absorbance\ of\ test}{Absorbance\ of\ \text{standard}} \times Concentration\ of\ \text{standard}\ (mg/dl)$$

Estimation of superoxide dismutase (SOD) activity

Superoxide dismutase activity was carried out using the method described by [28].

Principle

Auto-oxidation of heamatoxylin (with increase in absorbance at 560 nm) is inhibited by SOD activity at the assay pH 7.8; the percentage of inhibition is linearly proportional to the amount of SOD present within a specific range. SOD activity in the sample was determined by measuring the amount of heamatin formed at 560 nm.

$$0_2 + HTH_2 \xrightarrow{SOD} H_20_2 + HT\ \left(Abs\ 560nm\right)$$

(Hematoxylin) (Hematin)

Procedure

Assay buffer (phosphate buffer 0.05 M, pH 7.8) (920 µL) was added to a clean test tube followed by the addition of 40 µL of sample (tissue homogenate) which was indicated as sample test. A reagent test (sample blank) was also prepared by adding 40 µL of the assay buffer to another clean test tube. The mixtures were shaken and incubated for 2 minutes at room temperature. Also, 40 µL of hematoxylin was added to both sample test and reagent test tubes (sample blank) and were mixed quickly to start the auto-oxidation reaction. Following the addition of 40 µL of hematoxylin, absorbance of the sample test and reagent test was read at 560 nm every 30 seconds for 5 minutes against distilled water.

Calculation:

SOD activity was determined by measuring the ratios of auto-oxidation rates in the presence and absence of the sample. SOD activity in the sample was calculated thus;

Absorbance $_{\text{Reagent test}}$ (AR) = Absorbance $_{\text{Reagent test 2}}$ – Absorbance $_{\text{Reagent test 1}}$

Absorbance $_{\text{sample test}}$ (As) = Absorbance $_{\text{sample test 2}}$ – Absorbance $_{\text{sample test 1}}$

$$\left(\%\ SOD\ inhibition\ = \left[\frac{1 - As}{AR}\right] x100\right)$$

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$$SOD\ activity\ \left(U / ml\right) = \left[\frac{1 - As}{AR}\right] x\ 100\ x\ 1.25$$

1 unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the auto- oxidation of heamatoxylin to heamatin in 1 minute.

Estimation of catalase activity

Catalase activity was carried out using the method described by [29].

Principle

Catalase scavenges hydrogen peroxide (H2O2) converting it to water and molecular oxygen.

$$2H2O2 \xrightarrow{Catalase} 2H2O + O2$$

(Absorbance at 240 nm) (no Absorbance at 240 nm)

The activity of catalase in the sample was determined by following the rate of decrease in absorbance at 240 nm.

Procedure

Assay buffer 50 mM potassium phosphate buffer pH 7.0] (1000 µL) was added to a cuvette and used to zero the spectrophotometer at a wavelength of 240 nm. Also, 950 µL of working assay buffer [490 µL of50 mM potassium phosphate buffer (pH 7.0) and 460 µL of 30 mM hydrogen peroxide (H2O2)] and 50 µL of sample (tissue homogenate) were pipetted to another clean cuvette, mixed quickly. Also, a catalase standard was prepared by adding 50 µL of diluted catalase standard to 950 µL of working assay buffer and 460 µL of 30 mM H2O2. The decomposition rate of H202 was measured at 240 nm every 15 seconds for 5 minutes and a standard curve was ploted from the catalase standard. Decomposition rate is the (ΔA240nm/min) of the sample.

Catalase activity was determined using the prepared standard curve plot and expressed as (U/ml).

Estimation of glutathione peroxidase

Glutathione peroxidase assay was carried out using the method adapted by [30].

Test Principle:

Glutathione Peroxidase catalyzes the reduction of hydrogen peroxide (H2O2), oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming NADP+ (resulting in decreased absorbance at 340 nm) and recycling the GSH. However, because glutathione peroxidase is limiting, the decrease in absorbance at 340 nm is directly proportional to the glutathione peroxidase activity.

Procedure:

All reagents were brought to room temperature and samples (tissue homogenate) were placed on ice. The NADPH reagent (β-nicotinamide adenine dinucleatide phosphate and GSH reduced) was reconstituted with NADPH diluent (glutathione reductase in buffer with stabilizer and 4mM NaN3) and was labelled as working NADPH. 50 µL of samples were added to clean sample test tube followed by the addition of 50 µL working NADPH to the sample test tubes. Also, 50 µL of working H2O2 (0.3 ml of 3% H2O2 diluted to 10 ml with assay buffer) was added to the sample test tube and was allowed to equilibrate for 1 minute. Sample blank tube was prepared by replacing the sample with 50 µL of distilled water. The mixtures in both tubes were transferred to cuvettes and absorbance was read at 340 nm for 5 minutes with 30 seconds recording intervals against sample blank.

Calculations:

Glutathione Peroxidase activity was calculated from the net rate and expressed as mU/ml.

Estimation of thiobarbituric acid reactive substance (TBARS)

Thiobarbituric acid reactive substance (TBARS) in the tissues was carried out in the form of MDA using the method described by [31].

Principle The formation of malondialdehyde is the basis for the well-known thiobarbituric acid (TBA) method used for evaluating the extent of lipid peroxidation.At low pH of 2-3 and high temperature (600C), malondialdehyde (MDA) binds thiobarbituric acid to form a pink complex (MDA-TBA) adduct which absorbs maximally at 532 nm.

Procedure

Tissue homogenate (sample) (250 µL), 10 µL of BHT reagent (butylated hydroxyl toluene in ethanol), 250 µL acid reagent (1M phosphoric acid) and 250 µL of TBA reagent (2-thiobarbituric acid reconstituted with 10.5 ml distil water) were added to a clean sample centrifuge tubes, mixed vigorously. A sample blank test was prepared by replacing the sample with 250 µL of distilled water and the mixture in both tubes were incubated for 60 minutes at a temperature of 600C in a water bath, cooled and centrifuged at 10,000 xg for 3 minutes. The reaction mixture in both tubes were transferred to cuvettes and the absorbance of the pink colour produced was read at 532 nm for 5 minute against sample blank.

Calculations:

The concentration of TBARS is expressed in terms of Malondialdehyde (MDA) in µM.

Molar extinction of MDA 1.56 x $10^5 cm^{-1} m^{-1}$

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$$MDA\ concentration = \frac{Absorbance}{1.56 \times 10^{5}\ cm^{-1} m^{-1}}$$

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 deviation (SD). 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 (10.40±0.37%) and the various fractions have aqueous residue as the highest yield (39.34±6.67%), followed by n-butanol fraction (23.98±4.83%), ethylacetate fraction (11.58±2.49%) and n-hexane fraction which has the lowest yield (06.76±1.54%).

Phytochemical analysis of *G. latifolium* and *In vitro* antioxidant assay: The quantitative analysis of phytochemical constituents of the crude extract of Gongronema latifolium for triplicate determinations showed significantly (P<0.05) higher tannin content (1.64±0.30d) followed by glycosides (0.55±0.09a), alkaloids (1.29±0.04c) and saponin (0.95±0.11b). The results in (Table1 & 2) also showed that the n-butanol fraction has significantly (P<0.05) higher flavonoids, polyphenols and ascorbic acid content than the ethylacetate, n-hexane and aqueous residue fractions. Antioxidant potential is inversely proportional to Inhibitory concentration (IC50) value which was calculated from the linear regression of the % inhibition versus fraction concentrations. The Free radical scavenging ability of the fractions on DPPH was investigated and the IC50 values of n-hexane, ethylacetate, n-butanol and aqueous residue of Gongronema latifolium leafis represented in (Table 3). The result shows that the n-hexane fraction has the highest IC50 value (0.257 mg/ml), followed by the aqueous residue (0.245 mg/ml), ethylacetate (0.139 mg/ml) and n-butanol fraction which has the lowest IC50 value (0.082 mg/ml) and thus the highest antioxidant potential.

Table 1: Phytochemical Screening of Methanolic Leave Extract of *Gongronema latifolium.*

Phyto chemicals	Remarks
Carbohydrates	+
Glycosides	+
Cardiac glycosides	+
Saponin	+
Flavonoids	+
Tannins	+
Free Anthraquinone	-
Alkaloids	+

Table 2: Quantitative analysis of the phyto chemical constituents of fractions of *Gongronema latifolium* (mg/g).

Fractions	Polyphenols	Flavonoids	Ascorbic acid
n-Butanol	0.07±0.01^{c}	0.10±0.02^{d}	0.08±0.01^{c}
Ethylacetate	0.06±0.01^{c}	0.07±0.01^{c}	0.06±0.01^{b}
n- hexane	0.02±0.01^{a}	0.01±0.01^{a}	0.03±0.01^{a}
Aqeousresidue	0.04±0.01^{b}	0.03±0.01^{b}	0.04±0.01^{b}

Table 3: Inhibitory Concentration (IC50) of the fractions of *G. latifolium*

Fractions	IC50 values (mg/ml)
n-butanol	0.082
Ethylacetate	0.139
Aqueous residue	0.245
n-hexane	0.257

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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 and heamoglobin concentration: The effect of sub-chronic oral administration of n-butanol fraction of G. latifolium methanolic leaves extract and silymarin (Standard drug) at 100 mg/kg b.wt, 150 mg/kg b.wt and 200 mg/kg b.wt. on packed cell volume and haemoglobin (Hb) concentration in CCl4-induced liver damage rats after 28 days of treatment is shown in (Figure 1&2). The result showed that the packed cell volume (PCV) and Hb concentration of induced control group was significantly (P<0.05) lowered than the PCV and Hb concentration of induced treated groups.

Effects of n-Butanol Fraction of G. latifolium on body and organ weight change: Changes in body weight of CCl4 induced liver damage rats after 28 days of treatment with n-butanol fraction of G. latifolium methanolic leaves extract and silymarin (Standard drug) is represented in (Figure 3). The result shows a significant (P<0.05) decrease in body weight change for the CCl4-induced liver damage control group when compared with the induced treated groups. Changes in organ weight of CCl4-induced liver damage rats treated daily with n-butanol fraction of G. latifolium methanolic leaves extract for a period of 28 days is presented in (Table 4). The result shows a significant (P<0.05) increase in liver weight change for the CCl4-induced liver damage control group when compared with the induced treated groups.

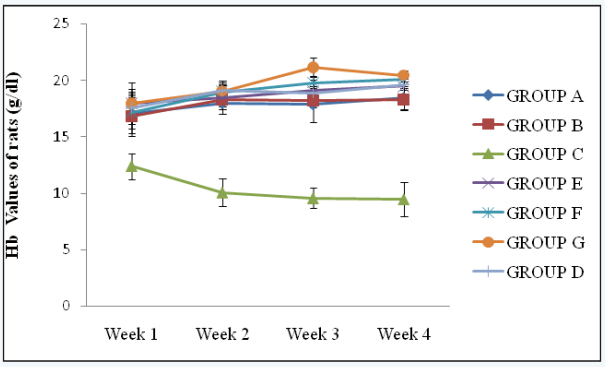

Figure 2: Mean changes in Hb values of CCl4-induced liver damaged rats after 28 days of treatment with oral administration of n-butanol fraction of *Gongronema latifolium*.
Values are presented as Means ± SD with six replicates for each group. Group A: Normal Control rats, Group B: Vehicle control rats, Group C: CCl4 Induced liver damaged control rats, Group E: CCl4 Induced liver damaged rats+100mg/kg b.wt. of n-butanol fraction, Group F: CCl4 Induced liver damaged rats+150mg/kg b.wt. ofn-butanol fraction, Group G: CCl4 Induced liver damaged rats+200mg/kg b.wt. of n-butanol fraction, Group D: CCl4 Induced liver damaged rats+100mg/kg b.wt. of Standard Drug (Silymarin). CCl4: Carbon tetrachloride, Hb: Heamoglobin.

Table 4: Mean changes in organ weights of CCl4-induced liver damaged rats after 28 days of treatment with oral administration of n-butanol fraction of *Gongronema latifolium.*

Groups (n=6)	% Change in Liver Weight
NC	4.31±0.34[a]
VC	4.58±0.78[a]
IC	6.94±0.92[b]
CCl_4+ BF	4.70±0.41[a]
CCl_4+ BF	4.21±0.53[a]
CCl_4+ BF	4.40±0.34[a]
CCl_4+ Std	4.64±0.35[a]

Biochemical studies

Assessment of liver function indices: Liver function indices of alanine amino transferases (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 damage rats after 28 days of daily oral administration of n-butanol fraction of G. latifolium represented in (Table 5 & 6). There was significant (P<0.05) increase in activities of all these liver marker enzymes (ALT, AST and ALP) in the CCl4-induced control group when compared with the induced treated groups. The n-butanol fraction significantly (P<0.05) increase the serum total protein levels of the induced treated groups compared with the induced control group. Also serum albumin concentrations of the induced control group was significantly (P<0.05) lower than the induced treated groups.

Table 5: Effects of n-butanol fraction of *Gongronema latifolium* after 28 days of treatment on serum liver function parameters (ALT, AST and ALP) of CCl4-induced liver damaged albino rats.

Group (n=6)	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
NC	45.83±6.96[a]	42.50±3.98[ab]	60.17±5.34[a]
VC	44.50±7.94[a]	41.33±4.63[a]	59.50±4.32[a]
IC	60.33±7.39[b]	56.83±5.34[c]	76.00±8.41[b]
CCl4 + BF	48.00±5.25[a]	47.33±3.77[b]	64.67±5.71[a]
CCl4 + BF	47.33±3.98[a]	43.83±4.26[ab]	62.17±2.71[a]
CCl4 + BF	45.50±4.23[a]	44.50±4.18[ab]	61.83±4.35[a]
CCl4 + Std	48.33±4.96[a]	46.67±4.17[ab]	64.00±4.42[a]

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In vivo antioxidant studies

Effects of n-butanol fraction of *Gongronema latifolium* on some endogenous antioxidant enzymes in the liver of CCl_4 - induced liver damaged albino rats: The effects of daily oral administration of n-butanol fraction of Gongronema latifolium 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 damaged rats is represented in (Table 7). There was a significant (P<0.05) increase in the level of Malondialdehyde (MDA) and a significant (P<0.05) decrease in the level of catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) of the CCl4 induced control rats compared with the induced treated groups.

Table 6: Effects of n-butanol fraction of *Gongronema latifolium* after 28 days of treatment on serum total protein, albumin and bilirubin levels of CCl4-induced liver damaged albino rats.

Group (n=6)	TP (g/dl)	ALB (g/dl)	TB (mg/dl)	DB (mg/dl)	IB (mg/dl)
NC	61.17±4.44cd	37.17±5.11c	11.98±2.03a	6.68±0.99a	5.30±1.68a
VC	60.33±4.76bcd	33.50±3.72bc	12.23±1.96a	7.13±1.66a	5.10±0.55a
IC	44.83±5.41a	24.83±4.26a	17.26±1.80b	10.50±1.39b	6.82±0.58b
CCl4 + BF	55.17±4.95bc	32.17±3.18bc	13.48±1.93a	7.70±2.18a	5.78±0.62ab
CCl4 + BF	59.50±3.61bcd	29.50±6.09ab	12.33±2.37a	7.622.33±a	4.72±0.57a
CCl4 + BF	63.33±5.04d	33.33±4.17bc	12.38±1.96a	6.83±1.91a	5.55±0.81a
CCl4 + Std	54.50±5.99b	33.00±4.73bc	13.06±2.08a	8.23±1.50a	4.83±1.24a

Values are Means ± SD. Values with different superscript down the columns are significantly different (P<0.05), NC: Normal Control rat, VC: Vehicle control rats, CCl_4: Carbon tetrachloride, IC: CCl_4 Induced liver damaged control rats, CCl_4 + BF: CCl_4 Induced liver damaged rats+100mg/kg b.wt. of n-butanol fraction, CCl_4 + BF: CCl_4 Induced liver damaged rats+150mg/kg b.wt. of n-butanol fraction, CCl_4 + BF: CCl_4 Induced liver damaged rats+200mg/kg b.wt. of n-butanol fraction, CCl_4 + Std: CCl_4 Induced liver damaged rats+100mg/kg b.wt. of Standard Drug (Silymarin), TP: Total protein, ALB: Albumin, TB: Total Bilirubin, DB: Direct bilirubin, IB: Indirect bilirubin.

Discussion

The preliminary phytochemical screening revealed the presence of carbohydrates, cardiac glycosides, glycosides, saponins, tannins, alkaloids, and flavonoids in the crude methanolic leave extracts of G. latifolium (Table 1). The presence of these phytochemicals in the plant, justifies its use as a medicinal plant [32]. The quantitative phytochemical analysis showed that tannins had the highest concentration in the crude extract whereas the n-butanol fraction had the highest concentration of flavonoids, ascorbic acid and polyphenols as well as the highest in vitro antioxidant potential [33-35] (Table 3&4). Plant phenolics, flavonoids and ascorbic acid constitute major groups of phytochemicals acting as primary in vitro antioxidants or free radical terminators [35,36]. Therefore, it was reasonable to determine their concentration in the n-butanol and ethylacetate plant fractions with the aim of utilizing the fraction with the highest concentration of in vitro antioxidant [35,37,38]. Polyphenols, flavonoids and ascorbic acid scavenging potentials and metal chelating ability is [39] dependent upon their unique chemical structure, the number and position of the hydroxyl groups [40-42]. 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 [35,43,44].

Haematological investigation provides information on the general pathophysiology of the blood and reticulo endothelial system [45-47] showed that xenobiotics causes low PCV and Heamoglobin (Hb) concentration which may be associated with the oxidization of sulphyldryl 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) and Hb values in rats exposed to CCl4 were low. The n-butanol fraction appeared to boost blood cells as the values of PCV and Hb approached the normal control in (Figure 1 & 2). The administration of the n-butanol fraction of the methanolic leave extract of G. latifolium to patient with remarkable low PCV and Hb level may lead to increase their PCV and Hb level. It implies that the n-butanol fraction may possess constituents that can elicit the production of more blood cells [35,48,49]. Changes in the body weight after CCl4 dosing have been used as a valuable index of CCl4-related organ damage by [35,50,51] 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 (Figure 3) and consequent increase in

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liver weights seen in CCl4-induced control group were considered to be as a result of direct toxicity of CCl4 and/or indirect toxicity that lead to liver damage (Table 4). This indicates that CCl4 may have caused hypertrophy of the cells of these organs as well as elicit remarkable tissue damage [35,52] 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 injury after induction with CCl4.

Assessment of liver can be made by estimating the activities of serum ALT, AST and ALP which are enzymes present originally at higher concentration in cytoplasm [53]. When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent of liver damage [54,55]. 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 to the induced treated group (Table 5). The elevated level of these marker enzymes with a corresponding decrease in serum proteins level observed in the CCl4-induced control group corresponded to the extensive liver damage induced by CCl4 which may lead to an impaired protein turnover. These results are in agreement with previous finding that the activity levels of serum ALT, ALP and AST were significantly elevated as well as a significant decrease in serum protein levels in rats after CCl4 administration [35,56-59].

Also, the significant ($P<0.05$) elevation of bilirubin levels (Table 6) in the CCl4-induced control group when compared to the normal control and the induced treated groups may be as a result of haemolytic anaemia 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 [35,53]. 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 [54,55]. However, since there are significant elevation of direct (conjugated) and indirect (unconjugated) bilirubin levels in the blood serum of CCl4-induced not treated group, this may be attributed to the inability of the hepatocyte to secrete conjugated bilirubin as envisioned in elevated direct bilirubin level as a result of liver necrosis or may be due to inability of the liver to conjugate bilirubin in the case of elevated indirect bilirubin which can be attributed to the inability of the necrotic liver to conjugate bilirubin or the inability of the hepatocytes to take up bilirubin [35,56-58]. Also, elevated bilirubin may also be due to obstruction in the flow of bile within the liver or in the bile duct as a result of severe liver damage [49].

There was a significant decrease of these liver marker enzymes activities as well as bilirubin levels with a consequent increase in serum proteins levels on administration of the n-butanol fraction for 28 days at a dose of 100 mg/kg b.wt., 150 mg/kg b.wt. 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 as observed in this study may be due to the prevention of the leakage of these intracellular enzymes as a result of in vitro antioxidants present in the n-butanol fraction as well as their membrane stabilizing activity which may be attributed to their ability to quench 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 [35,60,61]. 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 the regeneration of the hepatocytes by 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 [61,62]. It is therefore, a clear manifestation of the hepatocurative effects of the n-butanol fraction of G. latifolium [35,49,59].

Antioxidant scavenging activity of the generated free radicals is important in the curative effect of CCl4-induced hepatotoxicity. The body has an effective defense mechanism to prevent and neutralize free radicals-induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase. Decrease in enzyme activity of superoxide dismutase (SOD) is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in liver injury [63,64]. The increased level of malondialdehyde (MDA) in the liver tissue of CCl4 induced control rats (Table 7) 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 [56,65,66]. Also, the decreased activity of SOD, GPx and CAT in the liver tissues of CCl4-induced control 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 [67]. Treatment with n-butanol fraction of G. latifolium significantly ($P<0.05$) increased the activities of SOD, GPx and CAT as well as significantly ($P<0.05$) reduced MDA levels in the induced treated rats. 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 possess free radical scavenging activity due to the presence of in vitro antioxidants which could exert amelioratic effects against pathophysiological alterations caused by the presence of superoxide and hydroxide free radicals as well as hydrogen peroxide [2,35,59]. This results are in agreement with the report of [2,35,59,68-71].

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Table 7: Effects of n-butanol fraction of Gongronema latifolium after 28 days of treatment on some endogenous antioxidant enzymes in the liver of CCl4-induced liver damaged albino rats

Group (n=6)	MDA (µM)	SOD(U/ml)	CAT (U/ml)	GPx(mU/ml)
NC	1.32±0.15a	2.42±0.18b	50.00±7.18b	50.83±2.86b
VC	1.52±0.17a	2.25±0.19b	51.17±5.04b	49.50±4.64b
IC	2.53±0.21b	1.43±0.23a	40.33±2.88a	37.67±4.08a
CCl4 + BF	1.53±0.25a	2.23±0.23b	52.00±3.09b	47.17±4.02b
CCl4 + BF	1.52±0.26a	2.45±0.21b	53.00±3.23b	49.74±3.19b
CCl4 + BF	1.48±0.31a	2.43±0.39b	54.50±2.88b	51.00±3.74b
CCl4 + Std	1.58±0.24a	2.35±0.27b	52.50±3.27b	49.83±5.74b

Conclusion

The result of this study has scientifically verified the traditional use of G. latifolium in the management of various human diseases. The result showed that the n-butanol fraction of methanol leaves extract of G. latifolium possesses in vitro antioxidants which may have contributed to its significant anti-hepatotoxic properties. The n-butanol fraction of G. latifolium has comparable effects with 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/drug development industry.

Recommendation

The n-butanol fraction was able to increase the level PCV and Hb thus, will be therapeutically effective for treating anaemic patients with low PCV and Hb concentration. Also, owing to the high cost of liver and kidney treatment especially in Africa, the n-butanol fraction of the plant may offer an alternative and cheaper indigenous source of therapy for liver disease. Again, due to the rich anti oxidative properties of the n-butanol fraction of the plant, it may be very useful in the treatment of oxidative related diseases. Furthermore, more research studies can be carried out on the bioactivity-guided fractionation, isolation and identification of the bioactive constituents of the n-butanol fraction which is responsible for the observed pharmacological activities. Also, more research studies can be conducted on the chronic toxicity studies of the n-butanol fraction of the plant so as to ascertain its long term safety.

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