



PHYTOCHEMICAL CONSTITUENTS, ANTIOXIDANT ACTIVITY AND TOXICITY ASSESSMENT OF HYDROETHANOLIC LEAF EXTRACT OF *GRIFFONIA SIMPLICIFOLIA*

Ruby Ama Nyarko^{1*}, Christopher Larbie², Alexander Kofi Anning¹, Philip Kweku Baidoo¹

¹Department of Theoretical and Applied Biology, Kwame Nkrumah University of Science and Technology, Kumasi-Ghana.

²Department of Biochemistry and Biotechnology, Kwame Nkrumah University of Science and Technology, Kumasi-Ghana.

ABSTRACT

Griffonia simplicifolia is an African legume of significant pharmacological activity, with its leaves commonly used for the treatment of bladder and kidney problems, relieving constipation, and as an aphrodisiac. However, the leaves have not been subjected to scientific scrutiny and safety assessment yet. In this study, the phytochemical constituents, *in vitro* antioxidant activity, heavy metal concentration as well as the acute and sub-chronic toxicity of the hydroethanolic leaf extracts of *G. simplicifolia* were determined using standard methods. The results indicated the presence of glycosides, tannins, flavonoids, alkaloids, saponins and coumarins in the leaf extract of the plant. The highest total flavonoid and phenol contents recorded were 237.20 mg/100g QE in the hydro fraction and 8.71 mg/g GAE in the ethyl acetate, respectively. The highest 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity recorded was 24.82% in the hydro fraction. UV and FT-IR spectrometry also suggested the presence of phenolic compounds. Concentrations of all heavy metals analysed (Fe, Zn, Ni, Cu, Pb) were below WHO recommended limits. The median acute toxicity (LD₅₀) of the extract was determined to be <5g/kg body weight in mice. Sub-chronic use for 28 days resulted in significant weight gain, reduction in platelet large cell ratio and platelet count, and increase in low density lipoprotein (LDL) and blood glucose concentrations. The extract did not produce any toxic effects on vital organs except a slight decrease in liver weight of male rats. The hydroethanolic extract of *G. simplicifolia* could therefore be considered safe in moderate doses.

Key words: *Griffonia simplicifolia*, medicinal plants, phytochemicals, subchronic toxicity.

Corresponding Author : **Ruby Ama Nyarko** Email: rubynx06@yahoo.com

INTRODUCTION

Plants are a major source of medicine and form an integral part of primary health care for about 75-80% of the world's population (Abdel-daim *et al.*, 2016). They are one of nature's gifts to humans and have dominated the world's pharmacopeia for thousands of years (Ernst, 2005). Based on the ethnobotanic knowledge of medicinal

plants, several pharmaceutically active compounds have been discovered including aspirin, artemisinin, quinine and ephedrine (Kigen *et al.*, 2013). Over the past few decades in particular, there has been an upsurge in the interest and use of medicinal plants mainly due to the belief that these plants are intrinsically harmless (Neergheen-Bhujun, 2013). However, many adverse reactions to medicinal plants have been documented and with the global rise in their use, the safety and efficacy of medicinal plants have become a public health concern. According to WHO, out of about 35,000–70,000 plants used for medicinal purposes around the world, only about 5000 have been scrutinized scientifically (Zhang *et al.*, 2015). This calls for rigorous assessment of medicinal

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plants to ensure their quality, efficacy and safety prior to their acceptance and use (Larbie *et al.*, 2016).

Griffonia simplicifolia (M. Vahl ex DC.) Baill., commonly known as Griffonia, is an African legume of significant pharmacological activity. It is native to West and Central Africa, but it is primarily found in Ghana, Cote D'Ivoire and Togo (Esposito *et al.*, 2012). The seed of *G. simplicifolia* has occupied attention worldwide for several decades and has found modern therapeutic applications due to its high concentration of 5-hydroxyl-L-tryptophan (5-HTP), a direct precursor to serotonin (Weeks, 2009). It is used in the treatment of depression, obesity, insomnia, fibromyalgia, migraine, and improves cognitive functions (Esposito *et al.*, 2012; Wang *et al.*, 2017).

Apart from the seed, the leaf extract of *G. simplicifolia* is also reportedly used for treating malaria, bladder and kidney problems, for relieving constipation, as an aphrodisiac and a remedy for cough (Esposito *et al.*, 2012; Offoumou *et al.*, 2018; Pathak *et al.*, 2010). According to Pathak *et al.* (2010), the leaves of Griffonia contain volatile oils and coumarins, and about 0.1 % 5-HTP. Recent studies by Offoumou *et al.* (2018) also reported the presence of sterols, quinones, alkaloids and saponins in the aqueous and ethanolic extract of Griffonia leaf. Nonetheless, the toxicity and/or efficacy of the leaf extract have not been scientifically assessed. Primary studies are therefore required to help in clinical assessment and as a baseline for future research. The present study was, thus, aimed at determining the phytochemical constituents, antioxidant activity, heavy metal concentration, and UV-Vis and FT-IR spectra of the hydroethanolic leaf extract of *G. simplicifolia*, as well as its acute and sub-chronic toxicity in animals.

MATERIALS AND METHODS

Collection and Identification of Plant Materials

Leaves of *G. simplicifolia* were handpicked from the Kwame Nkrumah University of Science and Technology (KNUST) Botanic Garden, Kumasi-Ghana (latitude 6°35 N-6°40 N and longitude 1°30 W-1°35 W) before 9:00 am each sampling day, in November 2017. Plant identification was authenticated by a taxonomist at the Department of Theoretical and Applied Biology, KNUST, and a voucher specimen (KNUST/AB/2018/L9544) was deposited in the herbarium for reference purposes. The leaves were thoroughly washed with water and air-dried at room temperature under shade for three weeks. The dried samples were then milled, and packaged in zip-locks for storage.

Preparation of Plant Extract and Fractionation

A hydroethanolic extract of the milled leaves was prepared by suspending the leaves in 50% ethanol (50:50, ethanol: water, v/v) as previously described by Anim *et al.*

(2016). The extraction was done by cold maceration for 48 hours at room temperature on a shaker (Rocking Laboratory Shaker). The extract was filtered through cotton wool and the filtrate collected. The procedure was repeated twice. The extracts were concentrated using a rotary evaporator (Buchi R-205, Switzerland) under reduced pressure and freeze-dried (Labconco, England) to obtain the *Griffonia simplicifolia* ethanolic leaf extract (GSE). Forty grams (40 g) of the leaf extract was successively extracted with solvents of increasing polarity; petroleum ether, ethyl acetate and methanol. The residual portion was designated as hydro fraction. Each fractionation step was performed twice with 400 ml of solvent. The fractions were then concentrated by rotary evaporation, and air-dried at room temperature (25 °C).

Phytochemical screening

Qualitative determination of glycosides, tannins, flavonoids, alkaloids, saponins, coumarins and sterols was performed on the raw powder and the hydroethanolic crude extract of *G. simplicifolia* using standard procedures described by Harborne (1998) and Trease & Evans (1989).

In vitro Antioxidant activity

The free radical scavenging activity of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method was used to determine the antioxidant activity as described by Brand-Williams *et al.* (1995) with some modifications. Equal volumes of different concentrations of each extract (concentration range 0-5 mg/mL) were prepared and 0.5 mM Methanolic solution of DPPH was added to each solution. The mixtures were incubated at room temperature for 20 minutes and the absorbance read at a wavelength of 517 nm (Tecan Infinite M200 Pro plate reader, Austria). The inhibition concentration at 50% (IC₅₀) of each extract was calculated using the formula:

$$\% \text{ Scavenging Activity} = \frac{A_0 - A_1}{A_0} \times 100\%$$

Where A₀ is the mean absorbance of the wells containing negative control; A₁ is the mean absorbance of the wells with the test sample. Butylatedhydroxytoluene (BHT) was used as standard control. Analyses were performed in triplicates.

Total phenolic content determination

Total phenolic content (TPC) was measured using the Folin-Ciocalteu assay as described by Marinova *et al.* (2005). Ten microliters (10 µL) of 5.0 mg/ml of extract was diluted with 790 µL distilled water. The diluted sample was mixed with 50 µL of Folin-Ciocalteu reagent and incubated in a dark place for 8 minutes. Afterwards, 150 µL of 7% Na₂CO₃ was added and the mixture incubated for 2 hours in the dark. The absorbance was read at a wavelength of 750 nm using a microplate reader (Tecan Infinite M200, Austria). Analyses were performed in triplicates. Gallic acid (GA)

was used as the standard phenolic compound. The results were expressed in milligrams of GA equivalents per gram dry mass (mg GAE/g DM).

Total flavonoid content determination

The total flavonoid content was measured with an aluminium chloride colorimetric assay as described by Marinova *et al.* (2005). An aliquot (1 ml) of extracts or a standard solution of (+)-catechin (20, 40, 60, 80 and 100 mg/L) was added to a 10 ml volumetric flask, containing 4 ml of distilled deionized water (dd H₂O), and 0.3 ml 5 % NaNO₂ was added. After 5 min, 0.3 ml of 10% AlCl₃ was added. At the sixth minute, 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with dd H₂O. The solution was mixed well and the absorbance was measured against a prepared reagent blank at 510 nm with a UV-VIS Spectrophotometer Lambda 5. The data were expressed as milligrams of (+)-quercetin equivalents (QE) (mg QE/100 g DW). All samples were analysed in triplicates.

UV-Vis and Fourier transform infrared (FTIR) spectrophotometric analyses

The fractions of the leaf (10 mg/ml) were diluted in the ratio 1:10 using their respective solvents and analysed at a wavelength ranging from 200-800 nm. A double beam ultraviolet-visible spectrophotometer (Perkin Elmer, USA) was used to detect and record the characteristic peaks present in that range. Ten milligram (10mg) of the dried extract powder was encapsulated in 100 mg of KBr pellet to prepare translucent sample discs. The powdered sample of each extract was loaded in FTIR spectroscope (UATR Spectrum 2, Perkin Elmer) with a scan range from 400 to 4000 cm⁻¹ and a resolution of 4 cm⁻¹.

Heavy metal analysis

One gram (1.0 g) each of the extract and the raw sample was weighed into a 50 ml digestion tube. The sample was mixed with 1.0 ml of H₂O, 2.0 ml of conc. HCl, 5 ml of 1:1 conc. HNO₃: 60% HClO₄ and 2.0 ml of Conc. H₂SO₄. The mixture was allowed to stand for 20 minutes. At a temperature of 150 °C, the samples were heated in a digestion block. The digested samples were allowed to cool after which they were diluted with 50 ml of distilled water. All the samples were wet digested. The digests were analysed for the levels of lead, copper, nickel, zinc and iron using an Atomic Absorption Spectrometer (AnalytikjenaovAA 400P).

Animal selection and groupings

Swiss albino mice and Sprague-Dawley rats of both sexes were obtained from the animal house of the School of Medical Science, University of Ghana, Legon Accra. The animals were housed in aluminium rodent cages with bedding of wood shavings. They were

segregated according to sex to avoid any chance of mating, under standard conditions (25± 2°C, 40-60% humidity and ~12h light and dark cycle). They were fed with standard animal feed (AGRICARE, Kumasi, Ghana) and distilled water *ad libitum* throughout the period of the study except an overnight fast prior to sacrificing.

In grouping the animals, their body weights were taken into consideration to achieve approximately equal conditions among the groups. They were allowed to acclimatize to laboratory conditions for a week before the experiment began. The animals were identified by tail marks made with permanent markers. Animal studies were conducted in the animal holding facility of the Department of Biochemistry and Biotechnology (KNUST, Ghana) and in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA, New Delhi, India).

Acute oral toxicity

The acute toxicity study was carried out using three female Swiss albino mice (20-25 g). After an overnight fast, the animals were treated once with 5000 mg/kg bwt of the GSE administered orally by gavage. The dose was selected based on the OECD (2001) fixed dose method. The animals were observed for signs of toxicity (including paw-licking, stretching, respiratory distress, diarrhoea) and mortality for the first 4 hours and subsequently, daily for 7 days.

Sub-chronic oral toxicity

Sub-chronic toxicity study was performed using 16 male and 16 female rats. For each sex, the animals were divided into four groups of four animals each. Group I served as the control and received 1.0 ml of distilled water once daily. Groups II, III and IV were treated with 100, 250 and 500 mg/kg bwt of the GSE once daily in 1.0 ml of distilled water for 28 days. They were observed daily for general signs of toxicity and mortality.

Effect of treatment on body weight

Rats in all groups were weighed on the first day (D0) and subsequently at the end of every fourth day (i.e., D4, D8, D12, D16, D20, D24 and D28). The percent change in body weight was calculated using the formula:

$$\% \text{ Change in Body Weight} = \frac{W_n - W_0}{W_0} \times 100\%$$

Where W_n = weight on day 4 (D4), D8, D12, D16, D20, D24 and D28, and W₀ = weight on D0.

Collection of blood, serum and isolation of organs

At the end of the experiment, animals were fasted overnight and sacrificed by ether anaesthetization. The neck area was quickly cleared of fur to expose the jugular vein. The vein after being slightly displaced was sharply cut with a sterile surgical blade and an aliquot of the blood was collected in sample bottles containing EDTA for the

haematological analyses. Five millilitres of blood was then dispensed into gel-activated tubes and centrifuged at 3000 rpm for 5 minutes. The sera were aspirated with a Pasteur pipette into sample bottles for the various biochemical assays. The sacrificed animals were dissected and their liver, kidney, heart, stomach, spleen, testes or uterus were excised, freed of fat, washed with normal buffered saline, and blotted with clean tissue paper. They were observed macroscopically and weighed to obtain absolute organ weight (AOW). The relative organ weights (ROW) of the organs were calculated for each rat using the formula:

$$\text{ROW} = \frac{\text{AOW}}{W_{28}} \times 100\%$$

Biochemical and haematological analyses

Evaluation of biochemical parameters was done using the Cobas Integra 400 Clinical Chemistry Analyser (Roche, USA) and reagents from Fortress Diagnostics (UK). Parameters analysed included alanine aminotransferase (ALT), aspartate amino transferase (AST), total, direct and indirect bilirubin, creatinine, urea, sodium, potassium, chloride, total cholesterol (TChol), high density lipoproteins (HDL), total triglycerides (Trigs), glucose, and lactate dehydrogenase. Low density lipoprotein (LDL) concentration was calculated using the Friedewald's equation (Crook, 2006).

$$\text{LDL} = (\text{TC} - \text{HDL}) - \left(\frac{\text{TG}}{2.2}\right)$$

Haematological analyses were performed using Sysmex Haematology System (USA). Parameters included red blood cell count, haemoglobin concentration, and white blood cell count, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration (MCHC).

Data analyses

Data were analysed with GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). The percent change in body weight, haematological parameters and serum biochemistry were expressed as mean \pm standard error of mean (SEM). Data were assessed by one-way ANOVA followed by Tukey's multiple comparison test. All analyses were conducted assuming a significance value of 5%.

RESULTS

Phytochemical constituents

The raw leaf powder and hydroethanolic leaf extract of GS both contained glycosides, tannins, saponins and coumarins. In addition, the extract showed the presence of alkaloids and flavonoids (Table 1). However, none of the samples were observed to possess sterols.

Total flavonoid and phenol content

The total flavonoid content (TFC) did not differ among the various fractions analysed except the ethyl acetate fraction which was significantly lower compared to the other three extracts (Figure 2A; $p < 0.01$). Mean TFC of the hydro fraction was 237.2 mg QE, whilst the methanol fraction, crude extract and ethyl acetate fractions recorded values of 232 mg QE, 224 mg QE and 142 mg QE, respectively. The ethyl acetate fraction recorded the highest TPC (8.71 mg GAE; $P < 0.05$). The crude extract, hydro fraction and methanol fraction recorded mean TPC of 4.37 mg GAE, 3.98 mg GAE and 3.87 mg GAE, respectively. However, the TPC of the crude, methanol and hydro fractions were not statistically different from each other (Figure 2B).

In vitro antioxidant activity

There was no statistical difference in the scavenging activities of the hydro fraction, methanol fraction and crude extract. The hydro fraction recorded a scavenging activity of 24.82%. This was followed by the methanol fraction and crude extract with 24.49% and 23.30%, respectively. The ethyl acetate fraction had a lower scavenging activity compared to the other three extracts (14.07%; $P < 0.0001$) as shown in Figure 3.

Heavy metal content

The raw leaf powder of *G. simplicifolia* contained 6.74 mg/L of iron and 0.26 mg/L of zinc (Table 2). The extract, on the other hand, contained 1.41 mg/L of iron and 1.04 mg/L of zinc. The raw powder had higher iron (6.74 mg/kg) but lower zinc (0.26 mg/kg) contents than those of the extract. The levels of nickel, copper and lead in both the raw powder and extract were below detectable limits (< 0.00001 mg/L). Similarly, iron and lead concentrations in the samples were below the WHO limit for medicinal plants (WHO, 1998, 2007).

UV-VIS spectroscopic analysis

The crude, ethyl acetate and hydro fractions of *G. simplicifolia* each recorded five (5) major absorption peaks whereas the methanol fraction recorded three (3) peaks (Table 3). The wavelength generally ranged from 201.00 to 666.90.

FT-IR Spectra of fractions of *G. simplicifolia*

The FTIR spectra of *G. simplicifolia* leaf fractions predicted the functional groups corresponding to each peak as well as the nature of their bonds (Table 4). The frequencies and nature of the peaks were used to identify the functional groups by comparing the values with standards. Ethyl acetate, methanol and the hydro fraction recorded 10, 13 and 5 peaks, respectively.

Safety evaluation of the hydroethanolic leaf extract of *G. simplicifolia* Acute toxicity

In the study, no untoward changes were observed in the rats when they were dosed orally with 5000 mg/kg GSE. No changes in the nature of stool, urine and eye colour were observed. Also, there was no stretching, paw liking or diarrhoea. No mortality was recorded. Orally, 5000 mg/kg of GS was well tolerated in mice even after 7days. Hence, the LD₅₀ was estimated to be ≤ 5000 mg/kg.

Sub-chronic toxicity

Effect of treatments on body weight

In the sub-chronic study, the animals generally showed no exterior signs of toxicity to the *G. simplicifolia* treatment. As expected, the body weights of all test animals generally increased over time (Figure 4). There were significant increases in percent change in body weight in male rats treated with 100 mg/kg GS from days 8 to 28, in those treated with 250 mg/kg GS on day 8, 20 and 24, and in those treated with 500mg/kg GS on day 8 ($p < 0.001$) and day 24 ($p < 0.5$) compared to the control group. But, compared to the 100 mg group, significant decreases were observed in males treated with 250 mg and 500 mg from day 20 to 28 ($p < 0.5$). In female rats, however, there were highly significant increases in change in body weight in the 100 mg/kg treated group from day 8 through to 28 compared to the normal ($p < 0.0001$). Similarly, significant increases were observed in those treated with 250 mg and 500 mg from day 8 to 28. However, compared to the 100 mg group, they decreased in percent change in body weight from day 20 to 28 as observed in the male rats ($p < 0.5$).

Effect of treatments on relative organ weight (ROW)

Compared to the control group, a significant decrease in relative liver weight was observed at all doses in male rats, whereas that of females increased at 250mg/kg ($p < 0.5$). Uterus weight in females administered with 100 mg/kg GSE also decreased considerably ($p < 0.01$). The treatment had no significant effect on any of the other organs (Table 5).

Effect of treatments on haematological indices

With the exception of P-LCR and platelet count, the GSE showed no significant effects on haematological indices (Table 6). P-LCRs for all doses were lower relative to the control ($p < 0.001$). A significant decrease in platelet count was also observed in female rats ($p < 0.05$). All other parameters did not differ among groups.

Effect of treatments on serum biochemistry

The effects of GSE treatment on the serum biochemical parameters of the animals are shown in Table 7. In male rats, compared to the control, AST concentration increased at 100 and 500 mg ($P < 0.01$). Also, creatinine concentration increased significantly at 250 mg. Finally, significant increases were recorded in the concentrations of LDL at 500 mg and FBG at 100 mg. In the female rats, the levels of AST at all doses ($P < 0.01$), triglycerides at 500 mg, and lastly, LDL and FBG at all doses increased significantly relative to the control. All other parameters in both males and females showed no significant changes compared to the control group.

Table 1. Phytochemical constituents of raw powder and hydroethanolic leaf extract of *G. simplicifolia*

Phytochemical	Raw	Extract
Glycosides	+	+
Tannins	+	+
Flavonoids	+	+
Alkaloids	+	+
Saponins	+	+
Coumarins	+	+
Sterols	-	-

'+' indicates presence and '-' indicates absence.

Table 2. Level of heavy metals in the raw leaf and hydroethanolic leaf extract of *G. simplicifolia*

Metal	Mean concentration \pm S.D		
	Raw (n=3)	Extract (n=3)	WHO limit
Iron (mg/kg)	6.74 \pm 0.13	1.41 \pm 0.06	15.00
Zinc (mg/kg)	0.26 \pm 0.00	1.04 \pm 0.01	-
Nickel (mg/kg)	BDL	BDL	-
Copper (mg/kg)	BDL	BDL	-
Lead (mg/kg)	BDL	BDL	10.00

BDL means below detection limit; detection limit = 0.00001 mg/L

Table 3. UV-Vis peak characteristics of hydroethanolic crude extract and fractions of *G. simplicifolia* leaves.

	Peak	Wavelength (nm)	Absorbance
Crude	1	666.90	0.01
	2	341.90	0.37
	3	332.00	0.41
	4	329.00	0.42
	5	201.00	2.83
Ethyl acetate	1	203.90	0.31
	2	210.90	0.29
	3	206.90	0.27
	4	226.00	0.25
	5	209.00	0.17
Methanol	1	664.00	0.02
	2	204.20	2.07
	3	202.10	2.16
Hydro	1	329.90	0.08
	2	332.80	0.08
	3	337.00	0.07
	4	335.10	0.07
	5	341.80	0.07

Table 4. Functional groups in the crude and hydroethanolic crude extract and fractions of *G. simplicifolia* leaf

	Peak	X (cm ⁻¹)	Bond	Functional group
Ethyl Acetate	1	3385.97	O-H stretch, H-bonded N-H stretch	Alcohols, phenols 1°, 2° amines, amides
	2	2924.47	C-H stretch	Alkanes
	3	2853.08	C-H stretch	Alkanes
	4	1712.90	Unknown	Unknown
	5	1515.84	N-O asymmetric stretch	Nitro compounds
	6	1463.37	C-H bend	Alkanes
	7	1377.24	C-H rock	Alkanes
	8	1240.33	C-O stretch C-N stretch	Alcohols, carboxylic acids, esters, ethers Aliphatic amines
	9	1036.75	C-O stretch C-N stretch	Alcohols, carboxylic acids, esters, ethers Aliphatic amines
	10	721.66	C-H rock	Alkanes
Methanol	1	3305.26	O-H stretch, H-bonded N-H stretch	Alcohols, phenols 1°, 2° amines, amides
	2	2926.35	C-H stretch	Alkanes
	3	2850.70	C-H stretch	Alkanes
	4	1563.59	N-O asymmetric stretch	Nitro compounds
	5	1402.85	C-C stretch (in-ring)	Aromatics
	6	1347.27	N-O symmetric stretch	Nitro compounds
	7	1118.18	C-N stretch C-O stretch	Aliphatic amines alcohols, carboxylic acids, esters, ethers
	8	1070.04	C-N stretch C-O stretch	Aliphatic amines alcohols, carboxylic acids, esters, ethers
	9	1021.64	C-N stretch C-O stretch	Aliphatic amines alcohols, carboxylic acids, esters, ethers
	10	893.95	N-H wag C-H "oop" =C-H bend	1°, 2° amines Aromatics Alkenes
	11	647.73	-C≡C-H: C-H bend C-Br stretch	Alkynes Alkyl halides

			C-Cl stretch	Alkyl halides
Methanol	12	617.94	-C≡C-H: C-H bend C-Br stretch C-Cl stretch	Alkynes Alkyl halides Alkyl halides
	13	508.32	Unknown	Unknown
Hydro	1	3339.13	O-H stretch, H-bonded N-H stretch	Alcohols, phenols 1°, 2° amines, amides
	2	2108.12	-C≡C- stretch	Alkynes
	3	1634.4	N-H bend	1° amines
	4	418.64	Unknown	Unknown
	5	403.53	Unknown	Unknown

Table 5. Effect of GS on ROW of animals in sub-chronic toxicity study

Organ	ROW (%)			
	Control	100 mg	250 mg	500 mg
Male				
Liver	3.18 ± 0.22	2.86 ± 0.02*	2.67 ± 0.07***	2.67 ± 0.06***
Testes	1.16 ± 0.01	1.32 ± 0.03	1.25 ± 0.02	1.26 ± 0.03
Lung	0.63 ± 0.07	0.68 ± 0.07	0.71 ± 0.06	0.63 ± 0.05
Kidneys	0.55 ± 0.02	0.60 ± 0.02	0.58 ± 0.02	0.59 ± 0.01
Stomach	0.60 ± 0.03	0.63 ± 0.01	0.62 ± 0.04	0.56 ± 0.01
Heart	0.32 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	0.33 ± 0.01
Spleen	0.19 ± 0.01	0.25 ± 0.01	0.20 ± 0.02	0.20 ± 0.01
Female				
Liver	2.95 ± 0.10	2.99 ± 0.04	3.11 ± 0.03*	2.76 ± 0.02
Lungs	0.73 ± 0.07	0.83 ± 0.03	0.78 ± 0.06	0.68 ± 0.01
Kidneys	0.58 ± 0.00	0.58 ± 0.01	0.61 ± 0.03	0.55 ± 0.03
Stomach	0.65 ± 0.04	0.70 ± 0.01	0.62 ± 0.03	0.58 ± 0.01
Heart	0.35 ± 0.02	0.37 ± 0.01	0.38 ± 0.01	0.33 ± 0.02
Uterus	0.26 ± 0.03	0.18 ± 0.01**	0.26 ± 0.01***	0.22 ± 0.00
Spleen	0.23 ± 0.01	0.25 ± 0.01	0.22 ± 0.01	0.21 ± 0.00

Mean±SEM (n=4); Statistical significance; *p<0.05, **p<0.01, ***p<0.001 compared with Control, +++p<0.001 compared with 100 mg group

Table 6. Effect of treatments on the haematological parameters of animals in sub-chronic toxicity study

Parameter	Mean ± SEM			
	Control	100 mg	250 mg	500 mg
Male				
WBC (10 ³ /μL)	6.10 ± 0.50	6.68 ± 0.56	7.53 ± 0.51	6.05 ± 0.80
RBC (10 ⁶ /μL)	8.09 ± 0.47	8.31 ± 0.16	8.11 ± 0.22	8.33 ± 0.21
HGB (g/dL)	14.10 ± 0.67	14.55 ± 0.31	14.63 ± 0.16	14.63 ± 0.42
LYM (%)	61.20 ± 3.92	65.95 ± 1.43	68.98 ± 4.01	62.20 ± 4.22
HCT (%)	58.27 ± 3.45	56.83 ± 1.05	57.05 ± 2.31	58.75 ± 2.58
MCV (fL)	72.03 ± 0.74	68.40 ± 0.62	70.38 ± 2.06	70.45 ± 1.59
MCHC (g/dL)	24.23 ± 0.35	25.60 ± 0.32	25.73 ± 0.89	24.98 ± 0.38
MCH (pg)	17.43 ± 0.20	17.50 ± 0.07	18.05 ± 0.49	17.53 ± 0.23
P-LCR (%)	13.57 ± 1.01	8.48 ± 0.62***	8.00 ± 1.04****	8.25 ± 0.69****
PLT (10 ³ /μL)	1058.67 ± 80.76	1065.00 ± 105.83	844.00 ± 160.20	909.50 ± 76.77
Female				
WBC (10 ³ /μL)	7.30 ± 0.31	8.10 ± 1.27	7.45 ± 0.37	5.50 ± 0.60
RBC (10 ⁶ /μL)	7.20 ± 0.07	8.028 ± 0.39	7.95 ± 0.19	7.62 ± 0.30

HGB (g/dL)	13.43 ± 0.09	13.90 ± 0.24	14.28 ± 0.35	14.35 ± 0.55
LYM (%)	63.47 ± 0.48	69.98 ± 1.89	69.88 ± 1.69	66.10 ± 5.10
HCT (%)	52.07 ± 0.88	55.30 ± 1.85	54.00 ± 1.27	54.30 ± 1.80
MCV (fL)	72.30 ± 0.60	69.13 ± 2.10	68.00 ± 1.62	71.25 ± 0.45
MCHC (g/dL)	25.80 ± 0.32	25.23 ± 0.96	26.45 ± 0.27	26.45 ± 0.15
MCH (pg)	18.63 ± 0.12	17.43 ± 0.87	18.00 ± 0.34	18.85 ± 0.05
P-LCR (%)	11.90 ± 0.56	7.65 ± 0.72 ^{****}	6.50 ± 0.32 ^{****}	8.70 ± 1.40 [*]
PLT (10 ³ /μL)	1323.00 ± 156.52	1052.50 ± 84.19	891.75 ± 46.22 [*]	821.50 ± 66.50 [*]

Mean±SEM (n=4); Statistical significance; *p<0.05, **p<0.001, ****p<0.0001 compared with Control. WBC – white blood cell, RBC-red blood cell, HGB-haemoglobin, LYM-lymphocyte, HCT-haematocrit, MCV-mean corpuscular volume, MCH-mean corpuscular haemoglobin concentration, P-LCR-platelet large cell volume, PCT-plateletcrit, PLT-platelet

Table 7. Effects of treatments on the serum biochemical parameters of animals in sub-chronic toxicity study

Parameter	Mean concentration ± SEM			
	Control	100 mg	250 mg	500 mg
Male				
ALT (U/L)	63.87 ± 5.27	51.23 ± 4.55	50.35 ± 4.25	54.15 ± 6.32
AST (U/L)	148.00 ± 2.55	196.93 ± 3.32 ^{**}	181.53 ± 10.67	202.48 ± 16.18 ^{**}
Creat (μmol/L)	28.50 ± 6.22	28.38 ± 1.28	47.53 ± 7.93 ^{****}	29.48 ± 4.87 ^{##}
Urea (mmol/l)	11.04 ± 0.85	7.63 ± 0.55	8.40 ± 1.10	7.22 ± 1.38
TChol (mmol/L)	1.87 ± 0.07	2.36 ± 0.13	2.288 ± 0.12	2.39 ± 0.11
Trigs (mmol/L)	1.19 ± 0.15	1.41 ± 0.10	1.27 ± 0.34	1.19 ± 0.15
HDL (mmol/L)	0.58 ± 0.02	0.47 ± 0.08	0.64 ± 0.03	0.43 ± 0.06
LDL (mmol/L)	0.74 ± 0.03	1.26 ± 0.22	1.08 ± 0.12	1.42 ± 0.08 ^{**}
FBG (mmol/L)	1.72 ± 0.32	5.84 ± 0.26 ^{**}	3.57 ± 1.11	4.40 ± 0.44
Female				
ALT (U/L)	56.70 ± 4.22	48.83 ± 4.08	47.38 ± 3.27	51.17 ± 3.13
AST (U/L)	149.77 ± 8.24	202.13 ± 3.54 ^{***}	231.60 ± 12.00 ^{****}	199.70 ± 16.30 ^{**}
Creat (μmol/L)	28.50 ± 0.67	25.15 ± 4.74	25.70 ± 4.53	25.43 ± 4.55
Urea (mmol/l)	10.61 ± 0.46	8.21 ± 0.41	7.30 ± 0.66	8.13 ± 1.25
TChol (mmol/L)	2.15 ± 0.03	2.42 ± 0.09	2.36 ± 0.11	2.63 ± 0.01
Trigs (mmol/L)	1.27 ± 0.22	1.47 ± 0.17	1.02 ± 0.14 ⁺	0.60 ± 0.10 ^{****}
HDL (mmol/L)	0.87 ± 0.11	0.58 ± 0.11	0.46 ± 0.10	0.51 ± 0.09
LDL (mmol/L)	0.70 ± 0.13	1.18 ± 0.12 [*]	1.43 ± 0.23 ^{***}	1.81 ± 0.05 ^{****}
FBG (mmol/L)	1.20 ± 0.10	5.68 ± 0.01 ^{***}	5.24 ± 0.46 ^{***}	3.46 ± 0.87 ^{*+}

Mean±SEM (n=4); Statistical significance; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared with Control; +p<0.05, ++p<0.01, +++p<0.001, ****p<0.0001 compared to the 100 mg group; ##p<0.01 with respect to 250 mg group. ALT-alanine aminotransferase, AST-aspartate aminotransferase, Creat-creatinine, TChol-total cholesterol, Trigs-triglycerides, HDL-high density lipoproteins, LDL-low density lipoproteins, FBG-fasting blood glucose.

Fig 1. Griffonia simplicifolia plant (Source: Field work, 2017).



Fig 2. Total polyphenol content (mean±SD; n=3) of the extract and fractions of Griffonia leaves. A represents TFC, B represents TPC. Different letters indicate significant difference at P≤0.05. Crude - hydroethanolic extract, EthylAc – ethyl acetate, MetOH –methanol, Hydro – hydro fraction.

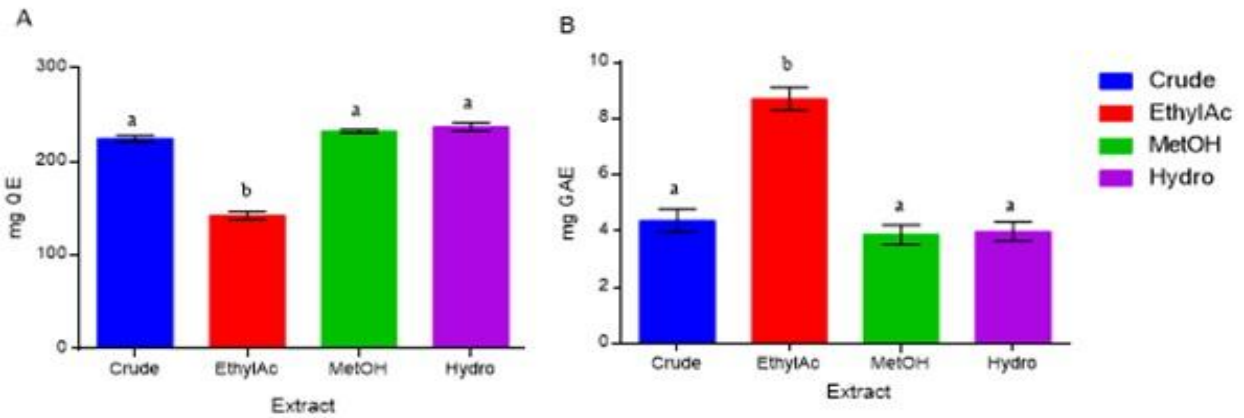


Fig 3. Scavenging activity of hydroethanolic crude extract and fractions of *G. simplicifolia* leaves (mean±SD (n=3)). Different letters indicate significant differences. Crude - hydroethanolic extract, EthylAc – ethyl acetate, MetOH –methanol, Hydro – hydro fraction.

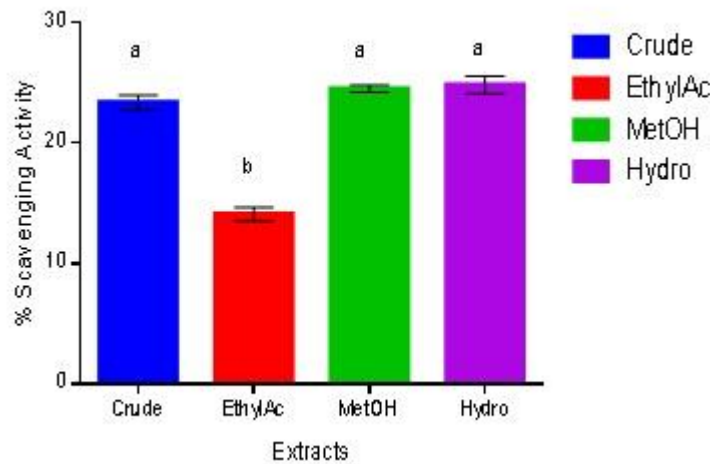
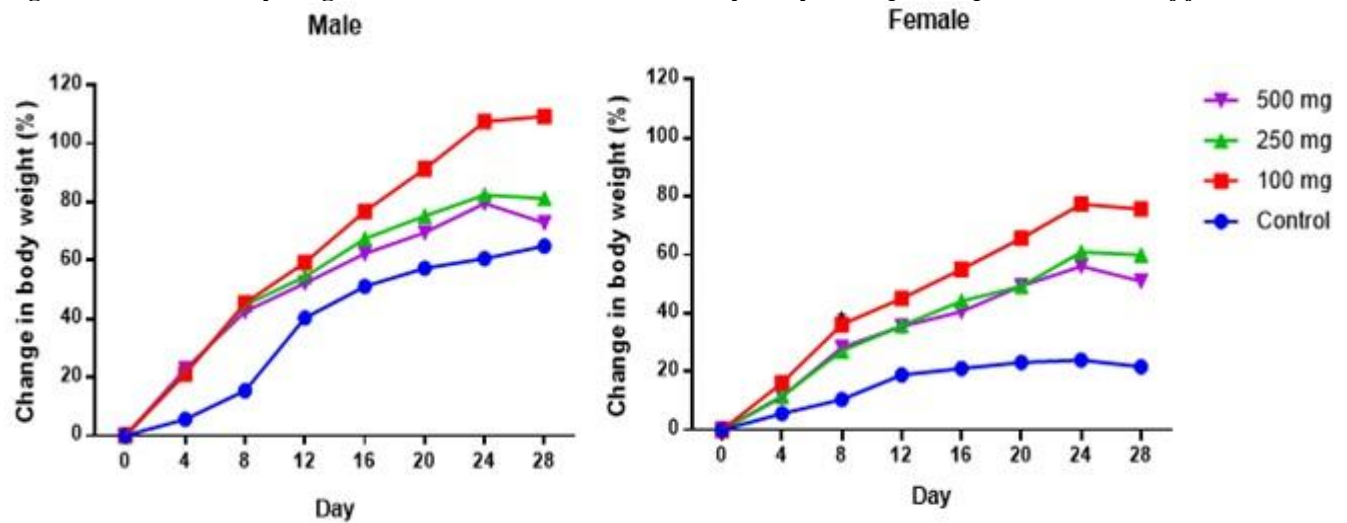


Fig 4. Variation in body weight of animals in sub-chronic toxicity study. Each point represents a mean of four animals.



DISCUSSION

The presence of phytochemicals in a plant material is an indication of the medicinal value of the plant. Phytochemicals such as phenols and polyphenolic compounds like flavonoids, tannins, saponins and alkaloids have been shown to possess good antioxidant and anti-inflammatory properties (Anim *et al.*, 2016; Iwalewa *et al.*, 2007). In this study, the crude leaf extract of *G. simplicifolia* was shown to possess phytochemicals such as glycosides, tannins, flavonoids, alkaloids and saponins, and coumarins. These phytochemicals could be partly responsible for the ethnomedicinal activity of the leaves of this plant species (Miller *et al.*, 2010). A recent study by Offoumou *et al.* (2018) in Cote d'Ivoire reported the presence of alkaloids and saponins in the ethanolic extract of Griffonia leaves. Contrary to the results of this present study, these authors reported the presence of sterols but the absence of flavonoids in the extracts. These variations could reflect differences in the extracts used, geographical sources, genetic makeup and/or level of maturity of the leaves used (Giurleo, 2017). Similarly, the varying concentrations of polyphenols in the different fractions in the present study could be accounted for the different solvents used and their polarity (Medini *et al.*, 2014). This observation is also consistent with the findings of Chebil *et al.* (2007) that the solubility of polyphenols and bioactive compounds differ in different solvents. TFC ranged from 142.3 mg QE in ethyl acetate fraction to 237.20 mg QE in the hydro fraction and TPC ranged from 3.87 mg GAE in methanol fraction to 8.71 mg GAE in ethyl acetate fraction. A study conducted in Ghana by Giurleo (2017) reported a total polyphenol content range of 9.78 to 29.83 GAE/gram in 60% methanol/water extract of *G. simplicifolia* leaves, which differed considerably among the four different regions of the country studied. Thus, the relatively lower TPC observed in our study might reflect differences in sampling location. However, few reports exist on the polyphenol content of leaves related to Griffonia, making effective comparisons difficult at this time.

Free radicals play vital physiological roles in the human body, including signal transduction, smooth muscle relaxation, cell growth and immune response. However, at high concentrations, they result in oxidative stress, which leads to the deterioration of cellular biomolecules (Verma *et al.*, 2017). The antioxidant defence system helps eliminate the excess free radicals to protect the body from oxidative insults. The antioxidant activities of medicinal plants are thus measured to determine their ability to scavenge free radicals. In the current study, the antioxidant activity of Griffonia leaves ranged from 14.07 % in the ethyl acetate fraction to 24.82 % in the hydro fraction which is much lower than the scavenging activity of ethanolic extract of the milk thistle (*Silybummarianum*) seeds, the source of silymarin (a nephroprotective drug), which has a scavenging activity of 92.0 %. This suggests

that Griffonia might not be a very potent antioxidant as compared to *Silybum*. Giurleo (2017) also measured the antioxidant activity of Griffonia leaves in his study. However, he used the ABTS assay instead of the DPPH method; thus his results are not comparable to those obtained from this present study.

Heavy metals such as iron, copper, zinc and nickel function as micronutrients in human body, although excessive concentrations in food or medicine may cause health risks (Darko, 2010). The generally low levels of heavy metals observed both in the raw powder and the hydroethanolic extracts of Griffonia leaves relative to the WHO guideline values in this study suggest that there is no potential negative side effects. Nonetheless, given the likely spatio-temporal variation in heavy metal concentrations in a plant species (Annan *et al.*, 2013; Haider *et al.*, 2004), continuous monitorings of these contaminants in plant materials is absolutely essential to ensure the quality of herbal medicine.

Most phenolic compounds such as flavones and flavonols, exhibit two major absorption bands in the ultraviolet-visible region; one in the 300-500 nm range and the other below 280 nm (Markham, 1989). The UV-Vis spectra of all the Griffonia extracts showed similar bands, suggesting the presence of flavones and flavonols.

The Organisation of Economic Co-operation and Development of France recommends four classes of acute systemic toxicity based on oral LD₅₀ values (OECD, 2001), namely: very toxic (≤ 5 mg/kg), toxic ($> 5 \leq 50$ mg/kg), harmful ($> 50 \leq 500$ mg/kg), and no label ($> 500 \leq 2000$ mg/kg). Based on this classification, the estimated LD₅₀ of the extract of ≤ 5 g/kg bwt suggests no behavioural changes and no adverse gastrointestinal effects. This indicates that the leaf extract of Griffonia is safe for oral use. The gram equivalence of an LD₅₀ ≤ 5 g/kg bwt in an average adult man with an approximate body weight of 60 kg would translate to 750 g dose of the extract.

One of the key indicators of the general health status of an animal is the changes in its body weight (Salawu *et al.*, 2009). In this study, there was a general weight gain in both males and females at all doses, indicating that the extract did not interfere with normal metabolism and nutritional benefits such as weight gain and stability of appetite expected of animals supplied with food and water *ad libitum*. However, at higher doses, the extract may interfere with gastric functions and decrease food conversion efficiency (Arthur *et al.*, 2011). Thus, GSE can be used for weight gain, but it will be more effective at a lower dose.

Organ weight is one of the most sensitive drug toxicity indicators (Bailey *et al.*, 2004; Piao *et al.*, 2013). In this study, Changes in relative organ weight in response to the GSE treatments were observed to be sex-dependent, with the liver and uterus of female rats showing no apparent changes over the duration of the experiment

although the male rates showed considerable reduction in liver weight during the same period. This does not conclusively indicate liver or uterus toxicity in females, but it would be safer to avoid using the extract during pregnancy.

In both males and females, the extract caused a marked reduction in platelet-large cell ratio (P-LCR). P-LCR is defined as the percentage of platelets that exceed the normal platelet volume of 12 fL in the total platelet count (Gawlita *et al.*, 2016). P-LCR is a prognostic factor for coronary artery disease and myocardial infarctions; the lower the percentage, the lower the chance of developing cardiovascular problems. Furthermore, the extract caused a reduction in platelet count. This indicates that GSE is a potent blood thinner and can reduce the incidence of thrombotic complications such as myocardial infarction and sudden cardiac arrest (Gawlita *et al.*, 2016). GS should therefore be further investigated for its potential as a treatment for cardiovascular diseases.

The GSE treatment increased plasma concentration of AST. Unlike ALT, AST is not specific to the liver because it is also found in the heart, skeletal muscles, kidneys, brain and red blood cells and thus, an increased concentration is not necessarily an indication of liver damage. When the plasma concentration of AST is greater than ALT, the injury may be due to muscle necrosis. However, this can only be confirmed if there is an increase in creatine kinase concentration (Aragon & Younossi, 2010). Treatment with GS extract also resulted in hyperbilirubinemia especially at the lowest dose. The extract also caused an increase in LDL (low-density

lipoproteins, commonly known as ‘bad cholesterol’) at the highest dose in males and at all doses in females. High concentration of LDL in the blood is often associated with an increased risk of atherosclerosis and heart diseases (Ai *et al.*, 2010). This means that the extract could potentially be dangerous for patients who already have high blood cholesterol levels, especially females. It may also cause hypercholesterolemia in normal individuals. Finally, the increase in blood glucose in both males and females suggests the presence of hyperglycaemic components in the GS extract.

CONCLUSION

In general, the study demonstrates that *G. Simplicifolia* leaf extract is safe for oral use due to the high LD₅₀. It can also be used for weight gain and for reducing platelet large cell ratio. Sub-chronic use may, however, cause side effects such as blood thinning and an increase in LDL and blood glucose concentration.

AUTHOR CONTRIBUTIONS

This work was carried out by collaboration among all authors. Authors AKA and CL conceived of the study. Authors RAN and CL did the experimental design, crude extract preparation, laboratory work, data and statistical analyses. Author RAN drafted the manuscript. Author AKA also participated in the design and coordination of the study, as well as the data analyses. He also helped to draft the manuscript. Author PKB participated in the design and coordination of the study. All authors read and approved the final manuscript.

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