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ACUTE AND SUB-ACUTE TOXICITY OF *UTHULI LWEZICHWE*™, A TRADITIONAL MEDICINE USED IN THE MANAGEMENT OF DIABETES MELLITUS IN KWAZULU NATAL, SOUTH AFRICA

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Abstract

Background: The number of people using traditional medicines in the management of chronic diseases is increasing. The safety profile of some of the formulations, however, has not been scientifically demonstrated. This study assessed the acute and sub-acute toxicity of *Uthuli Lwezichwe™ (UL)*, a traditional medicine used by a traditional healer in the management of diabetes mellitus.
Materials and Methods: In the acute toxicity assay, one female *Sprague dawley* rat was administered 1000mg/kg body weight (BW) of *Uthuli lwezichwe™* and observed for 14 days. In the sub-acute assay, 24 *Sprague dawley* rats were randomized into four groups. With one group as the control, the other groups were administered varying daily doses (100 mg, 300 mg, 1000 mg/kg BW) of *UL* for 28 days. Phytochemical analysis of *UL* was done using Liquid Chromatography – Quadrupole Time of Flight - Mass Spectrometer (LC-QTOF-MS).
Results: There were no significant differences in liver function biomarkers and kidney function biomarkers between the control and all the treatment groups. Histological examination of the kidney however, showed enlarged bowman's space and distal convoluted tubule diameter and area in the 1000mg/kg *UL* treated group compared to the control group. Similarly, histological examinations of the liver showed increased sinusoidal space and decreased central vein area in the 1000mg/kg *UL* treated group. A number of phytoconstituents were identified in *UL*.
Conclusion: The maximum safe dose was determined to be between 300 – 1000 mg/kg BW. We recommend a chronic toxicity study to assess the long-term effects.

Key words: *Uthuli lwezichwe*, acute toxicity, sub-acute toxicity, diabetes mellitus

List of abbreviations: *UL- Uthuli lwezichwe;* LC-QTOF-MS-Liquid Chromatography – Quadrupole Time of Flight - Mass Spectrometer; OECD- Organisation for Economic Co-operation and Development; MTA- Material Transfer Agreement; ALT- Alanine transaminase; ALP- Alkaline phosphatase; AST- Aspartate transaminase; CK- Creatine kinase; LDH- Lactate dehydrogenase; RBC- Red blood cell; WBC- White blood cell; Hct- Hematocrit; Hb- Haemoglobin; MCV-Mean corpuscular volume; MCH-Mean corpuscular hemoglobin; MCHC- Mean corpuscular hemoglobin concentration; MPV- Mean platelet volume; BC- Bowman's corpuscle; BS- Bowman space; PCT- Proximal convoluted tubule; DCT- Distal convoluted tubule; CV- Central vein

Introduction

The global burden of chronic non-communicable diseases has been increasing over the last decade (Baldwin and Amato, 2016; Mayosi *et al.,* 2009; World Health Organisation, 2013a, 2016). This has resulted in increased use of herbal or traditional medicines whose safety has not been scientifically validated (Mollaoğlu and Aciyurt, 2013; Peltzer *et al.,* 2016; Puataweepong *et al.,* 2012; Saydah and Eberhardt, 2006; Tulunay *et al.,* 2015). A common perception by users that traditional herbal medicine, being from natural sources, is safe is unfounded (World Health Organisation, 2003). Hence, it is important to establish safe dosage ranges for traditional medicines that are used in the management of the various conditions (World Health Organisation, 2013b).

The toxicity or safety profile of the traditional medicines can be assessed using in-vitro and in-vivo models. In-vitro models are designed to establish a concentration-effect relationship on cell lines, stem cells and primary cells in culture media (World Health Organization, 2000). The in-vivo models using rodents such as rats and mice are designed to assess the effects of traditional medicines on behavior, organ integrity, cell integrity, water and food intake, urine output, excretions, biochemical and hematological parameters.

Toxicity studies in animal models can either be single dose (acute toxicity) or repeated dose toxicity studies. Acute toxicity is the toxicity produced by a substance when administered in one or more doses during a period not exceeding 24 hours. Acute toxicity studies are necessary to determine the doses to use for repeated dose studies. Repeated dose toxicity studies can be sub-acute (28 days or 30 days), sub-chronic (less than 90 days) and chronic (>90 days) (Organisation for Economic Co-operation and Development, 2008; Woutersen *et al.,* 1984).

The present study was conducted to evaluate the acute and sub-acute toxicity of *Uthuli lwezichwe*™ (*UL*), a traditional herbal medicine used by a traditional healer in the management of diabetes mellitus in KwaZulu Natal, South Africa.

Materials and Methods

Preparation of UL

UL is a polyherbal mixture of six indigenous plants whose identity is withheld to protect intellectual property and indigenous knowledge. The traditional healer and the University of KwaZulu Natal (UKZN) entered into a material transfer agreement (MTA). The traditional healer was trained to collect and preserve relevant parts of the plants for later botanical verification. *UL* was obtained as an aqueous formulation, the dosage form which the traditional healer dispenses to patients. The aqueous mixture was centrifuged and freeze-dried to powder. The powder was stored at -20°C until use. Immediately, before dosage, *UL* powder was dissolved in Phosphate Buffered Saline (PBS) at a pH of 7.4 and filter sterilized.

Phytochemical analysis of UL using Liquid Chromatography-Mass Spectrometer

The aqueous extract of UL was analyzed using the Liquid Chromatography-Quadrupole time of flight-Mass Spectrometry (LC-QTOF-MS) system. Both the HPLC and the mass spectrometer were from Agilent Technologies. The HPLC was a 1260 Infinity equipped with binary pumps. The mass spectrometer was a Q-TOF 6530 model. Identification of the compounds was performed by comparing with spectral library.

The HPLC conditions

The HPLC column was a poroshell (120, (100x2.1, 2.7µm) maintained at 40°C in the thermostatted column compartment. The flow rate was 0.25ml/min. The mobile phase consisted of aqueous mobile phase A (water with 0.1% formic acid) and the organic phase mobile phase B (acetonitrile with 0.1% formic acid. Gradient elution was employed to separate the different phytochemicals of varied polarities. The gradient started with 20% mobile phase B, increasing to 30% in 3 minute. At 7 minutes mobile phase B was at 35%, this increased to 40% at 10 minutes, and at 13 minutes, it was 60%. Mobile phase continued to increase such that at 17 minutes it was 100% and was maintained at 100% up to 20 minutes before it dropped down to 20% at 20.50 minutes. It remained at 20% mobile phase B for 25 minutes to ensure column equilibration.

The Mass Spectrometry Conditions

Mass spectrometry was performed using high resolution Agilent 6530 Q-TOF mass spectrometer with mass hunter software version B. Electrospray ionization (ESI) was used in both positive and negative modes for data acquisition. Full scan was employed with a scan range from m/z (100-1000). Nitrogen gas from the peak scientific generator was used for both nebulization and sheath gas. The ion source gas temperature was maintained at 300°C with a flow rate of 8ml/min, Nebulizer gas pressure was 35psi, while sheath gas temperature was 350°C with a flow of 11ml/min. The capillary and nozzle voltages were 3500V and 1000V respectively. The fragmentor and skimmer voltages were maintained at 175V and 65V respectively.

Experimental animals

Male and female Sprague Dawley rats 5-6 weeks old weighing between 140-200 g were obtained from the Biomedical Resources Unit (BRU) of the UKZN. The rats were housed in transparent polycarbonate cages with stainless steel mesh lids and wood shavings as bedding under a 12-hour light/12-hour dark cycle. Animal rooms were set to maintain temperature and humidity at 22±2ºC and 55±10%, respectively. The rats were fed commercial standard rat pellets and tap water *ad libitum.* Animal handling and treatment was carried out following the principles outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences published by the National Institute of Health (USA). All animals were acclimatized in the animal rooms for at least seven days before dosing (Canadian Council on Animal Care, 1993). The study received ethical approval from the Animal Research Ethics Committee of the University of KwaZulu Natal (Reference Number: AREC/076/016D).

Acute toxicity study

One female rat was used in the acute toxicity study. The acute toxicity study was performed following the Organisation for Economic Co-operation and Development (OECD) guidelines for acute toxicity testing with modifications (Organisation for Economic Co-operation and Development, 2001). The rat was fasted for two hours prior to dosing. A dose of 1000mg/kg body weight (BW) *UL* was given via oral gavage and the animal was further fasted for two hours. The dose of 1000mg/kg BW is 10 times higher than the human equivalent dose the traditional healer dispenses to patients. The dose was chosen based on guidelines for industry by the United States Food and Drug Administration which recommends a dose ten times higher than the maximum effective dose (Food and Drug Administration, 2010). Xu et al, 2015, also used a maximum dose 10 times the effective dose in the acute toxicity assay (Xu *et al.,* 2015). The dose was converted from human to rat using a conversion formula from the FDA which takes into consideration allometric scaling (Food and Drug Administration, 2005).

General behavior was assessed on breathing, alertness, movement, posture, type of stool and secretions from body orifices were observed every 10 minutes for one hour after dosing, every one hour during the first 4 hours, and once every four hours for 24 hours and once daily for 14 days. Changes in body weight, external morphology and psychomotor activity were also recorded.

Sub-Acute toxicity study

Sub-acute toxicity testing was done according to the OECD guidelines for repeated dose toxicity testing (Organisation for Economic Co-operation and Development, 2008). The doses were based on the acute toxicity study, which did not show any acute toxicity effects at 1000 mg/kg BW. The animals were randomized into four groups (n=6; 3 females and 3 males for each group). The dosages were freshly prepared daily immediately before administration. The groups received phosphate buffer saline (control), 100 mg/kg BW *UL*, 300 mg/kg BW *UL* and 1000 mg/kg BW *UL* via oral gavage once daily for 28 days. General clinical observations were made at least once a day. All animals were weighed once weekly. Food consumption was recorded once weekly. Changes in physical appearance, psychomotor activity, stress symptoms and abnormal behavior were recorded.

At the end of the 28-day period, all animals were fasted for 12 hours, fasting glucose levels were measured using a portable glucometer (Accu-Chek, Roche Diabetes Care, Indiana) on whole blood from a tail prick. The rats were then anaesthetized using isoflurane by inhalation. Blood samples were collected by cardiac puncture into two tubes: one with ethylenediamine-tetraacetate (EDTA) and the other without anticoagulant.

Biochemical tests

Blood samples collected in plain tubes were allowed to clot then centrifuged at 3500 rpm for 10 minutes and serum collected. The serum was analyzed using a biochemistry analyzer (Beckman Coulter, DXC, UK) for urea, creatinine, albumin, total protein, phosphorus, calcium, sodium, potassium, chloride, total bilirubin, cholesterol, triglycerides, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and creatine kinase (CK).

Hematological tests

The blood samples collected into tubes with EDTA were analyzed for erythrocyte (RBC), total leukocyte (WBC), hematocrit (Hct), platelet count, hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and mean platelet volume (MPV) using a Beckman Coulter hematology analyzer.

Histopathological tests

Following blood collection, rats were sacrificed by overdose of isoflurane and the livers and kidneys were collected, trimmed of adherent tissue, and fixed in 10% neutral buffered formalin. Samples were dehydrated through

changes of an ascending concentration of alcohol (0- 100%), cleared with xylene and embedded in paraffin wax. The blocks were sectioned at 5µm thicknesses, stained with haematoxylin-eosin on microscopic slides. The slides were scanned using a Leica SCN 400 (Leica Microsystems GmbH, Wetzlar, Germany) scanner and morphological measurements were taken using the Leica microsystem software 2.0.

For the kidney, the Bowman's capsule (BC) diameter and area, Bowman's space (BS), proximal convoluted tubule (PCT) and distal convoluted tubule (DCT) diameters and areas were measured. For the liver, the central vein (CV) diameter and area as well as the sinusoidal spaces were measured.

Data Analysis

GraphPad® Prism Version 5.0 (California, USA) software was used for data analysis. Data were compared across the treatment groups using one away ANOVA. Where there was statistical significant differences, Tukey's post-hoc test was used during paired comparisons. The significance level was set at α=0.05.

Results

Phytochemical analysis of UL using LC-QTOF-MS

Phytochemical analysis using LC-QTOF-MS revealed the presence of several classes of phytoconstituents. The classes include alkaloids, flavonoids, quinones, quinolones, terpenoids, saponins, coumarins and glycosides. The LC-QTOF-MS chromatogram is shown in Figure 1. The phytoconstituents are grouped according to their class in Table 1.

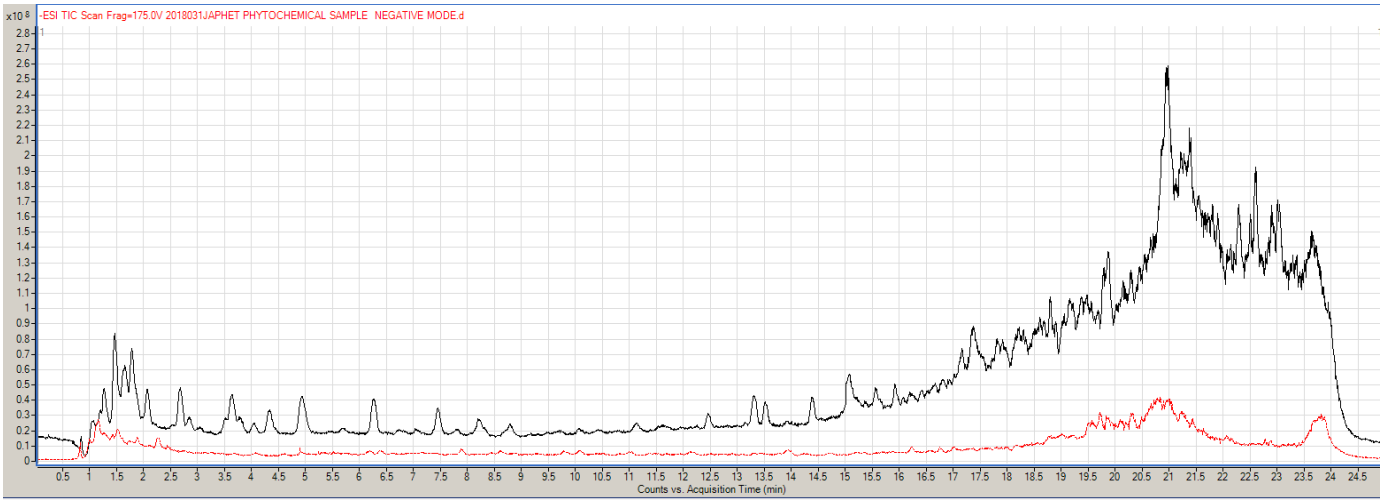

Figure 1: Overlaid negative ion mode and positive ion mode chromatograms. Positive ion mode is the one with high counts and negative ion mode has lower counts.

Table 1: Phytoconstituents in *UL* as revealed by LC-QTOF-MS

Class	**Phytoconstituent**	**Class**	subclass	URL
Glycoside	(1alpha,3beta,20S,22R,24S,25S)-Pubescenin	Steroid	steroid glycoside	http://foodb.ca/compounds/FDB001302
Glycoside	(1r,2r,4s)-p-menthane-1,2,8-triol 8-glucoside	Terpene	terpene glycoside	
Other	(2E,8E)-Piperamide-C9:2	Alkaloid	pyrrolidine	https://pubchem.ncbi.nlm.nih.gov/compound/131752411#section=Top
Flavonoid	(2R,3R)-3,3',4',7-Tetrahydroxyflavanone 7-O-alpha-L-Rhamnopyranoside	Flavonoid	flavanone glycoside	https://pubchem.ncbi.nlm.nih.gov/compound/131752847#section=Top
Terpenoids	(2S,4R,6S)-2-[2-(4-hydroxy-3-methoxyphenyl)ethyl]tetrahydro-6-(4-hydroxy-3,5-dimethoxyphenyl)-2H-pyran-4-ol	Diarylheptanoids	linear diarylheptanoid	http://foodb.ca/compounds/FDB002373
Other	1-(beta-d-glucopyranosyloxy)-3-octanone	fatty acyls	fatty acyl glycoside	http://foodb.ca/compounds/FDB003373

Alkaloid	1,2-Dihydrovomilenine	Alkaloid	indole	https://pubchem.ncbi.nlm.nih.gov/compound/11953964
Flavonoid	1,2-Dimethoxy-13-methyl-[1,3]benzodioxolo[5,6-c]phenanthridine	Alkaloid	benzoquinoline	http://foodb.ca/compounds/FDB020938
Other	10,20-dihydroxyeicosanoic acid	fatty acyls	eicosanoid	
Alkaloid	11 - methyoxy-vinorine	Alkaloid	indole	http://www.genome.jp/dbget-bin/www_bget?C12073
Terpenoids	11,12-dimethylrosmanol	Terpene	terpene lactone	http://foodb.ca/compounds/FDB020292
Glycoside	1alpha,3beta,22R-Trihydroxyergosta-5,24E-dien-26-oic acid 3-O-b-D-glucoside 26-O-[b-D-glucosyl-(1->2)-b-D-glucosyl] ester	Steroid	steroid glycoside	http://foodb.ca/compounds/FDB020131
Other	1-benzyloxy-1-(2-methoxyethoxy)ethane	Benzenoid	dialkyl ether	http://foodb.ca/compounds/FDB016130
Glycoside	1-Octen-3-yl glucoside	fatty acyls	fatty acyl glycoside	http://foodb.ca/compounds/FDB010945
Glycoside	2'-oxoaloesol 7-glucoside	Phenol	phenolic glycoside	http://foodb.ca/compounds/FDB014462
Flavonoid	4'-O-methyl-(-)-epicatechin-7-O-beta-glucuronide	Flavonoid	flavanol glycoside	
Other	5-Hexyl-1,4-dioxan-2-one	Dioxane	1,4-dioxane	http://foodb.ca/compounds/FDB019212
Terpenoids	6,10,14-trimethyl-5,9,13-pentadecatrien-2-one	Terpene	terpene derivative	https://pubchem.ncbi.nlm.nih.gov/compound/Farnesyl_acetone#section=Top
Flavonoid	6'-Hydroxy-O-desmethylangolensin	Flavonoid	isoflavonoid	http://foodb.ca/compounds/FDB029860
Flavonoid	7,8-Dihydro-3b,6a-dihydroxy-alpha-ionol 9-glucoside	Terpene	aliphatic alcohol glycoside	
Flavonoid	7-Hydroxy-2',4',5'-trimethoxyisoflavan	Flavonoid	isoflavonoid	http://foodb.ca/compounds/FDB019120
Other	7-hydroxy-2,5-dimethyl-4h-1-benzopyran-4-one	Benzopyran	chromone	http://foodb.ca/compounds/FDB020049
Other	aflatoxin exb2	Unclassified		http://foodb.ca/compounds/FDB018230
Other	AFN911	Alkaloid	pyridinylpyrimidine	http://www.hmdb.ca/metabolites/HMDB0013863
Terpenoids	all trans decaprenyl diphosphate	prenol lipids	isoprenoid phosphate	http://foodb.ca/compounds/FDB023878
Glycoside	alpha – antiarin	Steroid	steroid glycoside	https://pubchem.ncbi.nlm.nih.gov/compound/alpha-Antiarin#section=Other-Identifiers
Terpenoids	arabsin	Terpene	terpene lactone	http://foodb.ca/compounds/FDB015002
Flavonoid	aromadendrin 4'-methyl ether 7-rhamnoside	Flavonoid	dihydroflavonol glycoside	http://foodb.ca/compounds/FDB020337
Glycoside	caffeic acid	Phenol	hydroxycinnamic acid	http://foodb.ca/compounds/FDB002558
Glycoside	canesceol	Steroid	steroid lactone	http://foodb.ca/compounds/FDB012343
Alkaloid	cinnamoylcocaine	Alkaloid	tropane	https://pubchem.ncbi.nlm.nih.gov/compound/5281863
Other	citreovirenone	Phenol	methoxyphenol	http://foodb.ca/compounds/FDB011570
Glycoside	corchorusoside A	Steroid	steroid lactone	http://foodb.ca/compounds/FDB010799
Other	coriandrone E	Benzopyran	2-benzopyran	http://foodb.ca/compounds/FDB001258
Glycoside	coroloside	Steroid	steroid lactone	http://foodb.ca/compounds/FDB011818
Alkaloid	coronalidine			

Alkaloid	coronaridine	Alkaloid	tryptamine	https://pubchem.ncbi.nlm.nih.gov/compound/coronaridine#section=Top
Alkaloid	corydaline	Alkaloid	quinoline	https://pubchem.ncbi.nlm.nih.gov/compound/Corydaline#section=Top
Terpenoids	cucurbitacin P	Steroid	cucurbitacin	https://pubchem.ncbi.nlm.nih.gov/compound/cucurbitacin_p#section=Top
Quinones and quinoline	Cularine	Alkaloid	isoquinoline	https://pubchem.ncbi.nlm.nih.gov/compound/Cularine
Flavonoid	desmethylxanthohumol	Flavonoid	chalcone	http://foodb.ca/compounds/FDB002506
Glycoside	dide-o-methyl-4-o-alpha-d-glucopyranosylsimmondsin	Nitrile	nitrile glycoside	http://foodb.ca/compounds/FDB017664
Alkaloid	diferuloylputrescine	Phenol	cinnamic acid amide dimer	http://foodb.ca/compounds/FDB011510
Other	dihydrodaidzin	Flavonoid	isoflavonoid	http://foodb.ca/compounds/FDB019563
Quinones and quinoline	dihydrocoriandrin	Benzopyran	2-benzopyran	http://foodb.ca/compounds/FDB011357
Terpenoids	Dimethylaminoethyl reserpilinate	Alkaloid	indole	https://pubchem.ncbi.nlm.nih.gov/compound/70687281#section=Top
Other	dukunolide C	Naphthopyran	naphthopyran	http://foodb.ca/compounds/FDB013850
Alkaloid	erysopine	Alkaloid	isoquinoline	http://foodb.ca/compounds/FDB002080
Terpenoids	esculentoside E	Steroid	steroid glycoside	http://foodb.ca/compounds/FDB013158
Other	eupatoriochromene	Benzopyran	chromone	https://pubchem.ncbi.nlm.nih.gov/compound/Eupatoriochromene#section=Top
Alkaloid	gabunine	Alkaloid	indole	https://pubchem.ncbi.nlm.nih.gov/compound/11953929#section=Top
Flavonoid	gallocatechin-(4alpha->8)-catechin-(4alpha->8)-catechin	Flavonoid	proanthocyanidin	http://foodb.ca/compounds/FDB017718
Other	genipic acid	Furan	dihydrofuran	http://foodb.ca/compounds/FDB014903
Quinones and quinoline	geranylbenzoquinone	Terpene	terpene quinone	https://pubchem.ncbi.nlm.nih.gov/compound/Geranylbenzoquinone#section=Top
Other	gibberellin a105	Lipid	isoprenoid lipid	http://www.phytohormones.info/ga105info.html
Alkaloid	glaucine	Alkaloid	quinoline	http://foodb.ca/compounds/FDB001502
Saponins	Glucoconvallasaponin B	Steroid	steroid glycoside	http://foodb.ca/compounds/FDB012685
Terpenoids	gossyrubilone	Terpene	sesquiterpene	http://foodb.ca/compounds/FDB011289
Terpenoids	hiyodorilactone A	Terpene	terpene lactone	http://foodb.ca/compounds/FDB015620
Other	homodihydrocapsaicin	Phenol	methoxyphenol	http://foodb.ca/compounds/FDB015201
Alkaloid	isorhynchophylline	Alkaloid	indole	https://pubchem.ncbi.nlm.nih.gov/compound/Isorhynchophylline#section=Top
Other	khelloside	Benzopyran	furanochromone glycoside	https://pubchem.ncbi.nlm.nih.gov/compound/28404#section=Top
Other	lactariamide b	Sphingolipid	ceramined	http://foodb.ca/compounds/FDB018577
Alkaloid	Lamprolobine	Alkaloid	quinolizidine	https://pubchem.ncbi.nlm.nih.gov/compound/Lamprolobine#section=Top

Glycoside	licorice glycoside	Ambiguous		
Alkaloid	licorice glycoside A	Flavonoid	chalcone	http://foodb.ca/compounds/FDB008682
Alkaloid	lochinerine			
Other	LysoPE(0:0/20:5(5Z,8Z,11Z,14Z,17Z))	Lipid	glycerophospholipid	http://foodb.ca/compounds/FDB028205
Alkaloid	melostanin A			
Coumarins	methyl (2r*,3s*)-2,3-dihydro-3-hydroxy-2-isopropenyl-5-benzofurancarboxylate	Coumaran	benzofuran	http://foodb.ca/compounds/FDB011106
Other	N-hexadecanoylpyrrolidine	Alkaloid	pyrrolidine	http://foodb.ca/compounds/FDB010702
Coumarins	Nodakenin	Coumarin	psoralen	http://foodb.ca/compounds/FDB012422
Glycoside	Orientaloside	Phenol	naphthol	http://foodb.ca/compounds/FDB016090
Other	osmundalactone	Pyran	dihydropyranone	http://foodb.ca/compounds/FDB003355
Glycoside	PE(18:4(6Z,9Z,12Z,15Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Lipid	phosphatidylserine	http://www.hmdb.ca/metabolites/HMDB0112509
Flavonoid	phloridizin	Flavonoid	dihydrochalcone	http://foodb.ca/compounds/FDB015554
Terpenoids	picrasin C	Terpene	quassinoid	http://foodb.ca/compounds/FDB015499
Terpenoids	picrasin F	Terpene	quassinoid	https://pubchem.ncbi.nlm.nih.gov/compound/46173861#section=Top
Other	pterosin E	aryl akyl ketone	indanone	http://foodb.ca/compounds/FDB015521
Quinones and quinoline	pulmatin	Anthraquinone	anthraquinone glycoside	http://foodb.ca/compounds/FDB002585
Other	sapidolide A	Lactone	gamma butyrolactone	http://foodb.ca/compounds/FDB021574
Alkaloid	sarpagine	Alkaloid	indole	https://pubchem.ncbi.nlm.nih.gov/compound/44592554#section=Top
Alkaloid	senaetnine	Alkaloid	pyrrolizidine	https://pubchem.ncbi.nlm.nih.gov/compound/Senaetnine#section=Top
Terpenoids	simalikilactone D	Terpene	quassinoid	https://pubchem.ncbi.nlm.nih.gov/compound/118701222#section=Top
Alkaloid	sinactine	Alkaloid	quinolizidine	https://pubchem.ncbi.nlm.nih.gov/compound/5321317#section=Top
Alkaloid	skimmianine	Alkaloid	quinoline	https://pubchem.ncbi.nlm.nih.gov/compound/Skimmianine#section=Top
Flavonoid	spinosin A	Flavonoid	flavone	http://foodb.ca/compounds/FDB016530
Terpenoid	tokoronin	Steroid	steroid glycoside	https://pubchem.ncbi.nlm.nih.gov/compound/Tokoronin#section=Top
Terpenoid	trilobolide	Terpene	sesquiterpene lactone	https://pubchem.ncbi.nlm.nih.gov/compound/Trilobolide#section=Top

Acute toxicity testing

The rat used in the study did show any abnormalities with normal breathing, normal stool and no abnormal discharge/secretions from body orifices during the study. The animal gained weight from 140g at the time of dosing to 170 g at day 14 post-dosing.

Sub-acute toxicity testing
Effect of UL on food intake

There were no statistically significant differences in weekly food intake between the treatment groups compared to the control (ANOVA, p=0.9745). As expected, average food intake increased over the four week period in all groups. Calculated daily food consumption per 100g animal was between 6.85-8.75g across the weeks.

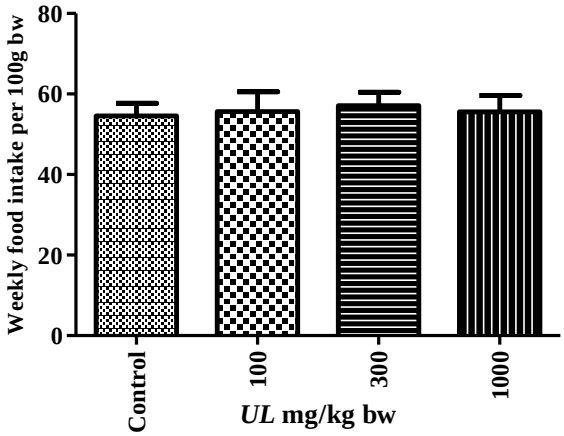

Figure 2: Mean weekly food intake. Data are expressed as Mean ± standard error of the mean (SEM).

Effect of UL on weight, day 0 to day 28

There was no significant difference in the percentage change in weight between the control group and all the *UL* treated groups (p = 0.5181).

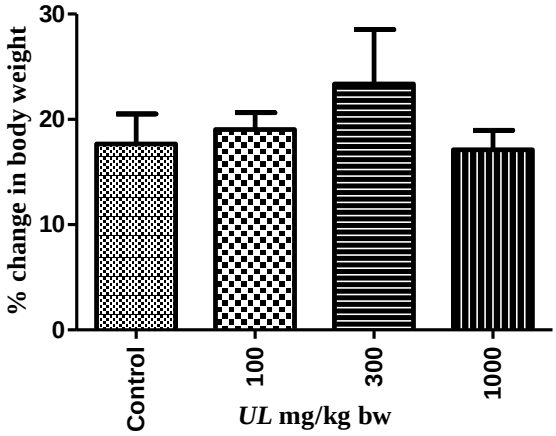

Figure 3: Percentage change in live body weights from day 0 to day 28. Data are expressed as Mean ± SEM.

Biochemistry analysis

There were no significant differences in AST, ALT, LDH, total bilirubin, cholesterol, albumin, total protein, creatinine, CK, sodium, potassium, chloride, calcium, phosphorus, tryglycerides and urea between the control and 100, 300 and 1000 mg/kg BW *UL* treated groups (Table 2). However, there was a significant difference (p=0.0097) in the glucose levels between the control and the *UL* treated rats. The Tukey's post hoc analysis showed that the significant difference was due to higher glucose level in the control group (5.68 mm/L) compared to the 1000 mg/kg BW *UL* (5.03 mm/L) group.

Table 2: Effect of *UL* on selected biochemical parameters. Data are expressed as Mean ± SEM

	Units	Treatment				
		Control	100 mg/kg *UL*	300 mg/kg *UL*	1000 mg/kg *UL*	p-value
AST	IU/L	84.17 ± 2.29	82.5 ± 4.33	80 ± 4.39	79.67 ± 2.63	p=0.7661
ALT	IU/L	42.5 ± 3.24	38 ± 0.82	44.5 ± 2	37. 17 ± 2.21	p=0.0904
ALP	IU/L	139.83 ± 15.33	118.83 ± 21.02	105.83 ± 4.37	105.5 ± 16.18	p=0.3776
LDH	IU/L	505 ± 75.36	444 ± 98.78	409.33 ± 71.28	370.16 ± 82.05	p=0.6999
Total Bilirubin	µmol/L	5 ± 0.73	4.73 ± 0.48	5.5 ± 1.18	5 ± 0.82	p=0.9481
Cholesterol	mmol/L	1.33 ± 0.11	1.07 ± 0.10	1.35 ± 0.10	1.33 ± 0.1	p=0.1785
Albumin	g/L	19 ± 0.52	19.5 ± 0.34	19.33 ± 0.42	19.33 ± 0.21	p=0.8317
Total protein	g/L	64.67 ± 1.28	65.5 ± 0.67	64.67 ± 0.67	65 ± 0.68	p=0.9236
Glucose	mmol/L	5.68 ± 0.06	5.38 ± 0.12	5.2 ± 0.14	5.03 ± 0.15	p=0.0097
Creatinine	µmol/L	26.8 ± 4.04	28.83 ± 2.12	21.83 ± 2.27	25.67 ± 1.28	p=0.3168
CK	IU/L	471 ± 62.98	401 ± 69.55	340.83 ± 26.09	331.5 ± 45.93	p=0.2628
Sodium	mmol/L	138.67 ± 0.56	139.17 ± 0.60	138.67 ± 0.42	138.67 0.42	p=0.8651
Potassium	mmol/L	6.68 ± 0.33	6.98 ± 0.43	6.67 ± 0.22	6.08 ± 0.33	p=0.3130
Chloride	mmol/L	102 ± 0.45	102.33 ± 0.99	102 ± 0.52	101.67 ± 0.49	p=0.9118
Calcium	mmol/L	2.52 ± 0.05	2.6 ± 0.03	2.55 ± 0.03	2.55 ± 0.03	p=0.4654
Phosphorus	mmol/L	2.54 ± 0.12	2.71 ± 018	2.89 ± 0.12	2.80 ± 0.19	p=0.4621
Tryglycerides	µmol/L	0.61 ± 0.11	0.53 ± 0.08	0.85 ± 0.12	0.57 ± 0.17	p=0.2851
Urea	mmol/L	6.93 ± 0.5	6.8 ± 0.36	6.85 ± 0.32	6.43 ± 0.69	p=0.8913

Hematological analysis

There were no significant differences in all the full blood count parameters between the control group and the *UL* treated groups (Table 3).

Table 3: Effect of *UL* on full blood count. Data are expressed as Mean ± SEM

	Units	Treatment				
		Control	100 mg/kg *UL*	300 mg/kg *UL*	1000 mg/kg *UL*	p-value
Hemoglobin (Hb)	g/dL	16.2 ± 0.46	16 ± 0.66	16.05 ± 0.30	16.03 ± 0.55	p=0.9927
Red blood cell count	E+6/µL	8.46 ± 0.39	8.14 ± 0.34	8.47 ± 0.18	8.26 ± 0.27	p=0.8414
Heamatocrit	%	43.81 ± 1.38	43.03 ± 1.79	43.55 ± 0.89	43.55 ± 1.53	p=0.9838
Mean Corpuscular Hb	pg	19.25 ± 0.51	19.65 ± 0.31	18.9 ± 0.38	19.4 ± 0.25	p=0.5889
Mean Corpuscular Hb Concentration	g/dL	37.03 ± 0.22	37.15 ± 0.21	36.90 ± 0.19	36.83 ± 0.14	p=0.6539
Mean Corpuscular Volume	fL	52 ± 1.18	53 ± 0.68	51.5 ± 0.72	52.67 ± 0.76	p=0.6165
White Blood cell count	E+3/µL	5.40 ± 0.43	5.70 ± 0.98	6.17 ± 0.51	5.75 ± 0.68	p=0.8866
Neutrophils	%	10.12 ± 0.94	10.2 ± 1.35	9.62 ± 1.49	9.65 ± 1.40	p=0.9830
Lymphocytes	%	72.73 ± 1.93	71.07 ± 3.50	70.28 ± 2.62	75.87 ± 3.71	p=0.5815
Monocytes	%	15.82 ± 1.83	17.43 ± 2.36	18.52 ± 1.97	12.05 ± 2.07	p=0.3938
Eosinophils	%	0.22 ± 0.07	0.28 ± 0.17	0.32 ± 0.12	0.16 ± 0.06	p=0.7933
Basophils	%	1.12 ± 0.14	1.01 ± 0.20	1.27 ± 0.16	1.1 ± 0.17	p=0.7752
Platelets	E+3/µL	813 ± 44.7	848 ± 25.7	845 ± 39.8	859 ± 21.4	p=0.7741
Mean Platelet Cell volume	fL	5.92 ± 0.04	5.93 ± 0.18	5.73 ± 0.10	5.58 ± 0.05	p=0.1098

Effect of UL on organ weight

UL did not have statistically significant effects on the liver, kidney and pancreas weight compared to control (Table 4).

Table 4: Organ weight to body ratio in percentage. Data are expressed as Mean ± SEM

	Treatment				
	Control	100 mg/kg *UL*	300 mg/kg *UL*	1000 mg/kg *UL*	p-value
Liver	2.94 ± 0.0013	2.82 ± 0.0018	3.03 ± 0.0012	2.88 ± 0.0020	p=0.8203
Kidneys	0.72 ± 0.0003	0.72 ± 0.0003	0.75 ± 0.0002	0.72 ± 0.0002	p=0.8090
Pancreas	0.29 ± 0.0003	0.32 ± 0.0003	0.34 ± 0.0004	0.32 ± 0.0002	p=0.6314

Histopathological analysis of Kidney

Animals administered 100 and 300 mg/kg BW *UL* showed normal nuclei with no cellular perturbations in the kidney when compared with control animals. However, animals treated with the high dose (1000 mg/kg BW *UL*), showed cellular swelling, and Bowman's space dilatation and vacuolation (Figure 4).

The morphological measurements of the kidney are shown in Table 5. There were no significant differences in the BC diameter, BC area, PCT diameter, PCT area and DCT area between the control group and all the UL treated groups. However, ANOVA revealed a significant difference in the Bowman's space between the groups ($p<0.0001$). Tukey's post hoc revealed that the 1000 mg/kg treated rats had increased Bowman's space compared to the animals in all the other groups. Further, a significant difference ($p=0.0024$) was also observed in DCT diameter between the groups. This was due to larger DCT diameter in the rats treated with 1000mg/kg (42.22±1.15 µm) compared to those treated with 100 mg/kg (37.26±1.20 µm) and the controls (37.61±0.69 µm).

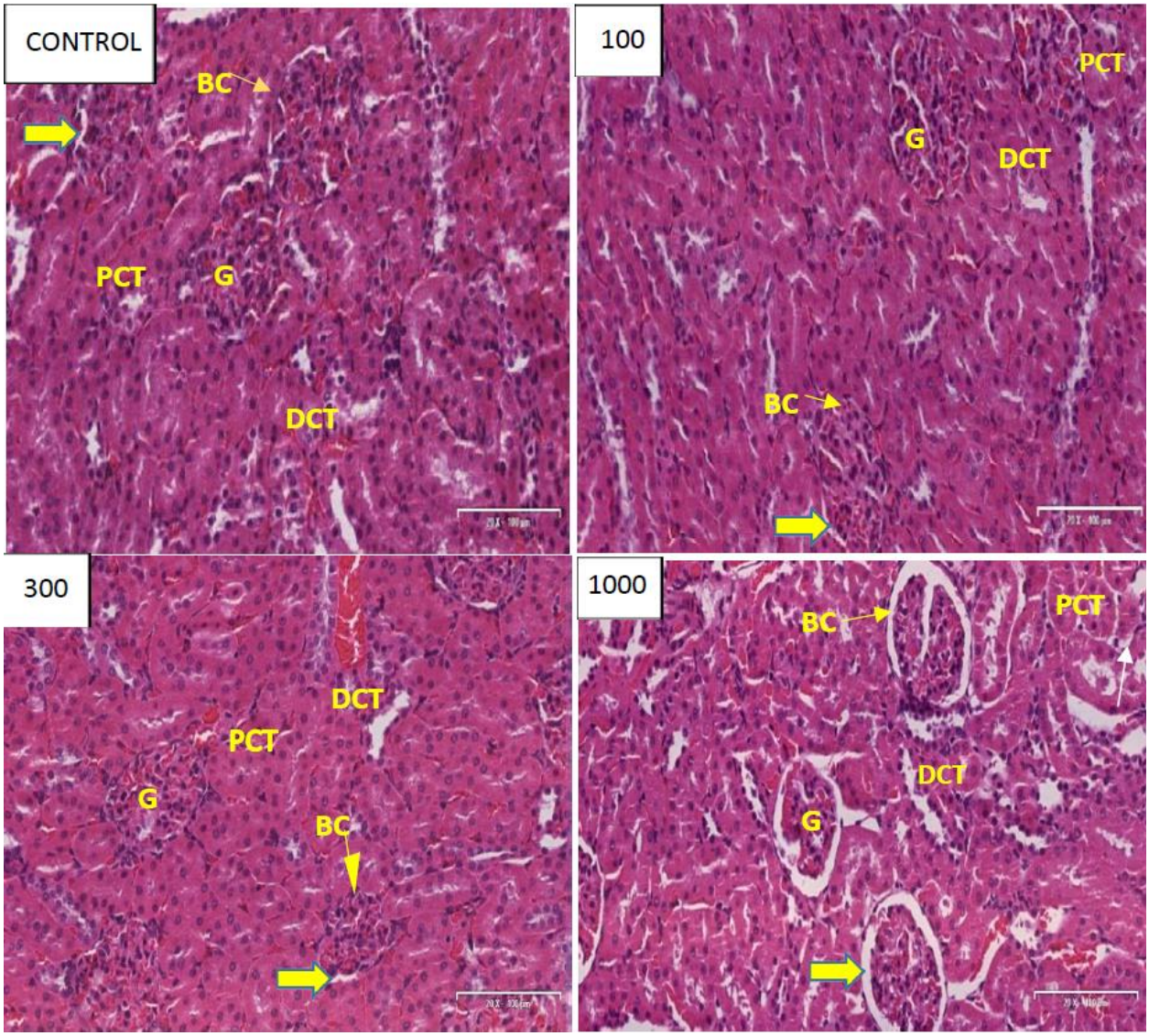

Figure 4: Photomicrograph of the renal cortex of rats treated with 0, 100, 300 and 1000mg/kg *UL*; showing Bowman's capsule (BC), Glomerulus (G), proximal convoluted tubule (PCT), distal convoluted tubule (DCT) and Bowman's space (arrowed).

Histopathological analysis of Liver

Photomicrograph of liver in control, 100mg/kg and 300mg/kg groups appeared healthy with no inflammatory cells and cellular degeneration. However, there was swelling of hepatocytes, constriction of the central vein and dilatation of sinusoidal space in animals treated with 1000mg/kg of *UL*. Cellular architecture was preserved for all doses administered (Figure. 5).

The morphological measurements of the liver are shown in Table 5. Overall, ANOVA showed that there was a significant difference in mean central vein diameter between the groups ($p=0.0179$). Post-hoc analysis showed significant reduction in central vein diameter in the 1000 mg/kg treated animals compared to the control and 100 mg/kg groups. ANOVA also showed a significant difference in sinusoidal space ($p<0.0001$). This was due to a larger sinusoidal space in the 1000 mg/kg treated animals compared to the other three groups.

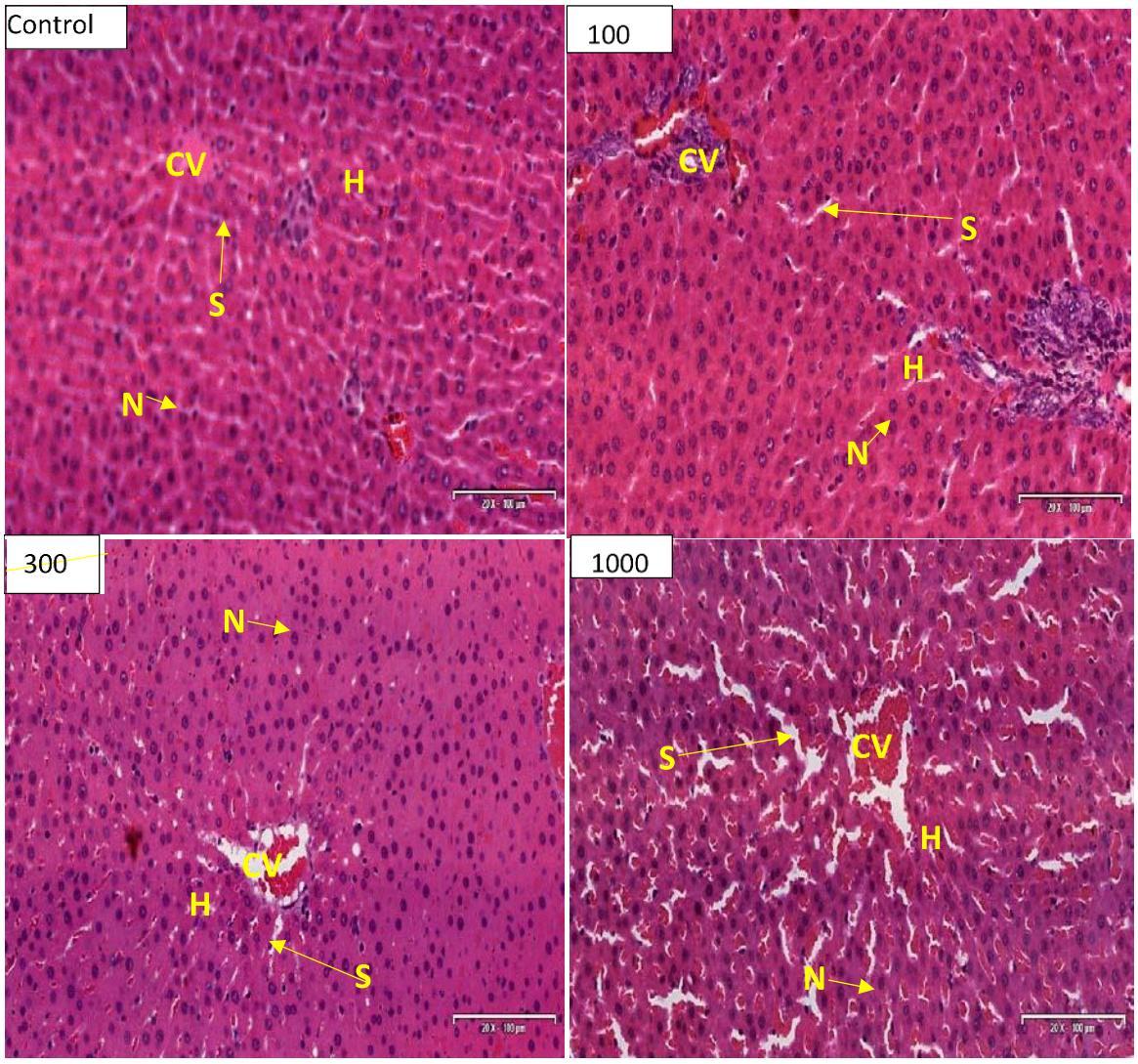

Figure 5: Photomicrograph of the liver of control, 100, 300 and 1000 mg/kg *UL* treated animals showing the central vein (CV), nucleus (N), sinusoidal space (S) and hepatocyte (H).

Table 5: Morphological measurements of selected kidney and liver parameters. Data are expressed as Mean ± SEM.

	Unit	Treatment				
		Control	100mg/kg	300mg/kg	1000mg/kg	P-value
Kidney						
Bowman capsule diameter	µm	73.29±2.05	75.87±4.44	64.45±4.38	66.18± 3.30	p=0.0916
Bowman capsule area	µm²	4987.66±315.20	4419.43±535.55	4449.76±413	4305.01±360.89	p=0.6564
Bowman space	µm	4.66±0.29	6.03±0.54	5.79±0.52	10.48±0.75	p<0.0001
Proximal convoluted tubule diameter	µm	38.76±0.90	38.92±0.90	38.50±0.94	40.15±0.65	p=0.5346
Proximal convoluted tubule area	µm²	1236.00±57.60	1171.33±43.42	1131.86±60.86	1236.10±46.66	p=0.4206
Distal convoluted tubule diameter	µm	37.61±0.69	37.26±1.20	40.53±0.99	42.22±1.15	p=0.0024
Distal convoluted tubule area	µm²	1069.84±43.03	1006.05±58.54	1115.05±81.18	1175.04±69.27	p=0.3212
Liver						
Central vein diameter	µm	35.04±1.88	37.77±2.50	30.82±1.46	30.84±0.92	p=0.0179
Central vein area	µm²	932.88±88.94	920.54±73.22	738.69±41.71	675.16±23.30	p<0.0001
Sinusoidal space	µm	3.81±0.37	4.48±0.36	3.87±0.30	7.02±0.40	p<0.0001

Discussion

In drug development, toxicological evaluation is carried out in experimental animals to predict toxicity and to provide guidelines for safe doses in humans. While, the sequence for pharmaceutical drug development moves from animal studies to human studies, traditional medicine research is usually vice versa because the herbal medicines are already being consumed by patients. The dose of herbal medicines patients take may determine the doses to use in animal studies by calculations based on allometric scaling. The maximum dose used in acute toxicity testing can be ten times the expected maximum effective dose (Food and Drug Administration, 2010). In this study, we used up to ten times the dose prescribed to patients by the traditional healer who provided the traditional medicine.

The administration of *UL* up to a dose of 1000 mg/kg body weight daily for 28 days was not associated with any abnormalities in general condition, growth, movement, behavior and food consumption of the animals. The differences in weekly food consumption and weight gain over the 28 day period between the control and *UL* treated animals were not significant suggesting that the doses are safe with regards to food consumption.

There was no change in liver function as assessed by AST, ALT, ALP and LDH between control and all the treatments of *UL* used in this study. This may suggest that *UL* does not promote degeneration or oncotic necrosis of liver cells (Cattley *et al.,* 2013). There was also no change in kidney function as assessed by urea, creatinine and the electrolytes (sodium, potassium and chloride). *UL* did not affect blood cell counts. This may suggest that *UL* does not interfere with hematopoiesis and hence may not be associated with any form of anemia.

UL did not have significant effects on organ/body weight ratio for the kidney, liver and pancreas. The liver/body weight ratio ranging between 2.82 – 3.02%, is within the expected liver/body weight ratio of 3% (Hubrecht and Kirkwood, 2010). We observed a slight increase in the pancreas/body weight ratio with increasing concentration of extract. This may explain the observed significant decrease in blood glucose levels with increasing concentration of *UL***.** Panigrahi et al (2016), reported significant increases in pancreas/body weight ratio with metformin and extracts of *Sesbania grandiflora* and this was accompanied by a proportional decrease in blood glucose levels (Panigrahi *et al.,* 2016). Possibly the herbal extracts increase pancreatic β cell mass and/or increase insulin production.

There was no alteration in the renal function parameters (urea, electrolytes and creatinine) in all the animals. The histology of kidney in animals administered with 100 mg, 300 mg *UL* and control appeared healthy. However, we observed histomorphological perturbations in the kidney of animals administered 1000 mg/kg BW. The cellular swelling, Bowman's space dilation and vacuolation observed corresponded with stereological measurements showing increased Bowman's space and diameter and area of Bowman's capsule. This may suggest a fluid hemodynamic mechanistic effect of *UL* at this dose (Frazier and Seely, 2013). Prolonged administration at the high dose may lead to irreversible tissue injury. Acute kidney injury has historically been diagnosed by monitoring blood urea nitrogen and serum creatinine (Frazier and Seely, 2013). However, these two markers may be less sensitive since they are only elevated when more than half of the kidney function has been compromised in humans (Bonventre *et al.,* 2010). Measurements of specific proteins in the urine offer an advantage over serum measurements to detect renal injury

(Sahota *et al.,* 2013). In this study, however, we did not assess for specific proteins in urine. The kidney is a vital organ in drug elimination (Anders, 1980; Gattone, 2007) and as such a good indicator of potential drug toxicity.

The histology of the liver in animals administered with 100 mg, 300 mg *UL* and control appeared normal with no cellular distortions. We observed swelling of hepatocytes, dilatation of central vein and dilatation of sinusoidal spaces in animals administered 1000 mg/kg BW. This may suggest that histological perturbations will occur before biochemical response to cellular injury. The liver is very sensitive to drug toxicity as drugs are mainly metabolized in the liver (Almazroo *et al.,* 2017).

In this study, treatment related histological renal and liver lesions were observed mostly at the highest dose without significant changes in biochemical parameters. Travlos *et al,* (1996) also observed histological kidney and liver lesions without biochemical parameter abnormalities (Travlos *et al.,* 1996). Some studies have however, reported the opposite while others have reported a direct relationship between biochemical parameters and liver and kidney lesions (Khoza *et al.,* 2017; Rhiouani *et al.,* 2008; Tang *et al.,* 2017). This suggests that there is no clear sequence between biochemical parameter changes and histologic lesions; and this may depend on the compound being tested or the species of animal being used.

Phytochemical analysis revealed the presence of several classes of phytoconstituents including alkaloids, flavonoids, quinones, quinolones, terpenoids, saponins, coumarins and glycosides. The positive mode run in the LC-QTOF-MS revealed more peaks and hence identified more phytoconstituents than the negative ion mode. The review of literature did not identify toxicity profiles of any of the phytoconstituents identified in *UL*.

Conclusion

The maximum safe dose of *UL* is between 300 – 1000 mg/kg body weight. Phytochemical analysis revealed a number of phytoconstituents, essentially making *UL* a combination therapy, an attribute which may be an advantage in the management of the metabolic syndrome. The multiple phytoconstituents however, may give rise to multiple organ toxicities. In the acute and sub-acute toxicity assays conducted, organ toxicities increased dose, with significant differences identified at a dose that is ten times more than the human equivalent dose prescribed by the traditional healer. These results suggest that *UL* has a large safe dose window. We conclude that the kidney is more sensitive to increasing doses of *UL* compared to the liver. Chronic toxicity studies are however, needed to assess the effect of *UL* over long-term treatment.

Competing interests: The authors declare that there are no competing interests.

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