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Toxicity and gas chromatography-mass spectrometry analyses of a polyherbal formulation commonly used in Ibadan metropolis, Nigeria

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ABSTRACT

A polyherbal formulation mainly composed of *Hibiscus sabdariffa* and Aloe barbadensis commonly patronized by some staff and students of a College Hospital in Ibadan, Nigeria was evaluated for its toxicity status and bioactive constituents. Its safety was assessed using acute and sub-chronic toxicity models in Wistar rats while Gas Chromatography-Mass Spectrometry (GC–MS) was used to identify the bioactive constituents. Findings showed that oral administration of the polyherbal formulation did not cause any notable behavioral changes or mortality in the animals during the acute toxicity testing. Similarly, statistical analysis of the data obtained showed that sub-chronic administration of the polyherbal formulation did not cause any significant changes in the serum activities of liver-function enzymes, hematological markers, serum electrolytes and other evaluated blood-chemistry indices in the experimental animals compared to those of their control counterparts. These observations were substantiated by the liver photomicrographs which showed that treatment of animals with the polyherbal mixture did not induce any visible lesions or derangements in the examined organ. Besides, GC–MS screening of the polyherbal formulation revealed compounds with known biological effects which are associable with the locally acclaimed therapeutic applications of the formulation. The outcome of this study therefore suggests high safety for the investigated polyherbal mixture and thus supports its usage in folklore medicine.

1. Introduction

More than ever before, humans through environment and diet are constantly exposed to high level of stress (physical, physiological and oxidative) beyond what the body`s restorative or protective mechanisms can cope with. Unfortunately, stress is arguably and evidently an underlying factor for most pathological conditions [1,2]. The import of good health and physique to man in enjoying life and accomplishing its purpose is largely evident across the globe [3]. For this reason, individuals are naturally and logically inclined to every possible means to stay hale and healthy. The use of conventional treatments as remedies for ailments and diseases has been associated with some limitations including cost and side effects. Besides the fear of damaging side effects, people simply cannot afford to pay for their medications month after month. This condition has grossly encouraged the use of herbal drugs or formulations which are relatively cheaper, believed to be more potent with lesser or no side effects. Presently, there is an enormous increase in the patronage of polyherbal formulations, and this treatment option is gaining huge popularity and acceptance in both developing and developed countries across the globe [4].

Plant medicines though, are generally believed to be safe, however some are indeed naturally toxic and harmful [5] and others may be toxic at high doses or have potentially adverse effects sequel to prolonged use. As it is with other drugs, the risk of unexpected effects may be associated with the use of polyherbal mixtures [6]. Toxicological evaluation of polyherbal mixtures with acclaimed therapeutic relevance is therefore important and extremely needful.

Large chunk of the global populace (particularly Africans) are quite unaware that the use of certain polyherbal mixtures could have adverse health effects, stemming from inherent toxicity of the herbs of choice, overdosing, or contamination during processing and formulation [7]. In this regard, this study sought to screen a commonly used polyherbal formulation (Gbogbonise) for its herbal safety (toxicity status) and bioactive constituents. The poly herbal mixture is usually sold in plastic bottles in the city of Ibadan, Nigeria, particularly at the University College Hospital (UCH) Ibadan, patronized by ''all and sundry'' across

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different educational and social boundaries for prevention and treatment of various ailments (malaria, typhoid, waist pain, back pain etc). Findings from this investigation will no doubt provide useful and helpful information to the public; as the societies of the world are gradually tilting towards alternative therapy or folklore medicine.

2. Material and methods

2.1. Collection and management of animals

Female Wistar rats (150−200 g BW) were obtained from the Department of Anatomy (Animal Breeding Unit), University of Ibadan. The handling and care of the animals were carried out according to the guidelines of the National Academy of Science published by the National Research Council [8]. The procedure was approved by the Ethics Committee on animal care and use in research, Faculty of Basic Medical & Applied Sciences, Lead City University, Ibadan Nigeria. The animals were kept in plastic cages, subjected to natural photoperiod of 12 h light and 12 h dark cycle in a well ventilated and hygienic rat house. They were provided rats pellets (Top feeds) and water *ad libitum* under suitable conditions of temperature and humidity. Animal acclimatization was done for a period of two weeks prior to commencement of the study. All animal experiments were carried out without anesthesia during the study.

2.2. Collection of poly herbal formulation

The polyherbal mixture which is usually sold in plastic bottles was purchased at one of its major vendor points, University College Hospital (UCH) in the city of Ibadan, Nigeria. It is a combination of two or more plant materials (mainly *Hibiscus sabdariffa* and A*loe barbadensis)* and is acclaimed to be a highly effective treatment for various ailments including malaria, typhoid, waist pain, back pain etc.

2.3. Lethal dose determination (Acute toxicity test) of the polyherbal formulation

Lethality studies (acute) to determine the LD_{50} of the polyherbal mixture was performed according to the combined procedures described by Lorke [9] and OECD guidelines-425 [10]. It was assessed through oral route of administration. The assessment was done in two phases. The first phase involved three rats respectively administered 10, 30 and 40 mL/kg body weight (BW) of the polyherbal mixture. The animals were critically observed for a period of 24 h. When no mortality was recorded, a second phase involving four groups (A to D) of rats containing two rats each was set up. The animals in the different groups were respectively administered 40, 50, 60 and 70 mL/kg BW of the polyherbal mixture. They were closely observed for negative behavioral changes and mortality within 24 h of the experiment. Animals were observed individually once during the first 30 min. after dosing, periodically during the first 24 h (with more attention during the first 4 h). The lethal dose of the polyherbal mixture was calculated using the formula; $\mathbf{LD50 = \sqrt{Do \times D_{100}}}$. Where Do = Maximum dose that cause 0% mortality, D_{100}= Minimum dose that cause 100 % mortality.

2.4. Sub-chronic toxicity assessment of the polyherbal formulation

Sub-chronic toxicity study was carried out to determine the effect of repeated administration of the polyherbal mixture on blood chemistry and soft tissues. Twenty-four rats were randomly assigned to three groups (n = 8), labeled Control, Dose 1 and Dose 2. The control group animals were administered distilled water (2 mL) while Dose 1 and Dose 2 groups of animals were given 10 and 20 mL/kg BW of the polyherbal mixture for a period of 30 days at 48 h interval. After the last dose of administration, the animals were weighed, fasted overnight (12 h) and sacrificed by cervical dislocation. Blood samples were collected from the retro sinus of the eye of each animal by ocular puncture into plain and heparinized tubes for different analyses. Part of the collected blood samples were centrifuged at 3000 revolution per minute (rpm) for 5 min., and the resultant supernatant in each case was used for selected blood chemistry analyses. The liver and kidney were quickly removed from each rat. Each organ was preserved in 10 % formalin solution for histopathological processing and examination. These organs were first gross-examined for any observable lesion or tissue derangement before they were processed using the automatic tissue processor. The technique involves dehydrating the fixed tissues placed in tissue baskets with their respective labels and passing them through graded alcohol (70 %, 90 %, 95 %, and 100 %) solutions. The tissues were removed after dehydration and moved into xylene solution baths to clear the alcohol and to facilitate molten wax impregnation. The tissues were finally sectioned using rotary microtome (at 5μ thickness), stained with Haematoxylin and Eosin (H&E) and then examined microscopically using standard techniques.

2.5. Estimation of serum activities of Liver function-Enzymes

Liver function markers were assayed in the serum using end point colorimetric diagnostic kit (Randox Laboratories Limited, England). Activities of Alanine amino transferase (ALT) formerly known as glutamate pyruvate transaminase (SGPT) and Aspartate amino transferase (AST) formerly known as glutathione-oxaloacetate transaminase (SGOT) were estimated according to the procedure described by Reitman and Frankel [11]. The method of Englehardt [12] was used in estimating activity of alkaline phosphatase (ALP). Gamma-glutamyl transferase (GGT) activity was determined by the method of Szasz and Persiyn [13]. Bilirubin level (Direct and Indirect) was estimated according to the procedure described by Jendrassik and Grof [14].

2.6. Estimation of serum concentrations of Kidney function parameters

Urea and creatinine concentrations were determined using the method of Bartels and Bohmer [15] as described in the Randox kit manual. Electrolytes (sodium, potassium and chloride ions) were respectively estimated by the methods of Trinder [16], Maruna [17], Terri and Sesin [18] and Skeggs and Hochstrasser [19] as described in Teco diagnostic kits manual.

2.7. Estimation of hematological parameters

A complete blood count and differential count was performed on full blood using Sysmex KX-21 N, an automated 3-part differential hematology analyzer. The machine automatically dilutes whole blood sample of 50 mL in the CBC/differential mode, lyses and enumerate white blood cells (WBC), red blood cells (RBC), hemoglobin concentration, packed cell volume (PCV), platelets, lymphocytes and neutrophils.

2.8. Estimation of serum total protein and albumin concentrations

The concentrations of total protein and albumin were estimated using sigma diagnostic kits (Sigma diagnostic, USA).

2.9. Gas chromatography–mass spectroscopy (GC–MS) analysis of the polyherbal formulation

The polyherbal formulation sample for GC–MS analysis was prepared using solid-phase extraction manifold. The C18 cartridge was first

Table 1
: Effects of the polyherbal mixture on liver enzymes activity in serum of rats.

GROUPS	ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)
CONTROL	11.75 ± 0.47	14.50 ± 0.65	48.50 ± 3.32	8.50 ± 0.64
DOSE 1	10.50 ± 1.70	14.50 ± 1.93	49.00 ± 5.40	7.75 ± 1.10
DOSE 2	7.50 ± 1.32	11.50 ± 0.65	37.75 ± 6.25	5.50 ± 1.04

Values are expressed as a mean of eight rat ± standard error of mean. * =significant difference when compared to control ($P < 0.05$). **Significant difference when compared to Dose 2.

Table 2
Effects of the polyherbal mixture on kidney-function indices in serum of rats.

GROUPS	UREA (mg/dL)	CREATININE (mg/dL)	TOTAL BILIRUBIN (mg/dL)	DIRECT BILIRUBIN (mg/dL)
CONTROL	25.25 ± 1.88	0.58 ± 0.05	0.53 ± 0.08	0.25 ± 0.06
DOSE 1	26.00 ± 2.55	0.60 ± 0.04	0.47 ± 0.10	0.23 ± 0.06
DOSE 2	28.50 ± 5.04	0.65 ± 0.12	0.45 ± 0.06	0.25 ± 0.03

Values are expressed as a mean of eight rat ± standard error of mean. * =significant difference when compared to control ($P < 0.05$). **Significant difference when compared to Dose 2.

Table 3
Effects of the polyherbal mixture on serum electrolytes of rats.

GROUPS	SODIUM ION (mmol/L)	POTTASSIUM ION (mmol/L)	CHLORIDE ION (mmol/L)
CONTROL	139.0 ± 1.08	3.85 ± 0.15	105.0 ± 2.04
DOSE 1	139.3 ± 1.25	3.85 ± 0.16	105.0 ± 2.04
DOSE 2	138.8 ± 1.89	3.83 ± 0.24	105.0 ± 2.04

Values are expressed as a mean of eight rat ± standard error of mean. * =significant difference when compared to control ($P < 0.05$). **Significant difference when compared to Dose 2.

conditioned with 5 mL methanol to make the solid phase active. The polyherbal formulation was then loaded on the cartridge, and allowed to drain through it. After which the trapped active compounds of the polyherbal formulation were eluded with methanol; and an aliquot was injected into the mobile phase of the GC which is typically composed of a gas such as helium or argon and the system is allow to run according to the procedure of Stashenko and Martínez, [20]. Once at the stationary phase, the GC sample separates. This occurs because the GC column is ramped, or gradually heated, and compounds with lower boiling points are eluted first. Likewise, the pressure of the mobile phase can be varied to fine-tune separation. Finally, chemical interactions occur between the sample compounds and the stationary phase; weaker interactions will dissociate faster and elute earlier than stronger ones. A compound can be measured from the time of its injection until the time of its elution from the GC column; this is called the compound's retention time. Lower molecular weight compounds will elute from a GC column sooner than those with a higher molecular weight because of boiling point differences. After the process of elution is complete, compounds undergo electron ionization (EI) or chemical ionization (CI) and become charged. They then undergo mass analysis within a mass spectrometer, and their unique mass (m) and charge (z) information is reported as numerical m/z ratios. Once these values are reported, they are typically displayed as ion peaks. The values are also compared against previously compiled libraries of known mass spectra using analytical software programs. Matching spectra are identified and characterized. Mass spectrometry

Table 4
Effects of the polyherbal mixture on serum proteins of rats.

GROUPS	TOTAL PROTEIN (mg/mL)	ALBUMIN (mg/mL)
CONROL	6.80 ± 0.17	3.83 ± 0.20
DOSE 1	6.85 ± 0.16	3.75 ± 0.16
DOSE 2	6.85 ± 0.28	3.83 ± 0.24

Values are expressed as a mean of eight rat ± standard error of mean. * =significant difference when compared to control ($P < 0.05$). **Significant difference when compared to Dose 2.

analysis enables the determination of compound molecular weight and formula, as well as functional groups.

3. Statistical analysis

Data analysis was performed using statistical software, Prism graphpad, version 6.4. The statistical significance of difference between groups was analyzed using the one-way analysis of variance (ANOVA), followed by independent-sample *t*-test. The level of significance was set at 95 % confidence level ($P < 0.05$). The results are presented as the mean ± SD

4. Results

Acute administration of the polyherbal mixture up to 70 mL/kg BW did not cause any notable behavioral changes or mortality in the treated animals. Compared to the control animals, Sub-chronic administration of the polyherbal mixture at a dosage of 10 mL/kg BW (Dose 1) did not cause any significant changes in the activity of the liver enzyme markers (ALT, AST, ALP and GGT) (Table 1), levels of kidney-function indices (urea, creatinine, bilirubin) (Table 2), serum electrolytes (Table 3), and serum proteins (Table 4), hematological parameters (red blood cell (RBC), packed cell volume (PCV), hemoglobin concentration (Hb), white blood cell (WBC) counts, platelet, lymphocytes and neutrophils) (Table 5). Also, photomicrographs of the examined organs (liver and kidney) of the this group of animals (Dose 1) did not show any visible lesion or tissue aberration (Fig. 2a and b)similarly to the control animals Fig. 1

However, administration of the polyherbal mixture at a dose of 20 mL/kg BW was observed in some cases, to have slightly altered the serum levels of the evaluated parameters relative to their corresponding values in the control animals. For instance, the polyherbal mixture at a dosage of 20 mL/kg BW caused a slight but non-significant increase in the level of urea, creatinine and total protein. The liver photomicrographs of the same group of animals also showed moderate paracentral hepatocellular atrophy Fig. 3a Fig. 3b(Fig. 3a).

Biologically active compounds detected in the polyherbal formulation using GC–MS technique are shown in Table 6 and the GC–MS chromatogram is shown in Fig. 4. Thirty (30) compounds were identified in the analyzed polyherbal formulation and the mass spectrometries of some of the bioactive compounds are shown in Figs. 5–10.

5. Discussion

The utilization of herbal medicines for treatment of different types of ailments and diseases is increasingly becoming popular in both developing and developed countries. A World Health Organization (WHO) survey indicates that about 70–80 % of the world's population rely on non-conventional medicine, mainly of herbal source for their primary healthcare [21]. Moreover, outcomes of experimental studies involving plant extracts tend to encourage the use of polyherbals [22]. Herbal medicines are usually regarded as safe and void of any serious side

Table 5
Effects of the polyherbal mixture on hematological indices.

Groups	PCV (%)	Hb (mg/dl)	RBC (x10^3/μL)	WBC (x10^3/μL)	PLATELETS (x10^3/μL)	LYMPH (%)	NEUTR (%)
Control	44.0 ± 0.82	14.5 ± 0.29	7.3 ± 0.80	5.3 ± 1.45	83.4 ± 6.41	72.7 ± 5.31	24.7 ± 5.57
Dose 1	44.2 ± 0.48	14.5 ± 0.27	7.37 ± 0.49	*5.1 ± 0.33	91.7 ± 5.61	75.0 ± 1.99	22.3 ± 1.32
Dose 2	44.0 ± 0.91	14.5 ± 0.22	7.4 ± 0.09	4.3 ± 0.9	101.8 ± 6.61	73.5 ± 4.56	22.8 ± 4.49

Values are expressed as a mean of eight rat ± standard error of mean. * =significant difference when compared to control ($P < 0.05$). **Significant difference when compared to Dose 2, Lymph = Lymphocytes, Neutr = Neutrophils.

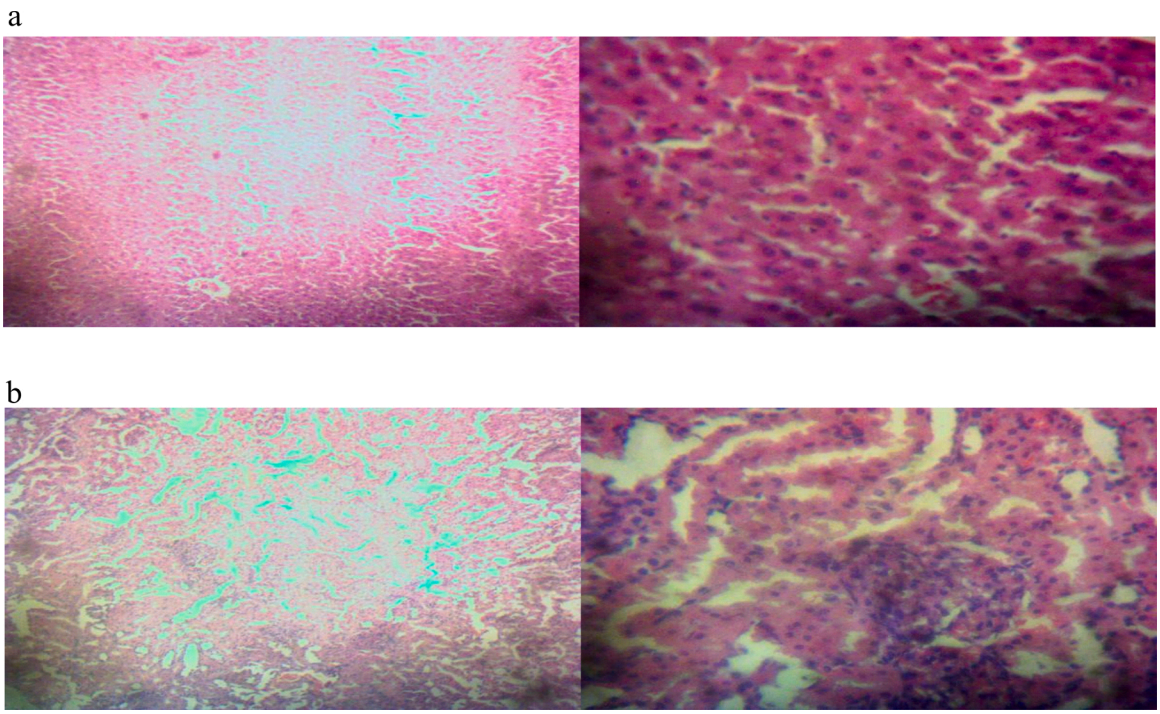

Fig. 1. (a) Photomicrographs of the liver sections of control animals (HE x100 & x400) showing mild atrophy of hepatic cords, spotty hepatocellular degeneration and accentuation of sinusoids. (b) Photomicrographs of the kidney sections of control animals (HE x100 & x400) Showing no visible lesion.

effects, but case reports suggest that herbal toxicity may result from toxic phytoconstituents if present in the herbs. More so, severe side effects may arise from pertinent interactions with other drugs or when consumed in inappropriate proportions [23]. Herbal toxicity remains major cause for concern in the therapeutic application of plant materials; hence, the knowledge of the toxicity profile of commonly consumed herbs or polyherbal mixtures is important, particularly in terms of lethal dose (LD50) (acute) and repeated dose toxicity (sub--chronic). The need for herbs and polyherbal mixtures to be evaluated not only for therapeutic efficacy, but also for safety is the background and motivation for this study.

The investigated polyherbal formulation which was obtained from the University College Hospital (UCH), Ibadan, Nigeria has been effectively employed in the prevention and treatment of ailments like malaria, typhoid, general body pains etc.

Blood-chemistry indices (hematological parameters, liver function enzymes, kidney-function markers, serum proteins and electrolytes which were analyzed in this study are excellent indicators of the physiological health status of living organisms, including man. They have normal physiological range of concentrations under healthy cellular conditions. These concentrations are usually altered or compromised in disease or toxic state. It is on this basis that they are used in diagnosis of diseases, assessment of drugs and plant extracts for safety and toxicity. The results obtained in this study, clearly showed consumption of the polyherbal formulation at the normal dose (10 mL/kg BW) of, did not compromise the physiological levels of the evaluated blood chemistry indices nor caused any visible tissue derangement or damage. This suggest that the polyherbal mixture when taking at the prescribed dose is neither hepatotoxic nor nephrotoxic.

Moreover, GC–MS screening of the polyherbal formulation revealed several compounds with known biological effects associable with the therapeutic applications of the formulation in local medicine. For instance, ***Cholest-22-ene-21-ol, 3, 5-dehydro-6-methoxy-pivalate*** is a Steroid with antimicrobial, anti-inflammatory, antiarthritic, antidiuretic, antiasthmatic effects. ***Octadec-9-enoic acid*** is a fatty acid with antihypertensive effects and health-benefit influence on the plasma levels of HDL (increased) and LDL (decreased). ***Pentadecane*** is an inhibitor of Sugar-phosphatase and chymosin and also has antibacterial potency. ***Nonadecane*** has both antimicrobial and cytotoxic effects. Hypoglycemic and antioxidant properties have been associated with ***Tetratetracontane***. ***Eicosane*** is known for antifungal, antitumor, larvicidal, antimicrobial, cytotoxic effects. ***Hexadecanoic acid, methyl ester*** has antioxidant; nematicide and insecticide properties [24]. The presence of these phytoconstituents in the polyherbal formulation is

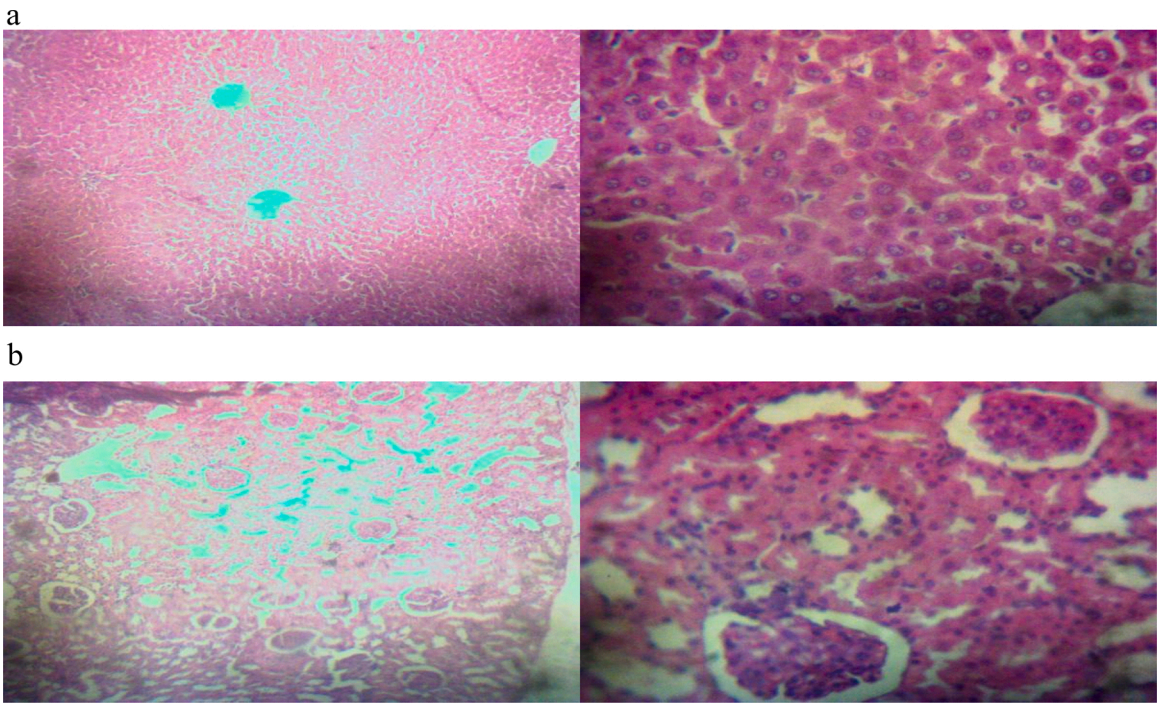

Fig. 2. (a) Photomicrographs of the liver sections of animals (HE x100 & x400) administered polyherbal mixture at a dosage of 10 mL/kg BW showing no observable lesions. (b) Photomicrographs of the liver sections of animals (HE x100 & x400) administered polyherbal mixture at a dosage of 10 mL/kg BW showing no observable lesion.

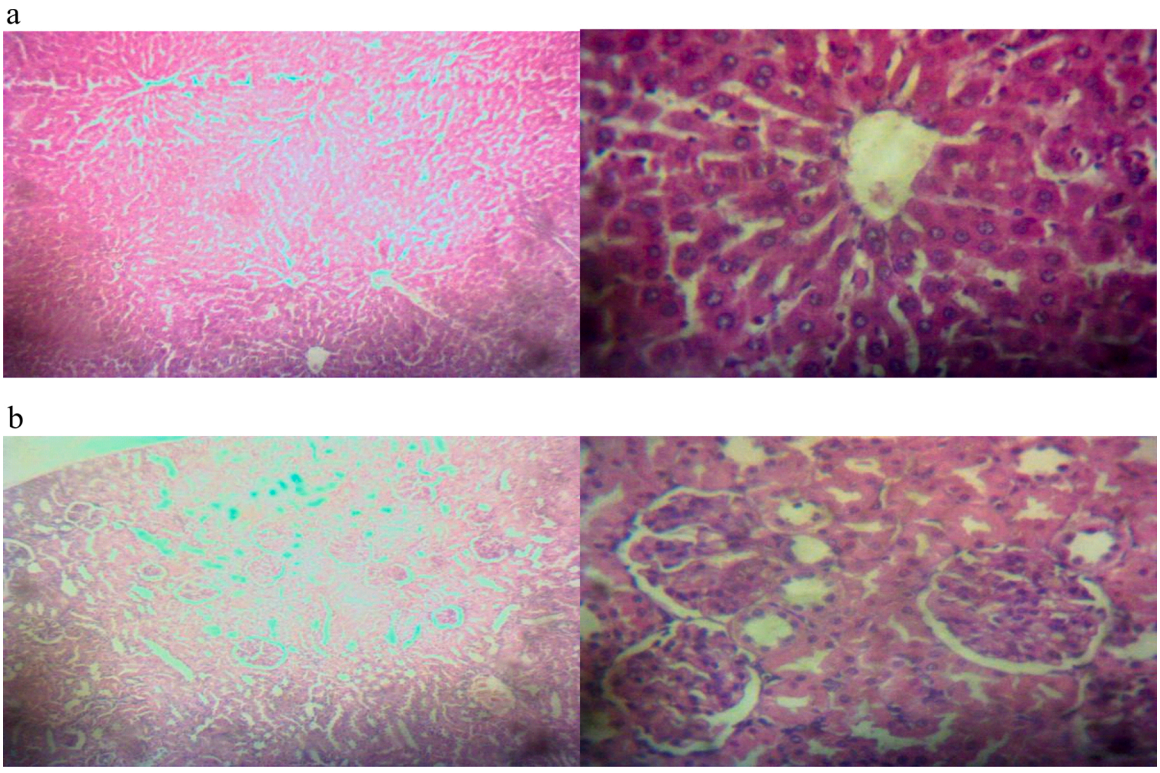

Fig. 3. (a) Photomicrographs of the liver sections of animals administered polyherbal mixture at a dosage of 20 mL/kg BW (HE x100 & x400) showing moderate paracentral hepatocellular atrophy. (b) Photomicrographs of the kidney sections of animals administered polyherbal mixture at a dosage of 20 mL/kg BW (HE x100 & x400) showing no observable lesion.

Table 6
Compounds detected in the Polyherbal formulation using GC–MS Technique.

S/N	Compound	Retention time (min:s)	Percentage abundance (%)
1	Cholest-22-ene-21-ol,3,5-dehydro-6-methoxy-pivalate	8.23	0.62
2	Cycloheptasiloxane	9.58	1.91
3	Pentadecane	9.75	0.43
4	1,3,5,2,4-Trioxadiborinane	10.81	1.88
5	Octadecane	11.15	2.19
6	Pentadecane	11.81	1.37
7	3,4-Dihydroxymandelic acid	12.09	4.17
8	10-Methylnonadecane	12.56	5.99
9	Ethyl 4-hydroxyphenylacetate	13.12	2.87
10	Tetratetracontane	13.87	3.45
11	Tritetracontane	13.95	3,98
12	1,1,1,5,7,7,7-Heptamethyl-3,3-bis- tetrasiloxane	14.27	2.33
13	Cyclohexane	14.42	3.03
14	Nonadecane (Methyl Nonadecane)	15.19	4.18
15	2,3,4,5-Tetrafluorobenzyl alcohol	15.27	2.12
16	Hexadecanoic acid, methyl ester	15.72	4.75
17	Cycloheptasiloxane	16.42	2.01
18	Eicosane	16.61	5.44
19	Octasiloxane	17.65	1.71
20	Heneicosane	18.16	7.42
21	9-Octadecenoic acid, methyl ester	18.26	13.0
22	Methyl stearate	18.77	3.04
23	Octacosane	19.84	4.59
24	1H-imidazole-2-methanol	20.29	1.27
25	1,1,1,5,7,7,7-Heptamethyl-3,3-bis-tetrasiloxane	21.42	3.03
26	Sulfurous acid, butyl dodecyl este	21.59	3.21
27	2-methyloctacosane	23.41	2.18
28	Trimethylsilyl-di- silane	24.05	4.00
29	Carbonic acid	25.21	0.52
30	Cyclodecasiloxane	26.70	2.68

arguably responsible for the several medicinal effects associated with it. Moreover, none of the compounds detected in the polyherbal formulation has a worrisome toxicity status. This probably explains the herbal safety profile demonstrated by the polyherbal formulation in this study particularly when consumed at the recommended dose.

However, it is important to explicitly state that though the alterations in the serum levels of some of the evaluated parameters were slight and non-significant In animals administered 20 mL/kg BW and more so, that the altered values remain within the boundaries of the normal physiological concentrations, a cause for concern exists with the intake of the formulation at higher doses as evidenced by the moderate paracentral hepatocellular atrophy associated with the liver of this group of animals. This observation suggests that the therapeutic use of the polyherbal formulation must be with great caution, particularly in

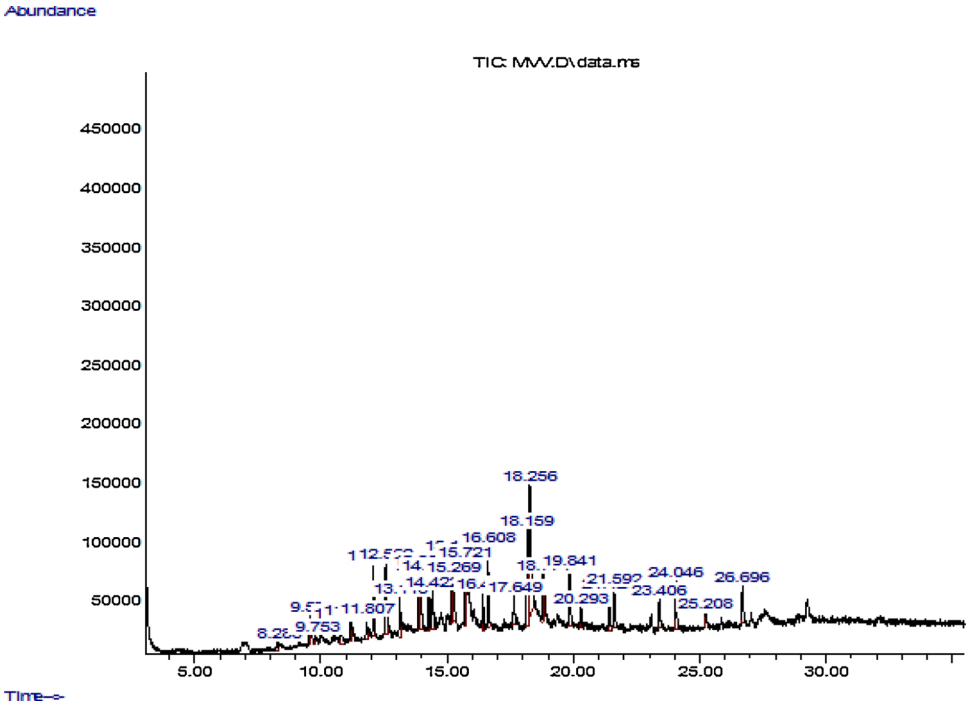

Fig. 4. GC–MS Chromatogram of the investigated polyherbal formulation.

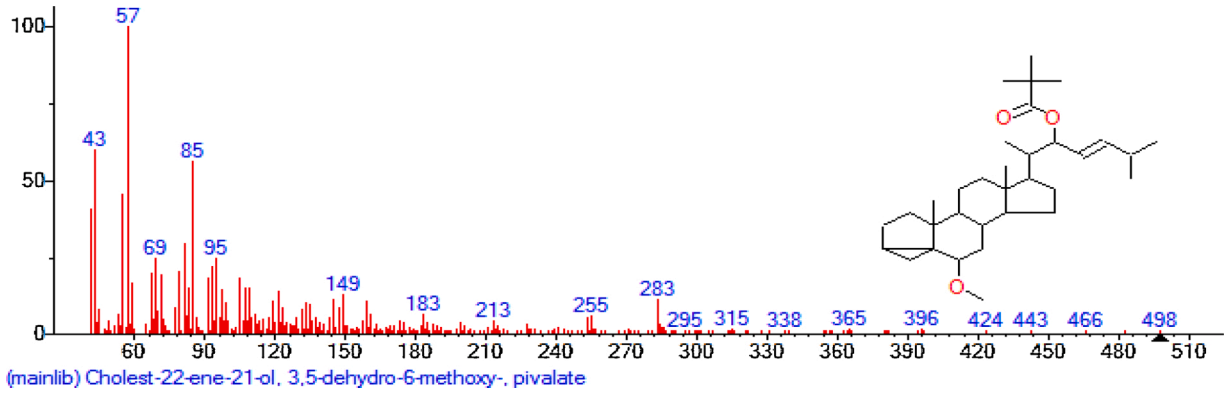

Fig. 5. Mass spectrometry of Cholest-22-ene-21-ol, 3, 5-dehydro-6-methoxy-pivalate.

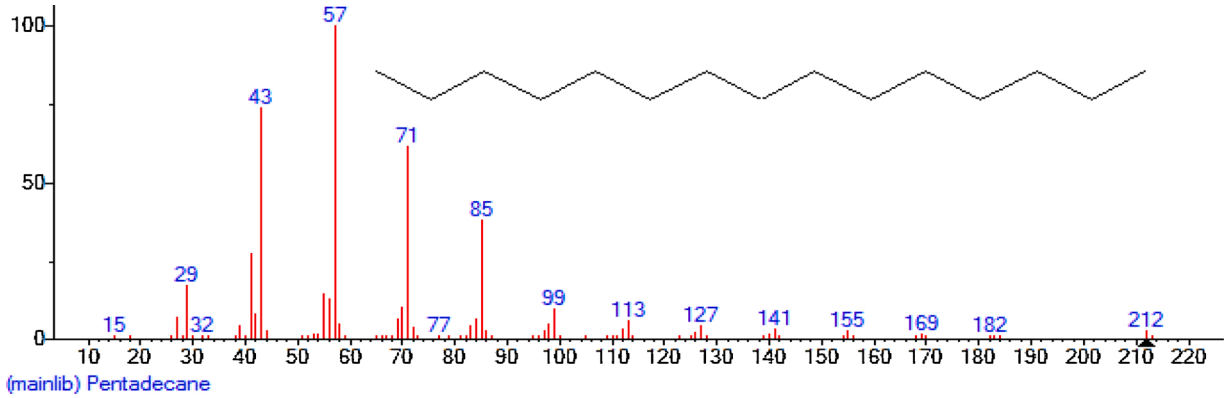

Fig. 6. Mass spectrometry of Pentadecane.

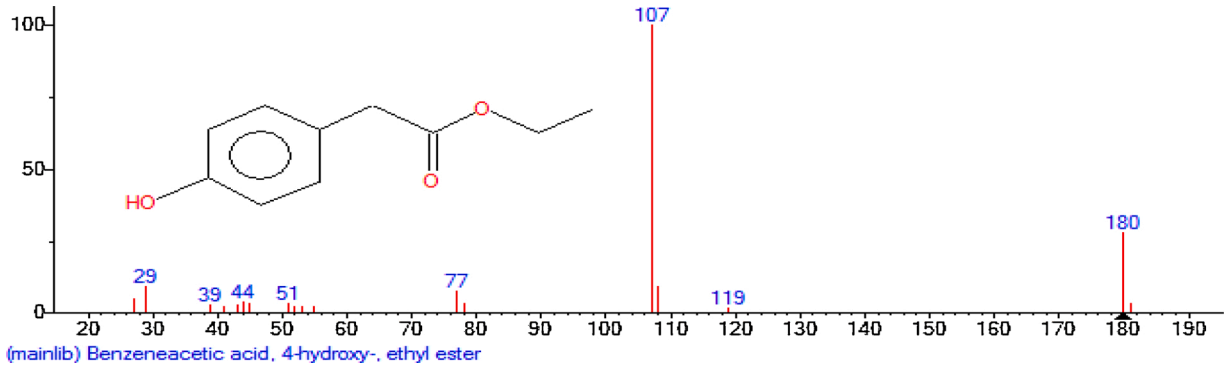

Fig. 7. Mass spectrometry of 4-hydroxy-ethyl ester Benzaen acetic acid.

terms of how much is consumed.

As to why the kidney of the 20 mL/kg BW group of animals did not show any visible derangements may be explained by the fact that the liver is the primary site of metabolism for xenobiotics, and hence more susceptible to their effects compared to other soft tissues. The observations made in this study are similar to those reported by Kpemissi et al. [25] and Ebbo et al. [26], Chaerunisaawho et al. [27] and Worasuttayangkurn et al. [28] respectively reported the safety of *Combretum micranthum*, *Diospyros mespiliformis,* Cassia fistula and Andrographis paniculata extracts to Wistar rats, following acute and sub-chronic toxicity testing.

5.1. Conclusion

The outcome of this study suggests high safety for the investigated polyherbal mixture at a dose of 10 mL/kg BW and thus supports its usage in folklore medicine. However, intake of the polyherbal formulation must be with great caution in terms of how much is consumed, particularly during long period of therapeutic application.

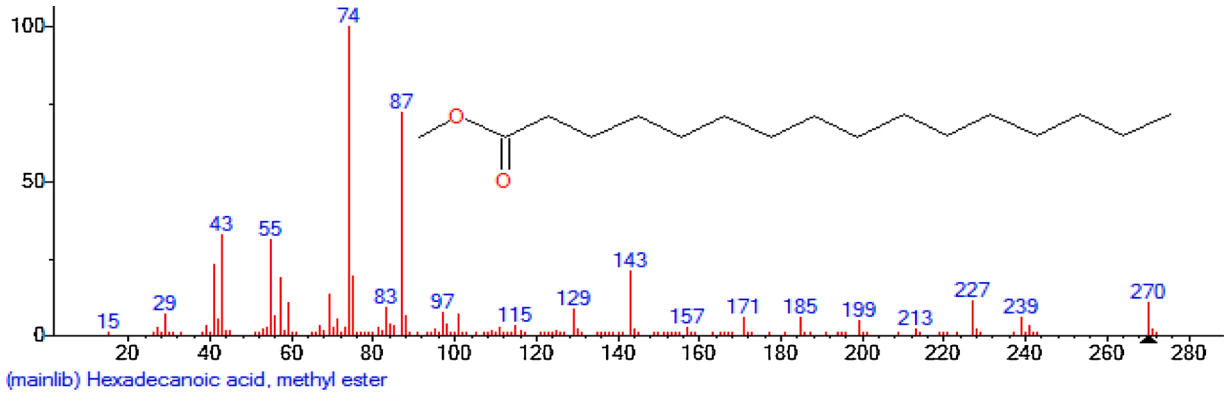

Fig. 8. Mass spectrometry of Hexadecanoic acid, methyl ester.

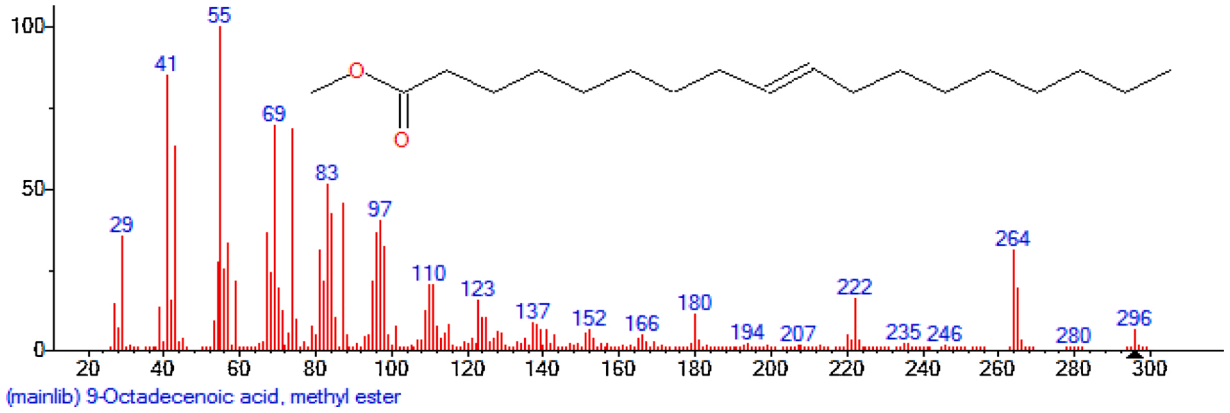

Fig. 9. Mass spectrometry of 9-Octadecenoic.

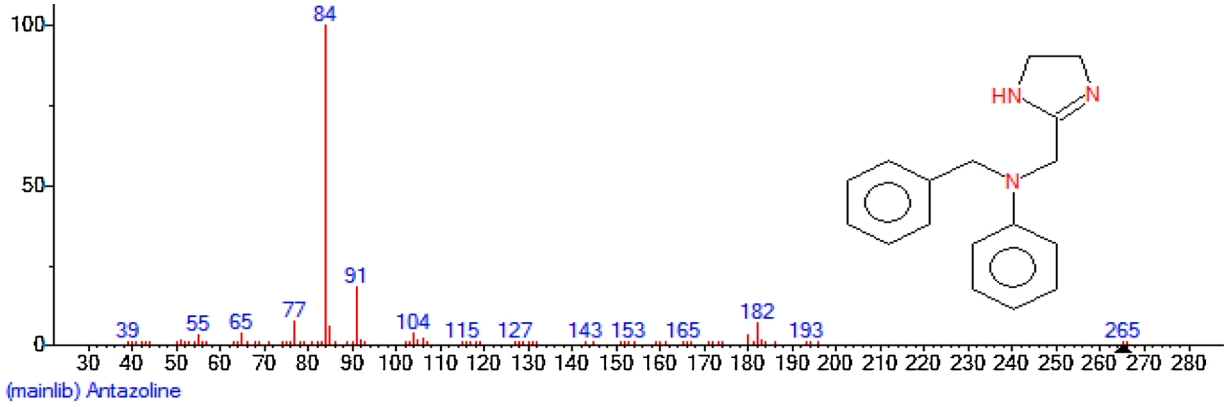

Fig. 10. Mass spectrometry of Atrazoline.

Declaration of Competing Interest

The authors report no declarations of interest.

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