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Anxiolytic-like effect of *Lippia alba* essential oil: A randomized, placebo-controlled trial

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ABSTRACT

Aim: This study was conducted to examine whether aromatherapy with essential oil of *Lippia alba* can reduce anxiety levels in a normal population.
Methods: The oil was extracted by hydrodistillation for 3 hours using a modified Clevenger-type apparatus and then chemical composition was investigated by a combination of gas chromatography analysis and gas chromatography-mass spectrometry. Moreover, a randomized, double blind, placebo-controlled trial was conducted, where 62 participants were divided into two groups, comprising a control group treated with placebo, and an experimental group (EG) treated with aromatherapy based on *Lippia alba* essential oil. The anxiety index was evaluated pretest by State-Trait Anxiety Inventory. Measures were taken twice: during pretest and posttest.
Results: The chemical analysis showed that carvone was the main component (62.8%). State and trait anxiety scores showed a decrease in the posttest study phase in comparison with pretest in the EG ($p < 0.005$ for state anxiety and $p < 0.05$ for trait anxiety). Cohen's d score was 1.06 in state anxiety, while it was 0.72 for trait anxiety. The percentages of change showed reductions of anxiety variable ranging between 15.50% for state anxiety and 12.25% for trait anxiety.
Conclusion: These results suggest that aromatherapy based on essential oil of *Lippia alba* may be useful as a means to counteract anxiety.

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Aromatherapy; essential oil; *Lippia alba*

Introduction

Nowadays, anxiety is one of the most common and prevalent health problems with high rates of relapse and recurrence [1]. According to the Global Burden of Disease Study, anxiety is one of the main causes of global disability [2], because it is associated with chronic conditions as migraine, chronic respiratory disorders, gastrointestinal problems, heart disease, among others [3]. It also impacts the existential aspects of individuals like employment; indeed, people with anxiety experience lower employment rates, higher absenteeism rates, as well as low productivity [2].

In spite of the success of short-term treatment of anxiety with medication, the rate of improvement on chronic treatment is disappointing. To this is added the fact that anxiolytic drugs produce various side effects with indiscriminate use [4]. In this context, complementary and alternative medicine (CAM) approaches have gained interest. This is due to the fact that CAM therapies are perceived as effective, natural, economical, and with fewer side effects [5]. One of these interventions is aromatherapy, which is based on the use of essential oils (EOs) as the main therapeutic agents. These volatile compounds are a mixture of saturated and unsaturated hydrocarbons, alcohol, aldehydes, esters, ethers, ketones, oxides phenols, and terpenes [6]. They are extracted from various parts of aromatic plants such as the flowers, leaves, stems, fruits, seeds, roots, rhizomes, barks, and resins [7].

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These oils are frequently isolated via steam distillation, hydrodistillation, and cold pressing methods [8]. They are administered via inhalation, massage, compress, and rarely, they are taken internally through gelatin capsules, honey water, vaginal pessaries, and suppositories [9].

Some researchers have found that aromatherapy can reduce anxiety [10], stress [11], and there is even some clinical evidence that EOs can be used as a promising treatment in preoperative anxiety and for reducing perioperative pain [12].

Lippia alba is an aromatic shrub that belongs to the Verbenaceae family. It has a wide distribution, especially in the tropical and subtropical as well as temperate zones of the Americas, Africa, and Asia [13]. *L. alba* is traditionally used to treat headaches, measles, rashes, stomachache, indigestion, diarrhea, pain, and for the nerves. Its essential oil has several chemotypes depending on the geographic location and the characteristics of the soil and climate [14,15]. Different laboratories have determined the antibacterial [16], antifungal [17], anesthetics [18], and anxiolytic [19] effects of *L. alba* EOs.

The present investigation was conducted to examine whether aromatherapy with EOs of *L. alba* can reduce anxiety levels in a normal population.

Materials and Methods

Plant material

The leaves of *Lippia alba* were collected from the Rosa Elena de los Rios Martinez Botanical Garden of Medicinal Plants National University of Trujillo at 34 meters elevation with sandy loam soil, located in Trujillo district, La Libertad Region, Peru. The sample collection was conducted in the months of May to June after the Niño Costero phenomenon in 2017. The voucher specimen was prepared and identified by Eric Frank Rodríguez Rodriguez, PhD, and deposited at the Herbarium Truxillense (HUT) of the National University of Trujillo, under registration number 59148.

Essential oils extraction

The powdered plant material (100 g) of the leaves of *L. alba* and 1,000 ml distilled water were placed in a round-bottomed flask and connected to a Clevenger-type apparatus. Hydrodistillation was completed after 3 hours of boiling. Then the oil was dried over anhydrous sodium sulfate and stored in a refrigerator in amber glass vials at 4 °C for further use in experiments [20].

Determination of essential oil composition

Gas chromatography analysis (GC) was performed in a Hewlett Packard 6890 gas chromatograph with a flame ionization detector, using the following conditions: column temperature, 40°C (8 minutes) to 180°C at 3°C/min, 180–230°C at 20°C/min, 230°C (20 minutes); injector temperature 250°C, detector temperature 250°C; split ratio 1:50; carrier gas H2 (34 KPa). Gas chromatography-mass spectrometry (GC-MS) was carried out using a Hewlett-Packard 6890 series gas chromatograph coupled with a mass selective detector Hewlett Packard MSD 5972. The system conditions were the following: fused silica capillary column HP-Innowax (30 m x 0.25 mm i.d., 0.25 μm film thickness), column temperature, 40°C (8 minutes) to 180°C at 3°C/min, 180–230°C at 20°C/min, 230°C (20 minutes); interface 280°C; split ratio 1:100; carrier gas He (56 KPa); EI mode 70 eV. Finally, the identification of essential oil constituents was accomplished by visual interpretation, comparing their retention indices and mass spectra with the literature data and with those in the NIST 2011 mass spectra library as well as Wiley library [17].

Study design and sample

An experimental study with measures at pre-test–posttest was conducted. 62 participants were divided into two groups of 31 participants, comprising a control group (CG) treated with placebo (sunflower oil was selected as placebo as it has similar texture and possesses no known therapeutic effect) [21], and an experimental group (EG) treated with aromatherapy based on *Lippia alba* essential oil. These two groups were compared in a randomized, double blind, placebo-controlled trial.

Study procedure

A free aromatherapy course was offered through local press to recruit participants. 79 people were enrolled and 62 took part in this research between July and August 2017. The inclusion criteria included male and female participants between the ages of 18 and 45 and they were required to have a State-Trait Anxiety Inventory (STAI) score of greater than 20 in both the scales; meanwhile the exclusion criteria were participants with previous practice of alternative therapies such as meditation, tai chi or yoga, psychiatric treatment, and pregnancy.

31 participants for each group were randomized by a person not involved in the study by

utilization of a random number table. After CG and EG were formed, a basic questionnaire consisting of social-demographic characteristics was applied in order to characterize the participants. After that, an anxiety self-report instrument was administered (pretest) and filled by all the participants. Two schedules were disposed for each intervention group (one in the morning and one in the afternoon). The *L. alba* essential oil and placebo (sunflower oil) were placed every session in identical amber glass vials marked with the code A and B, respectively. Both participants and researcher did not know the meaning of the codes. A psychotherapy room (4×4 m size) of Integral Psychotherapy Center was used for the experiments. The windows were closed hermetically during stimulus administration and the participants sat in ergonomic chairs forming a circle. Five environmental diffusers were used for administrating oil and placebo by inhalation. These were placed one in each corner of the therapy room and one in the middle of the circle of participants. The essential oil dose required to saturate the experimental room was four drops of 2% essential oil = 0.2 ml [22], and the placebo dose required was also four drops. All the groups had 30-minute intervention sessions from Monday to Saturday for two weeks (12 sessions). After that, an anxiety self-report instrument was administered (posttest) to 55 participants who remained to the end of the study (seven participants, three in EG and four in CG, were lost) (Fig. 1). When the offered course finished, all the participants were informed about the investigation program goals and signed a consent form in which confidentiality and anonymity were guaranteed.

The study was approved by the Ethics and Research Committee of the Postgraduate School of Cesar Vallejo University, as well as this investigation was performed in accordance with the Declaration of Helsinki.

Instruments

Anxiety was evaluated using the STAI, which consists of two self-report scales measuring two distinct types of anxiety: state (actual levels of intensity and anxiety states) and trait (selects individuals who vary in their tendency to react to psychological stress with varying degrees of intensity). Both the scales consist of 20 statements and respondents rate the intensity of their feelings about each at that moment from 1 (not at all) to 4 (very much so). The part that regards trait describes how the subjects generally feel, while the part that regards state describes how they feel at a given moment [23,24]. Inventory was validated for the local population in a previous study [22].

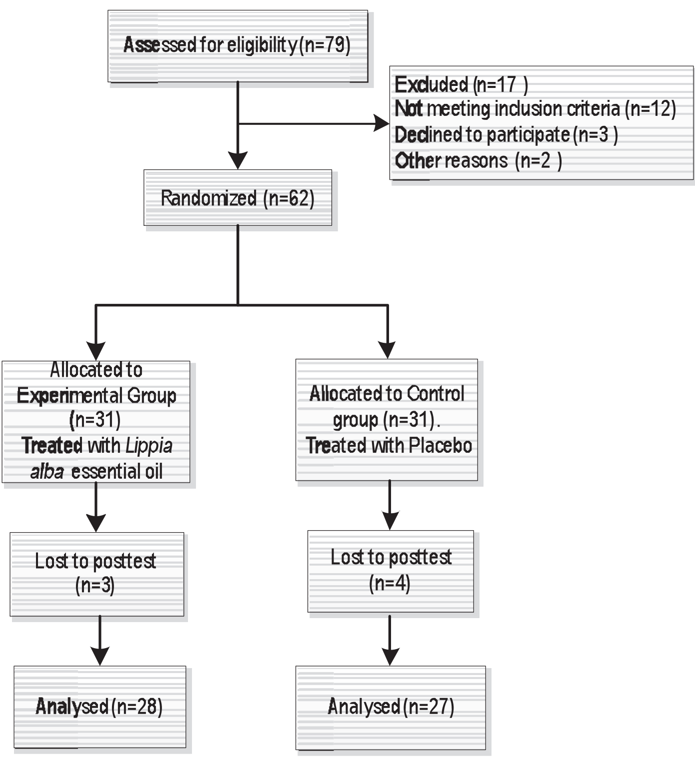

Figure 1. Flowchart of the study.

Table 1. Main chemical constituents (%) of the essential oil of *Lippia alba*.

Composition	RI	%
α-Thujene	922	0.2
α-Pinene	928	0.1
Sabinene	959	2.8
1-octen-3-ol	965	0.5
Myrcene	973	0.3
Limonene	1,027	6.8
Gamma-terpinene	1,042	0.9
Linalool	1,086	1.9
Terpinen-4-ol	1,151	T
Trans-dihydrocarvone	1,178	0.2
Carvone	1,223	62.8
Piperitone	1,241	1.1
Bornyl acetate	1,266	0.1
Thymol	1,285	0.3
Piperitenone	1,310	2.1
Eugenol	1,359	T
α-copaene	1,372	0.4
β-bourbonene	1,387	0.6
β-cubebene	1,394	0.5
β-elemene	1,399	0.5
β-caryophyllene	1,422	0.2
Aromadendrene	1,434	0.6
α- humulene	1,460	T
Germacrene D	1,473	6.1
α-muurolene	1,492	3.2
δ-cadinene	1,514	0.3
Germacrene B	1,559	T
Bulnesol	1,571	1.6
(E)-Nerolidol	1,584	2.4
α-muurolol	1,628	0.2
α-cadinol	1,654	0.1
14-Hydroxy-b-caryophyllene	1,660	0.2
Nd	1,720	0.1
(E)-Phytol	2,084	0.6
Nd	2,230	0.1
Total identified (%)		97.8

RI: Retention index; t = traces (<0.1%); Nd: Not determined

Data analysis

Data were presented as mean ± standard deviation (SD). Mann–Whitney U test was used to determine significant differences between CG and EG, while Wilcoxon test for paired samples was used to determine significant differences between the study phases. These tests were chosen because data did not conform to the normal distribution. Cohen's D and percentage change were calculated between the pretest and posttest scores. All statistical analysis was performed using SPSS v.23.0 (IBM Corp., Armonk, NY, USA).

Results

Chemical composition of the essential oil of *Lippia alba* is shown in Table 1, where 33 constituents were identified, representing 97.8% (area percent) of the total oil content, among which carvone (62.8%), limonene (6.8%), germacrene D (6.1%), α-muurolene (3.2%), sabinene (2.8%), (E)-nerolidol (2.4%), piperitenone (2.1%), linalool (1.9%), and bulnesol (1.6%) were the major components.

Table 2. Socio-demographic and clinical data of participants in study.

Socio-demographic data	Control group	Experimental group	Total
Gender			
Male	13 (48%)	12 (43%)	25 (46%)
Female	14 (52%)	16 (57%)	30 (54%)
Age(yr)			
18–24	5 (19%)	3 (11%)	8 (15%)
25–35	15 (56%)	16 (57%)	31 (56%)
36–45	7 (25%)	9 (32%)	16 (29%)
Level of education			
High school	2 (7%)	1 (4%)	3 (5%)
Undergraduate	11 (41%)	13 (46%)	24 (44%)
Graduate	9 (33%)	10 (36%)	19 (35%)
Postgraduate	5 (19%)	4 (14%)	9 (16%)
Marital status			
Married	9 (33%)	10 (36%)	19 (34%)
Unmarried	17 (63%)	16 (57%)	33 (60%)
Divorced	0 (0%)	2 (7%)	2 (4%)
Widowed	1 (4%)	0 (0%)	1 (2%)
Anxiety treatment provided			
Psychological	5 (19%)	4 (14%)	9 (16%)
Pharmacological	0 (0%)	0 (0%)	0 (0%)
None	22 (81%)	24 (86%)	46 (84%)

Table 2 presents the socio-demographic and clinical data of the analyzed participants, where 25 were male and 30 were female. CG was formed by 13(48%) males and 14(52%) females, while EG was formed by 12(43%) males and 16(57%) females. The majority of participants were between 25 and 35 years old (n = 31; 56%), followed by the participants between 36 and 45 years old (n = 16; 29%); and the rest between 18 and 24 years old (n = 8; 185%). Three people had (5%) attended high school, 24 (44%) were undergraduate students, 19(35%) were graduates, and nine (16%) were postgraduates. In relation to their marital status, 33 (60%) were unmarried, 19 (34%) were married, two (4%) were divorced, and just one participant was widowed. The majority of participants had never attended to treatment (n = 46; 84%), 9 (16%) attended to psychological treatment, and none had visited a psychiatrist for pharmacological treatment. Finally, both CG and EG were constituted by the same average number of participants by variable.

Table 3 shows the mean score and SDs for anxiety based on STAI, while EG does not present

Table 3. Group differences of anxiety variable according to State-Trait Anxiety Inventory.

Groups	Pretest		Posttest		*p*-value[b]
	Mean	SD	Mean	SD	
CG					
State anxiety	36.42	±5.64	36.71	±5.58	0.399
Trait anxiety	30.16	±6.32	30.94	±6.96	0.500
EG					
State anxiety	36.65	±6.48	30.97	±5.23	0.005*
p-value[a]	0.766		0.001**		
Trait anxiety	30.13	±7.31	26.44	±5.54	0.046*
p-value[a]	0.676		0.018*		

*$p < 0.05$, **$p < 0.005$
[a]*p*-value is calculated by Mann–Whitney U test between groups
[b]*p*-value is calculated by Wilcoxon test between study phases.

differences in the pretest scores in comparison with CG ($p > 0.05$ for state and trait anxiety); somehow, differences are shown in the posttest scores ($p < 0.005$ for state anxiety and $p < 0.05$ for trait anxiety).

Besides, anxiety scores show a decrease in the posttest study phase (30.97 and 26.44 for state and trait anxiety, respectively) in comparison with pretest (36.65 and 30.13 for state and trait anxiety, respectively) in EG ($p < 0.005$ for state anxiety and $p < 0.05$ for trait anxiety). These results show a change in STAI scores after the intervention. Meanwhile, CG shows a slight increase in the posttest scores in comparison with the pretest scores, but it does not show statistically significant differences to suggest considerable changes in anxiety levels ($p > 0.05$).

Regarding the amount of change in the mean scores at posttest, it is observed that Cohen's d score is $d = 1.06$ for state anxiety. Scores over 1 mean a large size effect, which suggests the intervention was effective for improving state anxiety. In the case of trait anxiety, Cohen's d score is $d = 0.72$. In this sense, scores over 0.50 and less than 1 indicate a medium size effect, suggesting that aromatherapy was moderately effective in improving trait anxiety. Finally, percentages of change between the pretest and posttest measures show a reduction of 15.50% for state anxiety and 12.25% for trait anxiety. All these results show a decrease in the anxiety scores (Table 4).

Table 4. Cohen's *d* and pretest–posttest percentages of change in intervention groups.

Group	Cohen's d posttest	% of change pretest-posttest
EG		
State anxiety	1.06	−15.50
Trait anxiety	0.72	−12.25

Discussion

The chemical analysis of *L. alba* essential oil showed that carvone is the main component and could be classified as carvone chemotype. This is in agreement with data from another study where this chemotype for Peru is also reported [25].

In relation to the socio-demographic and clinical data of participants, the majority were young adults and adults with university studies. This is in agreement with a previous study conducted by our research team, where participants were demographically similar [26]. This point constitutes one of the limitations, because there were no participants that represented low educational levels and our study does not show how these therapies can work in a different population. To this is added the fact that the participant number may not be enough to generalize the results. Another limitation is that due to homogeneity sample, correlations between socio-demographic and clinical data with anxiety scores are not displayed. It is appropriate to remark that in the Peruvian context, the main provider of CAM therapies is Essalud, a type of national health insurance, which covers only the salaried population. Their services are not well known by the general population, nor can the low income population afford it [27]. In fact, some researchers state that most educated sectors are more interested in CAM therapies [28].

There is some clinical evidence in favor of the EO use to reduce preoperative anxiety [29], as well as anxiety in dental treatments [30]. Indeed, findings confirm that aromatherapy can reduce anxiety levels [31]. These data are in agreement with our findings where participants show a decrease in the anxiety scores after intervention. Although more precisely speaking, state anxiety scores showed a large size effect in comparison with trait anxiety

scores that only exhibited medium size effect. This is in accordance with other studies which investigated the effectiveness of aromatherapy in reducing a state anxiety situation [11, 32]. This may be due to the fact that state anxiety is related to temporary situations that change every moment, and when they disappear, the person no longer experiences anxiety; on the other hand, trait anxiety is related to particular and permanent personality features [33]. Nevertheless, further investigations are needed to determine if the essential oil of *Lippia alba* can be useful in pathological anxiety.

Besides, other studies found that *L. alba* essential oil (carvone chemotype) show anxiolytic effect in rats, pointing that carvone could be responsible for its action as a tranquilizer, due to its effect as a depressor of the central nervous system, interacting with $GABA_A$ receptors in the brain after crossing the blood–brain barrier [19]. Therefore, carvone attenuate neurons in limbic and septo-hippocampal systems, structures involved in suppressing the response of "active-avoidance" [34]. Other components related to exert an anxiolytic effect similar to that seen with diazepam are limonene and linalool [35], also present in the *L. alba* essential oil analyzed in this study. In the case of limonene, a study suggests a non-benzodiazepine mechanism [36]. Meanwhile linalool has antagonistic action on glutamatergic receptors such as N-methyl-D-aspartate receptors, which may explain its sedative effect [37].

Conclusion

These results suggest that aromatherapy based on essential oil of *Lippia alba* may be useful as a means to counteract anxiety.

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Effects of aqueous leaf infusion of *Pterocarpus santalinoides* DC. on the serum lipid profile of guinea pigs (*Carvia porcellus*)

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ABSTRACT

Aim: This study investigated the effects of aqueous leaf infusion of *Pterocarpus santalinoides* on serum lipid profile (SLP) of guinea pigs (GPs).
Methods: Fresh leaves of *Pterocarpus santalinoides* were collected in February 2015. Aqueous leaf infusion was prepared daily by soaking dried ground leaves of *P. santalinoides* in hot water for 10 minutes. Twenty female GPs were randomly assigned to four groups of five GPs each, treated as follows: Group A—water as placebo (control), Groups B, C, and D—1.5, 3.0, and 4.5 g/kg body weight of ground *P. santalinoides* leaf soaked in 600 ml of hot water, respectively. Treatment was given orally daily for 28 days. Assay of SLP was done on days 0 (before treatment), 14, and 28 of treatment, following standard procedures.
Results: The mean serum high density lipoprotein cholesterol (HDLC) of Groups B, C, and D rose to almost double its baseline values and was significantly ($p < 0.05$) higher than that of Group A on day 28, while the mean serum low density lipoprotein cholesterol (LDLC) of Group D was significantly lower ($p < 0.05$) than those of other groups. The mean serum triglyceride and very low density lipoprotein cholesterol (VLDLC) of Groups B and C were significantly lower ($p < 0.05$) than that of Group A at days 14 and 28 of treatment.
Conclusion: Administration of *P. santalinoides* aqueous leaf infusion as used in this study led to significant positive effects of enhancement of serum HDLC and decrease of serum LDLC, triglyceride, and VLDLC.

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KEYWORDS

Pterocarpus santalinoides; aqueous leaf infusion; serum lipid profile; guinea pigs

Introduction

Cholesterol and triglycerides are the major clinically significant lipids commonly assayed for in the blood of humans and animals, because alterations in certain components of them have been found to be instrumental to the development of atherosclerosis and its clinical complications of cardiovascular diseases such as myocardial infarction (heart attack), cerebral infarction (stroke), and gangrene of the extremities [1–4]. The major components of serum total cholesterol (TC) associated with increased risk of atherosclerosis are low density lipoprotein cholesterol (LDLC) and very low density lipoprotein cholesterol (VLDLC), which play the physiologic role of vehicles for the delivery of cholesterol to peripheral tissues; in contrast, high density lipoprotein cholesterol (HDLC) mobilizes cholesterol from developing and existing atheromas and transports them to the liver for excretion in bile in a process known as "reverse cholesterol transport" [5,6]. Several studies have shown the critical roles that LDLC, VLDLC, and HDLC play in the development, progression, diminution, and/or management of atherosclerosis [6,7]. Thus, efforts/strategies aimed at prevention and management of atherosclerosis have been centered on the development of drugs, supplements, diets, and lifestyle adjustments that will reduce serum TC, LDLC, and VLDLC, and enhance serum HDLC [6,7].

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Pterocarpus santalinoides DC (Fig. 1) is an indigenous Nigerian plant in the family *Papilionaceae*. It is commonly referred to as "Red sandal wood" in English [8,9]. Leaves of *P. santalinoides* are traditionally used as food (vegetable) and as medicine in the treatment of various ailments, including inflammatory and cardiovascular diseases (heart attack and stroke) [10–12]. Aged people traditionally use *P. santalinoides* leaves for soup and as medicine because it is believed to help them cope with old age-related cardiovascular diseases such as weak/failing heart and stroke [10,12,13]. Scientific reports on the medicinal use of the leaves of *P. santalinoides* for the treatment or management of cardiovascular diseases is, however, lacking in the available literature. Based on the various medicinal uses of *P. santalinoides* especially in the treatment of cardiovascular diseases and the role that serum lipids play in the evolution, development, and progression of cardiovascular diseases, the objective of this study was to evaluate the effects of aqueous leaf infusion of *P. santalinoides* on the serum lipid profile (SLP) of guinea pigs (GPs).

Materials and Methods

Chemicals, reagents and assay kits

The clinical biochemistry assay kit for the evaluation of the SLP was procured from Quimica Clinica Applicada (QCA), Spain. All other routine reagents and chemicals were of analytical grade.

Figure 1. Picture showing leaves, flowers, and stem of *Pterocarpus santalinoides*.

Plant collection, identification, and preparation

This study was conducted in 2015. Fresh leaves of *P. santalinoides* used for the study were collected in February 2015 from Nsukka, Enugu State, Nigeria. The plant was identified by a plant taxonomist (Mr. A. O. Ozioko) at the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka (Voucher Specimen Number—University of Nigeria Herbarium no. 2). The leaves were dried under shade, and ground into coarse powder. The infusion was prepared by dissolving varied quantities [1.5, 3.0, and 4.5 g/kg body weight (BW)] of the ground leaves of *P. santalinoides* each in 600 ml of hot water (70°C–90°C) for 10 minutes. The resulting infusion was filtered using a domestic tea sieve (0.63 mm pore size) and allowed to cool.

Experimental animals

Thirty-two adult female GPs (*Carvia porcellus*) of 12 weeks of age, weighing between 300– and 400 g, obtained from the Laboratory Animal Unit of the Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka, were used for the study. The GPs were housed in a fly-proof animal house at room temperature (23°C–29°C), and allowed for 2 weeks to acclimatize before the commencement of the study. All through the study, the GPs were fed commercial pelletized feed (Grand Cereals Ltd, Jos, Nigeria), composed of 13% crude protein, 8% fat, 15% crude fiber, 0.9% calcium, 0.35% phosphorus, and 2,600 Kcal/kg metabolizable energy, and were provided with clean water *ad libitum*. Twelve of the GPs were used for the acute toxicity study, while 20 were used for the study of the effects of the infusion on the SLP.

GPs were chosen as the experimental animal model for this study because of the numerous documented metabolic similarities to humans especially in lipid metabolism and response to hypocholesterolaemic agents [14–16]. Like humans, GPs carry the majority of plasma cholesterol in low density lipoprotein (LDL) and have been shown to vary cholesterol and lipoprotein metabolism in response to dietary interventions [17–19]. GPs also have a tissue distribution of whole body cholesterol synthesis similar to that of humans and express plasma cholesteryl ester transfer protein activity [20,21].

The animal experimental protocol was approved by the Experimental Animal Ethics Committee of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka and in compliance with the Federation of European Laboratory Animal Science

Association and the European Community Council Directive of November 24, 1986 (86/609/EEC).

Experimental design

Acute toxicity study

The acute toxicity and median lethal dose (LD_{50}) of the infusion was determined following Lorke's two-step method of acute toxicity testing [22]. Nine of the 12 GPs used for the acute toxicity testing were used for the first step of the acute toxicity testing. These nine GPs were randomly assigned into three groups (A, B, and C) of three GPs each, and were given the 10, 100, and 1,000 mg/kg BW of the *P. santalinoides* leaf powder infusion *per os*. These were observed for 3 days, and in the absence of any signs of abnormalites or mortality, the remaining three GPs were used for the second step which involved these three being given 1,600, 2,500, and 5,000 mg/kg BW of the *P. santalinoides* leaf powder infusion according to Lorke [22]. The GPs used for the second step were also observed for signs of abnormality and mortality for up to day 14 post-administration [22].

Phytochemical analysis

Semi-quantitative phytochemical analysis was carried out on the infusion to test for the presence of tannins, flavonoids, alkaloids, saponins, glycosides, terpenes, and sterols following the standard procedures [23,24]. One gram of the *P. santalinoides* leaf powder was dissolved in 100 ml of distilled water in a beaker. The solution was filtered with Whatman Filter Paper No. 1 to obtain a clear filtrate, which was used to test for the presence and semi-quantity of the phytochemicals–high levels of specific phytochemicals were scored +++, moderate levels were scored ++, low level were scored +, while phytochemicals that were absent were not scored [23,24].

Evaluation of the effects of the aqueous leaf infusion on SLP of GPs

The 20 GPs used for the *in vivo* testing of the effect of the aqueous leaf infusion on SLP were randomly assigned into four groups (A, B, C, and D) of five each. The four groups of GPs were treated as follows: Group A was given 600 ml of water as placebo and served as control. Groups B, C, and D were given infusions made from soaking 1.5, 3.0, and 4.5 g/kg BW of ground *P. santalinoides* leaf in 600 ml of hot water, respectively. Fresh infusions were prepared daily in the morning for the GPs, and were made freely available to them all through the 28 days of the study. Blood samples were collected from the GPs after a 12-hour overnight fast before the commencement of treatments (day 0), and on days 14 and 28 of the treatment for the assay of the SLP. Blood sample collection was by the orbital technique [25], while the assay of the SLP was done using commercially available QCA test kits (QCA, Spain), following standard colorimetric methods [26].

Serum TC was determined based on the enzymatic colorimetric method [27], which involved the enzymatic hydrolysis and oxidation of cholesterol in the serum samples by cholesterol esterase and oxidase, respectively, contained in the QCA cholesterol working reagent, leading to formation of a colored quinonic derivative the optical density of which was measured at 505 nm wavelength and compared with that of a standard containing 200 mg/dl of cholesterol [28] using a Spectrum lab 23A spectrophotometer (HME Global Medical, England). The serum HDLC was evaluated based on the dextran sulphate-magnesium (II) precipitation method [29], which involved the precipitation of LDLC and VLDLC in the serum sample using dextran sulphate in the presence of magnesium acetate, leaving only the HDLC in the supernatant after centrifugation. The supernatant containing only HDLC was then subjected to cholesterol determination procedure as described above [30]. The serum triglyceride was determined based on glycerol phosphate oxidase enzymatic method [31]. In the triglyceride determination procedure, lipases, glycerol kinase, glycerol 3-phosphate oxidase, and peroxidase in the QCA triglyceride working reagent catalyzed the conversion of triglyceride in the serum sample to a colored indicator compound (quinoneimine), the optical density of which is measured at 505 nm wavelength and compared with standards containing 200 mg/dl of triglyceride [32] using a Spectrum lab 23A spectrophotometer (HME Global Medical, England). Diacal Auto (Dialab, Wiener Neudorf, Austria) lyophilized calibration serum was used as control for the quantitative *in vitro* clinical chemistry determinations. The serum VLDLC was calculated as 1/5 of the serum triglyceride, while the serum LDLC was calculated using the Friedewald formula [26].

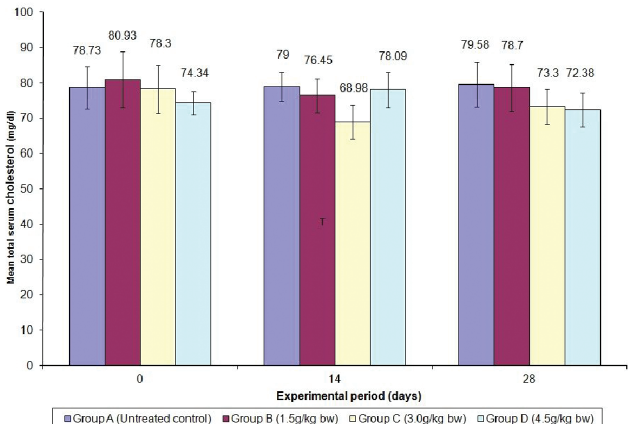

Figure 2. Serum TC of GPs given graded oral doses of *Pterocarpus santalinoides* leaf infusion.

Statistical analysis

Data obtained from the study were subjected to one-way analysis of variance, and variant means were separated post hoc using the least significant difference method. Significance was accepted at $p < 0.05$.

Results and Discussion

The infusion produced was golden brown in colour. It was well accepted by the GPs as there was no significant difference ($p > 0.05$) between the volume of the infusion consumed by GPs in the treatment Groups B (78.60 ± 3.45 ml/day), C (83.00 ± 2.37 ml/day), and D (79.30 ± 4.01 ml/day) and the volume of water consumed by the control group A (78.82 ± 4.16 ml/day) that were given plain drinking water.

In the acute toxicity study, no mortality was recorded even at the highest dose of 5,000 mg/kg BW. No signs of abnormality/toxicity were observed either, and there were no significant variations between the GPs when given the varied doses. This

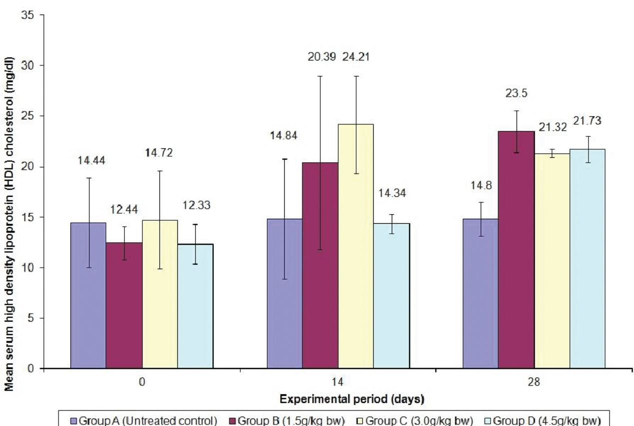

Figure 3. Serum HDL-cholesterol levels of GPs given graded oral doses of *Pterocarpus santalinoides* leaf infusion.

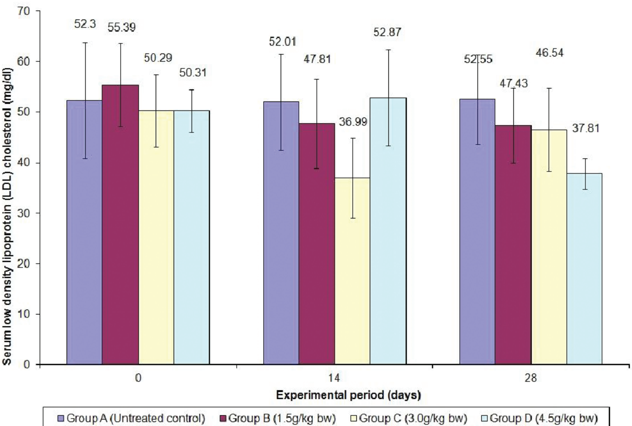

Figure 4. Serum LDL-cholesterol levels of GPs given graded oral doses of *Pterocarpus santalinoides* leaf infusion.

result of the acute toxicity study implied that the infusion is not acutely toxic. An LD_{50} above 5,000 mg/kg is within the World Health Organization's category of substances "unlikely to present acute hazard in normal use" [33]. Thus, the aqueous leaf infusion of *P. santalinoides* is considered safe. This is in agreement with the works carried out by Anowi et al. [9], and Eze et al. [34], who respectively reported that *P. santalinoides* leaf and stem bark extracts are acutely non-toxic.

The phytochemical analysis showed that the infusion contained high levels (+++) of glycosides, terpenes and sterols, moderate levels (++) of tannins, flavonoids and saponins, and low level (+) of alkaloids. The results of the phytochemical analysis in this study is in agreement with the reports of Anowi et al. [9] and Eze et al. [34] who reported on the phytochemical constituents of the ethanol leaf extract, and aqueous stem bark extract of *P. santalinoides*, respectively. Also, alkaloids, saponins, and flavonoids were present in varying quantities in the leaves of *P. santalinoides* as reported by Odeh et al. [35]. Heterogeneous phytoconstituents of crude extracts have been reported to have synergistic effect [36]. These phytochemicals, most especially flavonoids, have been reported to possess the ability to reduce free radical formation and scavenge free radicals *in vivo* [37,38], and this is important in the management of diseases associated with oxidative stress such as atherosclerosis and other cardiovascular diseases [39].

There were no significant ($p > 0.05$) variations between all the groups in their serum TC all through the study (Fig. 2). There were also no significant ($p > 0.05$) variations in the serum HDLC on days 0 and 14, but by day 28, the serum HDLC of Groups B,

C, and D had risen to nearly double their baseline values and were significantly ($p < 0.05$) higher than that of Group A (Fig. 3). The serum LDLC of all the groups did not significantly ($p > 0.05$) vary at day 0 and 14, but by day 28, the serum LDLC of the Group D GPs was significantly ($p < 0.05$) lower than those of all other groups (Fig. 4). The serum VLDLC and triglyceride of the different groups did not significantly ($p > 0.05$) vary at day 0, but at days 14 and 28, the serum VLDLC and triglyceride of Groups B and C were significantly ($p < 0.05$) lower than that of all other groups (Figs. 5 and 6).

Though the serum TC levels of the treated groups were not affected by treatment with the aqueous leaf infusion of *P. santalinoides*, the treated groups had significantly higher levels of HDLC and lower levels of LDLC, under which condition atheroma growth rate had been reported to be low, or even negative for any given TC concentration [6,7]. It is thought that the administered infusion may have modulated the lipoprotein synthetic capability of the liver in such a way that relatively more HDLC was synthesized by the liver, while more LDLC and VLDLC were catabolized by the liver. The significantly lower LDLC in the group treated with 4.5 g/kg BW aqueous leaf infusion of *P. santalinoides*, and significantly lower triglyceride and VLDLC recorded in the groups treated with 1.5 and 3.0 g/kg BW aqueous leaf infusion of *P. santalinoides* may partly be attributed to the antioxidant activity of the phytochemicals in the aqueous leaf infusion of *P. santalinoides*, as numerous studies had shown that antioxidant treatment protects against dyslipidaemia-induced atherogenesis and atherosclerosis [38,40,41]. The significantly higher HDLC recorded for all the aqueous leaf infusion of *P. santalinoides* treated groups is considered to be clinically relevant as low HDLC had been identified as an additional clinically important cardiovascular risk factor [6,7]. The beneficial effects on cardiovascular outcome of agents that act mainly by raising HDLC have been reported [6,7].

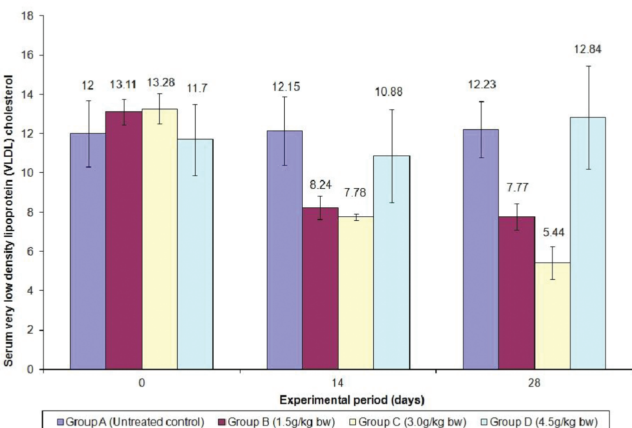

Figure 5. Serum VLDL-cholesterol levels of GPs given graded oral doses of *Pterocarpus santalinoides* leaf infusion.

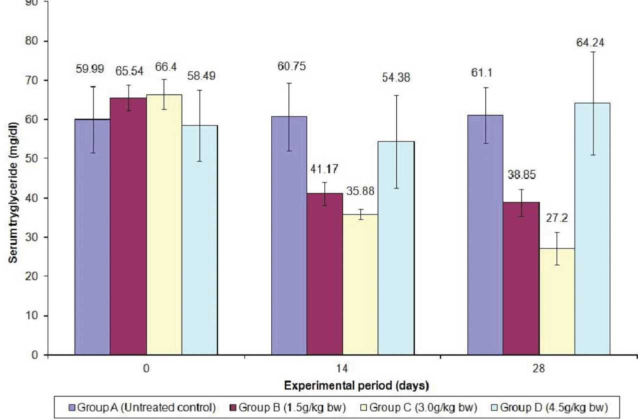

Figure 6. Serum triglyceride levels of GPs given graded oral doses of *Pterocarpus santalinoides* leaf infusion.

Conclusion

Based on the results of this study, it was concluded that oral administration of *P. santalinoides* aqueous leaf infusion as used in this study led to significant positive effects of enhancement of serum HDLC and decrease of serum LDLC, triglyceride, and VLDLC in the treated GPs. These are clinically relevant positive effects that can help in the prevention and management of dyslipidaemia-induced atherosclerosis and other cardiovascular diseases.

Acknowledgements

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ORIGINAL RESEARCH

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Medical plants used for treatment of gynecological disorders in Ottomans in the 15th century

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ABSTRACT

Aim: The aim of the present study is to give a brief evaluation about plants used for the treatment of obstetric and gynecological conditions by using Cerrâhiyyetü'l-Hâniyye book which has been written by Serefeddin Sabuncuoğlu who was a famous surgeon in Anatolia in the 15th century.

Materials and Methods: Based on the Cerrâhiyyetü'l-Hâniyye book, we selected plants that are used for women health, and systematically arranged plants according to diseases. Plant species that detected in this book were compared with the plants of three works (Soranus' On Gynecology, Paulus Aeginata's The Seven Books, and Zahavi's At Tasrif).

Results: Cerrâhiyyetü'l-Hâniyye book contains 24 different medical plants for women health. Six plants found in Cerrâhiyyetü'l-Hâniyye were different from the other books compared. Sabuncuoğlu wrote about same 18 plants, which are typed by other authors for women health.

Conclusion: This study shade light on the Ottoman herbal remedy. Regard to plant treatment, difference between Cerrâhiyyetü'l-Hâniyye book and other books were submitted by the present study.

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KEYWORDS

Gynecology; obstetrics; medicinal plants; history of medicine

Introduction

According to the literature on the history of medicine, humankind used plants for treatment of diseases. Ancient and folk medicine was incorporated plants, mineral, or animal products particularly in Anatolia located between Asia and Europe [1–5]. The way of using herbal medicine in obstetrics and gynecological conditions were similar to the treatment of other diseases [6–10]. Medicine men (monks, witch doctors, and shaman) maintained the health of people by herbal products and surgical procedures in prehistoric culture occurred before the invention of writing [5,10,11]. Then, special prescriptions for women's health are available in written medical literature in Anatolia, such as Ancient Greece and Roman medicine in the fifth century BC and until the 11th century AC [6,11–14].

Islamic medicine began to improve in 12th century in Anatolia. Islamic medicine is not at limited to religious medicine, but also includes all the medicine of the rich and varied cultures of the Islamic community. The authors wrote many scientific books in this period. They have been dominant since about the 13th century and strongly influenced the Ottoman physician of Turkey [2,6,11,15,16]. Serefeddin Sabuncuoğlu is one of the famous surgeons in Ottoman State. Şerefeddin Sabuncuoğlu (Amasya, 1385–1468) translated the 30th chapter of Zahravi's At-Tasrif dedicated to surgery into Turkish and wrote his book named Cerrâhiyyetü'l-Hâniyye. He wrote his experiences in his book by drawing

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miniatures (pictures) concerning the first medical illustrations in Islamic medicine in the 15th century (Fig. 1). "Cerrâhiyyetü'l-Hâniyye" was divided into chapters in a way similar to that was done in At-Tasrif [11,14]. Three copies of Cerrâhiyyetü'l-Hâniyye book were present today in Istanbul (two copies) and Paris (one copy). Cerrâhiyyetü'l-Hâniyye book consists of three chapters of surgical procedures and incisions, interventions, and herbal remedy. Sabuncuoğlu also wrote three books namely Mürecebname, Müfid (Nazmü't-Teshil), and Akrabaddin for physicians [11,12,17]. In this study, books of Sabuncuoğlu which were written in classical period were investigated. The medical plants used for obstetrics and gynecological conditions were compared to find similarities and differences between the Cerrâhiyyetü'l-Hâniyye book and scientific literature of ancient medicine.

Materials and Methods

Plants that were used for women health in Şereffedin Sabuncuoğlu's Cerrâhiyyetü'l-Hâniyye book were systematically arranged according to diseases. Plant species that detected in this book compared with the three famous physicians books in the history of medicine such as Soranus, Paulus Aegineta, and Zahravi [10,11,14,16].

Four physicians, who practiced in the classical medicine era, were selected as resources according to chronological order. In this study, Soranus's Gynekiea, which was translated by Temkin into English and was published in 1991 under the name

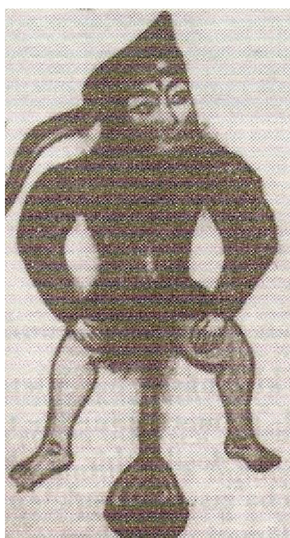

Figure 1. Miniature shows fumigations for gynecological conditions. Fumigation instrument had been used instead for medical plants to open delivery of the placenta. Şerefeddin Sabuncuoğlu drew a miniature painting of the procedure.

of "On Gynecology"; Zahravi's At Tasrif 's English version translated by Spink and Lewis; two volumes of Şereffedin Sabuncuoğlu's Cerrâhiyyetü'l-Hâniyye, prepared by İlter Uzel in a comparative way; and Paulus Aegineta's book, translated into English by Adams were used [10,11,14,16]. In addition, Mücerebbname and Acrabaddin written by Sabuncuoğlu referenced as additional resources [12,17]. Plants prescribed in the books that are not specific to women's diseases were not included in our study.

Results

Sabuncuoğlu's Cerrâhiyyetü'l-Hâniyye has been written in two volumes. The first volume of the book consists of 57 sections, drawings of operations scene and surgical procedures (54), instruments (7), and four incisions, one of them was about cauterization of the uterus. Eight of 98 issues in the second volume of this book has been written about women health. Sabuncuoğlu recommended surgical, dietetic, and pharmacologic treatment for obstetrics and gynecological conditions [11]. Commonly used Sabuncuoğlu's herbal formulas include decoctions, powders, pills, washes, fumigants, suppositories, and enemas. Decoctions and fumigation were dominant forms of pharmacologic therapy treatment of Sabuncuoğlu's works [11,12].

Descriptions of diseases, information on their treatment, and the names of plants are present in all the works investigated. Sabuncuoğlu used the terms of Greek physicians and discussed the subjects under the same titles. The plants present in his prescriptions were either a single or a combination of plants [10,11,14,16]. Cerrâhiyyetü'l-Hâniyye is a translation of Zahravî*'s At-Tasrîf* that is devoted to surgery [11,14].

Sabuncuoğlu's books for providing information for physicians contain nine chapters focused specifically on gynecology and obstetrics [11]. Şerefeddin Sabuncuoğlu described nine obstetrics and gynecological conditions in the Cerrâhiyyetü'l-Hâniyye. Title of these conditions are hermaphroditism (70th Section), clitoridectomy (71st Section), the treatment of the children with vaginal occlusion or atresia (72nd Section), the treatment of the vaginal and vulval lesions (genital warts and pustules) (73rd Section), extirpation of fibrous tissue in vagina (74th Section), about abnormal delivery (75th Section), about the removal of the dead fetus (76th Section), about the removal of the fetus that died in the maternal uterus (77th Section), and

about the removal of the placenta (78th Section) (11) (Table 1).

Regard to gynecological conditions, Soranus, Paulus Aeginata, Zahravi, and Sabuncuoğlu used 19, 12, 26, and 24 of 51 different plants, respectively (Tables 1–3).

Zahravi and Sabuncuoğlu similarly used acacia and Dragon's blood for vaginal occlusion or atresia. Paulus Aeginata, Zahravi, and Sabuncuoğlu similarly used pomegranate, also Zahravi, Şerefeddin Sabuncuoğlu similarly used oak galls, sumac, and barley for genital warts and pustules. Paulus Aeginata, Zahravi, and Şerefeddin Sabuncuoğlu similarly used mallow and rose for the extirpation of fibrous tissue in the vagina. Paulus Aeginata and Sabuncuoğlu used birthwort for the extirpation of fibrous tissue in the vagina. Zahravi and Sabuncuoğlu similarly used liquorices extirpation of fibrous tissue in vagina. Paulus Aeginata and Zahravi similarly used fenugreek used for abnormal delivery. Zahravi and Şerefeddin Sabuncuoğlu similarly used sesame, marsh mallow, and myrrh for abnormal delivery. Paulus Aeginata and Sabuncuoğlu similarly used figs for removal of the placenta. Soranus, Zahravi, and Sabuncuoğlu similarly used cassia for removal of placenta. Soranus and Zahravi similarly used artemisia for removal of the placenta. Zahravi and Şerefeddin Sabuncuoğlu similarly used cassia, chamomile, rue, and pennyroyal for removal of the placenta (Table 2).

In Cerrâhiyyetü'l-Hâniyye, 24 different plants are present for gynecological conditions. On the other hand, Sabuncuoglu used same plants for different gynecological conditions in Cerrâhiyyetü'l-Hâniyye.

Paulus Aeginata and Sabuncuoğlu used same seven plants (birthwort, figs, iris, mallow, pomegranate, and rose oil); Soranus and Sabuncuoğlu used same three plants (myrrh, rose, and cassia); Zahravi and Sabuncuoğlu used same 14 plants (acacia, dragon's blood, barley flour, cassia, liquorices root, mallow, marshmallow, oak galls, pennyroyal, pomegranate rind, rose oil, sesame, sumac, chamomile, and rue) for same or different gynecological conditions (Table 2).

Table 1. Titles of gynecological conditions and preferred plants by authors.

	Soranus*	Paulus Aeginata**	Zahravi***	Şerefeddin Sabuncuoğlu
Hermaphroditism (70th Section)		None	None	None
Clitoridectomy (71st Section)	-	None	None	None
The treatment of the children with vaginal occlusion or atresia (72nd Section)		Fenugreek Rosin Turpentine pp 640	Acacia Dragon's blood Dry linen (linseed) Olibanum	Acacia Dragon's blood Date Olive oil
The treatment of the vaginal and vulval lesions (genital warts and pustules) (73rd Section)		Rose oil Illyrian iris Pomegranate Turpentine	Acacia Dragon's blood Olibanum Sumac Pomegranate rind Thyme Oak galls Barley flour Plantain Eglantine	Oak galls Barley flour Bramble Sumac Pomegranate rind
Extirpation of fibrous tissue in vagina (74th Section)		Birthwort Iris Mallow Rose oil	Green oil Liquorices Mallow Rose oil	Liquorices Mallow Rose oil Olive oil Birthwort
About abnormal delivery(75th Section)	Anise Cedar resin Dittany Olive oil Southernwood Sweetbay Wild cucumber	Fenugreek Mallow Linseed Camphor	Fenugreek Gum Marsh Mallow Myrrh Ptarmica (green oil) Sesame	Ferula Sesame Pomegranate Marshmallow Myrrh St John's wort
About the removal of the dead fetus (76th Section)			74th Fenugreek Marshmallow Linseed	74th
About the removal of the Fetus that died in the maternal uterus (77th Section)			None	None
About the removal of the placenta (78th Section)	Black cumin Cassia Celery Dittany Galbanum Illyrian iris Lilies oil Myrrh Rose Salvia Soap wort Spikenard Wormwood (Artemisia)	Cardamum Figs	Anise Artemisia Cassia Chamomile Marshmallow Pennyroyal Rue Sesame	Cassia Chamomile Figs Pennyroyal Rue Speedwell St John's wort
Common title	19	12	26	24

*Sabuncuoğlu and Soranus wrote same two titles for gynecological situations.
**Sabuncuoğlu and Paulus Aeginata wrote same five titles.
***Sabuncuoğlu and Zahravi wrote same eight titles.

Table 2. Titles of gynecological conditions and the number of plants used by the authors.

	Soranus	Paulus Aeginata	Zahravi	Sabuncuoğlu	
The treatment of the children with vaginal occlusion or atresia (72nd Section),		3	4	4	*2
*Zahravi and Sabuncuoğlu similarly used Acacia, Dragon's blood.					
The treatment of the vaginal and vulval lesions (genital warts and pustules) (73rd Section)		4	10	5	*1 **3
*Paulus Aeginata, Zahravi, and Sabuncuoğlu similarly used Pomegranate.					
**Zahravi, Şerefeddin Sabuncuoğlu similarly used Oak galls, Sumac, and Barley.					
Extirpation of fibrous tissue in vagina (74th Section)		4	4	5	*2 **1 ***1
*Paulus Aeginata, Zahravi, and Şerefeddin Sabuncuoğlu; Mallow and Rose.					
**Paulus Aeginata, Sabuncuoğlu similarly used Birthwort.					
***Zahravi, Sabuncuoğlu similarly used Liquorices.					
About abnormal delivery (75th Section)	7	4	6	6	*1 **3
*Paulus Aeginata and Zahravi similarly used Fenugreek.					
**Zahravi and Şerefeddin Sabuncuoğlu similarly used Marshmallow, Myrrh, and Sesame.					
About the removal of the dead fetus (76th Section)	-	-	3	-	-
About the removal of the placenta (78th Section)	13	2	8	6	*1 **1 ***1 ****4

*Paulus Aeginata and Sabuncuoğlu similarly used figs.
**Soranus, Zahravi, and Sabuncuoğlu similarly used cassia.
***Soranus and Zahravi similarly used Artemisia.
****Zahravi, Şerefeddin Sabuncuoğlu similarly used cassia, chamomile, pennyroyal, and rue.

Table 3. Plants used for gynecological conditions in this study.

Common name	Local name	Name of the plant	Genus (Family)
Olive (oil)	Zeytin ağacı Akdeniz Zeytini Şifalı Zeytin	*Olea europaea* L.	Olea (Oleaceae)
Rose (oil)	Gül (Isparta gülü, yağ gülü, and fındık gülü)	*Rosa ×damascena* Herrm.	Rosa (Rosaceae)
Rue	Sedefotu	*Ruta chalepensis* L.	*Ruta* (Rutaceae)
Barley	Arpa	*Hordeum vulgare* L.	*Hordeum* (Poaceae)
Cucumber	Salatalık	*Cucumis sativus*	*Cucumis* (Cucurbitaceae)
Date Palm Tree	Hurma, Şeker Ağacı	*Phoenix dactylifera*	*Phoenix* (Arecaceae)
Fenugreek	Çemenotu Boyotu Buyotu Hulebe	*Trigonella foenum-graecum*	*Trigonella* (Leguminosae)
Camphor	Kafurun, kafur	*Cinnamomum camphora* (L.) J. Presl	*Cinnamomum* (Lauraceae)
Fig	İncir	*Ficus carica* L.	*Ficus* (Moraceae)
Linseed (linen) Linseed, Flax seed	Keten tohumu, zeyerek, bızıktan, zeylek, zerek	*Linum usitatissimum* L.	*Linum* (Linaceae)
Myrrh	Sarı Sakız, Mür, Mirra Mirrisafi Mürrüsafi Hakiki Mirra	*Commiphora myrrha* (Nees) Engl.	*Commiphora* (Burseraceae)
Pomegranate Rind	(Nar Kabuğu), Cülnar-I Mısri	*Punica granatum* L.	*Punica* (Lythraceae)
Turpentin	Çam Terebentin Kızılçam	*Pinus brutia* Ten.	*Pinus* (Pinaceae)
İris	Nevruz Çiçeği	*Iris persica* L.	*Irıs* (Iridaceae)
Anise	Anason (Büyük) Tekeotu Akanason	*Pimpinella anisum* L.	*Pimpinella* (Apiaceae)
Cassia	Çin Tarçını	*Cinnamomum cassia* (L.) J. Presl	*Cinnamomum* (Lauraceae)
Cumin	Kimyon Şifalı Kimyon	*Carum carvi* L.	*Carum* (Apiaceae)
Mugwort, Wormwood, Artemis	Pelin Acı Pelin Pelinotu Mideotu Ak Pelinotu	*Artemisia absinthium* L.	*Artemisia* (Co mpositae)
Pennyroyal	Yaban Fesleğeni Yarpuz Fesleğen Reyhan Fütenec	*Mentha pulegium* L.	*Mentha* (Lamiaceae)

(Continued)

Table 3. Plants used for gynecological conditions in this study. (Continued)

Common name	Local name	Name of the plant	Genus (Family)
Rosin weed	Reçine bitkisi	*Silphium integrifolium* Michx.	*Silphium* (Compositae)
Birthwort	Lohusa Otu Ziravend-İ Tavilin	*Aristolochia clematitis* L.	*Aristolochia* (Aristolochiaceae)
Mallow	Ebegümeci	*Malva sylvestris* L.	*Malva* (Malvaceae)
Liquorice (licorice) Root	Meyan Kökü	*Glycyrrhiza glabra* L.	*Glycyrrhiza* (Leguminosae)
Acacia	Akasya	*Acacia arabica* (Lam.) Willd.	*Acacia* (Leguminosae)
Dragon's Blood	Kardeş Kanı	*Daemonorops draco* (Willd.) Blume	*Daemonorops* (Arecaceae)
Olibanum (frankincense)	Günlük, buhur, tütsü	*Boswellia ameero* Balf. f.	*Boswellia* (Burseraceae)
Sumac	Sumak Sumak (Zehirli) Huzurotu Siyatikotu	*Toxicodendron pubescens* Mill.	*Toxicodendron* (Anacardiaceae)
Thyme	Kekik	*Thymus vulgaris* L.	*Thymus* (Lamiaceae)
Oak Galls	Mazı Meşesi	*Quercus infectoria* G. Olivier	*Quercus* (Fagaceae)
St. John's Wort	Sarı Kantaron Kılıçotu Binbirdelilikotu Mayasıl Otu Koyunkıran Yara Otu	*Hypericum perforatum* L.	*Hypericum* (Hypericaceae)
Plantain	Sinir otu, Damar Otu	*Plantago Major* L.	*Plantago* (Plantaginaceae)
Eglantine, Sweet Bier	Yaban Gülü (Kokulu)	*Rosa rubiginosa* L.	*Rosa* (Rosaceae)
Bramble (Blackberry)	Böğürtlen	*Rubus ulmifolius* Schott*	*Rubus* (Rosecease)
Cedar Resin	Sedir Reçinesi	*Cedrus libani* A. Rich	*Cedrus* (Pinaceae)
Sweet Bay, Trunk Bark, Magnolia Bark Extract	Manolya	*Magnolia virginiana* L.	*Magnolia* (Magnoliaceae)
Dittany Winter Savory, Herb	Geyik Otu	*Satureja Montana* L.	*Satureja* (Lamiaceae)
Southernwood	Miskotu	*Artemisia abrotanum* L.	*Artemisia* (Compositae)
Ferula	Çakşır Cavşir (Oğlan Aşı)	*Ferula elaeochytris* corovin	*Ferula* (Apiaceae)
Marshmallow	Tıbbi Hatmi Hatmi İbiskökü Şifalikök Tıbbikök Devegülü	*Althaea officinalis* L.	*Althaea* (Malvaceae)
Gum	Çadır Uşağı, Uşşak, Keleh El-Kanaveşak	*Dorema ammoniacum* D. Don	Dorema (Apiaceae)
Mastic (Gum)	Sakız Ağacı	*Pistacia lentiscus*	*Pistacia* (Anacardiaceae)
Ptarmica sneezewort, Yarrow (green oil)	Civanperçemi	*Achillea ptarmica* L.	*Achillea* (Compositae)
Sesame	Susam Sırık Sırlan Şırlagan Süsen	*Sesamum İndicum* L.	*Sesamum* (Pedaliaceae)
Celery	Kereviz	*Apium graveolens* L.	*Apium* (Apiaceae)
Galbanum	Kasnı otu Kasnısı	*Ferula szowitziana* DC.	*Ferula* (Apiaceae)
Lili	Ak Zambak (Misk zambağı)	*Lilium candidum*	*Lilium* (Liliaceae)
Salvia Sage Clary Sage Trilobed Sage	Yünlü Adaçayı	*Salvia aethiopis* L.	*Salvia* (Labiatae)
Soap Wort	Sabunotu Çöğen Otu Çövenotu, Çoğanotu Üşnan,Tahdik Otu, Köpürgen, Kargasabunu	*Saponaria Officinalis*	*Saponaria* (Caryophyllaceae)
Spikenard	Hint Sümbülü	*Nardostachys Jatamansi* (D. Don) DC	*Nardostachys* (Caprifoliaceae)
Chamomile	Papatya	*Chamaemelum nobile* (L.) All.	*Chamaemelum* (Compositae)
Speedwell	Şıh (Yavşan)	*Artemisia spicigera* K. Koch	*Artemisia* (Compasitae)
Cardamom	Kakule Malabar Kahvesi	*Elettaria Cardamomum* (L.) Maton	*Elettaria* (Zingiberaceae)

Sabuncuoğlu wrote same 19 plants which typed by other authors for gynecological conditions in Cerrâhiyyetü'l-Hâniyye book. These plants are acacia, barley flour, birthwort, cassia, chamomile, dragon's blood, figs, liquorices, mallow, marshmallow, myrrh, olive oil, oak galls, pennyroyal, pomegranate rind, rose oil, rue, sesame, and sumac (Table 2). Sabuncuoğlu used different five plants (St John's wort, date, bramble, ferula, and speedwell) similarly for medicinal purpose in Cerrâhiyyetü'l-Hâniyye (Table 2).

Discussion

In the present study, plants used to treat the gynecological conditions in Sabuncuoğlu's

Cerrâhiyyetü'l-Hâniyye book were systematically determined. Sabuncuoğlu's book had an effect on physicians in Islamic medicine literature [11,18–20].

The work of Sabuncuoğlu contains detailed information about the treatment of women diseases and obstetrics. Sabuncuoğlu presented the medical applications without focusing on the underlying mechanism of disease and the results of treatments [3,5,11,13,15].

The treatment modalities in Cerrâhiyyetü'l-Hâniyye consists of three groups: dietetic, surgery, and pharmacology. Three groups of treatment, which also used for difficult delivery, were used to cure diseases of women. Sabuncuoğlu written difficult delivery and placenta retention sections under the same title like other authors such as Soranus, Paulus Aeginata, and Zahravi [10,11,14,16]. Sabuncuoğlu repeats the procedures of these physicians about the diagnosis and treatment of gynecological conditions [10,11,14,16]. However, there are differences between other books and Cerrâhiyyetü'l-Hâniyye in term of the names and numbers of plants used for obstetrics and gynecological conditions. Submitted difference in the present study was about prescriptions written by Sabuncuoğlu and the lack of occurrence of same plant names is remarkable for the history of medicine [10,11,14,16]. Half of the plants used for obstetrics and gynecological conditions in the Cerrâhiyyetü'l-Hâniyye were different from the other books compared. Physicians in the present study lived in different regions and different periods.

In accordance with the period which the physicians lived, it is obvious in the works that there exists a lack of knowledge among the physicians with regard to anatomy, microbiology, and pathology and it is obvious that the physicians were not able to evaluate the etiopathogenesis of the diseases [10,11,14,16]. Therefore, selection criterions of plants for a disease were different compared to nowadays. Gynecology and obstetrics conditions diagnosed according to symptoms and observations by the physicians without determining the relationship of reason-result [4,9]. The chosen treatment performed according to the experience of the physician.

In addition, the relationship between the "active ingredient" in the plant and the expected effect had not been known by physician of that period. Therefore, the plants were either used alone or in mixtures, without knowing the active ingredients. The treatments were not changed according to the response to the treatment [10,11,14,16]. Sabuncuoğlu recommended the necessity of continuing to the treatment in the case of even if the disease was not cured. Nevertheless, Sabuncuoğlu used a considerable number of different plants for gynecological conditions. For that reason, it is difficult to reach a conclusion about the efficacy of the plants on the treatment of the diseases [10,11,14,16]. In the Sabuncuoğlu's Mücerebname work, it mentioned that he performed medical drug experiments [12]. Nevertheless, it was different from our current understanding of drug tests.

The historical period and its environment affected the attainability of the plant used to treat the illness. On the other hand, plants used by the physicians either grow in that area or it depends on the conduction of trade of the plants [4,13,15,21]. Anatolia is one of the richest regions on this subject.

Medical applications and recipes in the work of Sabuncuoğlu are same with the other works examined, but some of the plants used to prepare drugs are different. The difference that we detected could depend on the effect of the period that physician lived in or the environment that physician worked. One of the writers examined in the present study Soranus lived in the first century in Bergama and Alexandria city. Paulus Aeginata and Zahravi were the most important physicians in the sixth and nineth century, respectively. Paulus Aeginata lived in Aeginata, a city in Greece; Zahravi was a surgeon in Cordova, Spain; and Sabuncuoğlu had spent most of his life practicing medicine in Amasya and Bolu cities in Anatolia [10,11,14,16]. Therefore, physicians could have used the plants likening the appearance, shape, smell, or a property of the plant [4].

According to the modern modality of medicine, a relationship is established between the active ingredient used in drugs for the treatment of disease and the effect which the drug has [22,23]. Some of the plants used in the folk and traditional medicine continued to be used in Anatolia [5,20,24]. Experimental studies to uncover the benefits of this kind of plants are available [23,25,26].

Conclusion

Plants used as treatments for conditions related to gynecology and obstetrics in Cerrâhiyyetü'l-Hâniyye book have some differences from the other works compared. One of classic medicine representative Sabuncuoğlu who lived in 15th century in Anatolia did not copy the use of plant methods of preceding physicians but instead used his knowledge and experiences according to his conditions. Differences and similarities in the use of the plants we determined

in our study may contribute to the existing knowledge of medical history. In addition, botanists, geographers, and archeologists could examine these findings from their aspects and comparison of more works in a similar way will strengthen the result of our study.

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ORIGINAL RESEARCH

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Acute and sub-chronic toxicity evaluation of the aqueous extract of *Codiaeum variegatum* leaves on *Wistar albino* rodents of both sexes

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ABSTRACT

Background/Aim: The decoction of *Codiaeum variegatum* leaves is used by Cameroonian local population in the treatment of amoebic dysentery. The present study was carried out to investigate the safety of the aqueous extract of *Codiaeum variegatum* leaves on *Wistar albino* mice and rats of both sexes.
Methods: For the acute toxicity study, seven groups of eight mice (four males and four females) each received orally once distilled water (10 ml Kg^{-1}) and the aqueous extract in a single dose of 2, 4, 8, 12, 16, and 24 g Kg^{-1}. These mice were observed for mortality after 48 hours and thereafter, for clinical signs daily for 14 days. In the sub-chronic toxicity evaluation, four groups of 12 rats (six males and six females) each received distilled water (10 ml Kg^{-1}) and the aqueous extract at doses of 50, 100, and 200 mg Kg^{-1} by oral gavage for 28 consecutive days. The body weight was evaluated every 2 days for each animal and after sacrifice, the relative weight of vital organs was determined. Blood serum was used for the analysis of biochemical markers of renal and hepatic toxicity.
Results: The administration of the aqueous extract in both acute and sub-chronic toxicity evaluation did not cause neither significant visible signs of toxicity nor mortality and no significant changes were observed on body weight, relative organ weight and biochemical parameters in treated groups compared to the control groups.
Conclusion: These results demonstrated that the aqueous extract of *Codiaeum variegatum* leaves is non-toxic and may be safely used for its therapeutic application.

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Aqueous extract; *Codiaeum variegatum*; acute toxicity; sub-chronic toxicity; biochemical parameters; mice and rats

Introduction

Codiaeum variegatum, commonly known as "*garden croton*", belongs to the family of Euphorbiaceae and is an ornamental shrub with diverse beautiful and attractive foliage. This houseplant is native of tropical forests from Indonesia, the Philippines to New Guinea and Australia [1]. *Codiaeum variegatum* is more often used for decorations, and there are various species which are usually hybridized to produce the most decorative potted plants. More than 300 cultivars of *C. variegatum* known as mutants or hybrids are reported around the world; and these cultivars are grouped into nine species based on their leaf morphology [2]. Some cultivars of *C. variegatum* are also used for their medicinal properties in the treatment of various diseases. Freeze-dried leaves decoction of *C. variegatum* is taken as tea by Filipinos and eating crushed leaves cures diarrhea [3]. The root and bark are used against syphilis, constipation, stomach-ache, loss of appetite, and dysuria. In Cameroon, the decoction of *C. variegatum* (var. *mollucanum*) leaves is used by local population in the treatment of amoebic dysentery. In our previous study, it was reported that the

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aqueous extract of *C. variegatum* leaves (AECVL) exhibited significant anti-amoebic activity on polyxenic and axenic culture of *Entamoeba histolytica* [4,5]. Moreover, optimal conditions were defined for leaves' collection to maximize the anti-amoebic activity of the aqueous extract and a sub-fraction (SF9B) was identified with significant higher anti-amoebic activity compared to the unfractionated aqueous extract. Anti-amoebic activity of the most potent SF9B was confirmed with the morphological characteristics of induced death in trophozoites of *E. histolytica* through the destabilization of Gal/GalNAc lectin, an abundant parasite cell surface protein. Differential gene expression analysis using high-throughput RNA sequencing implies that these sub-fraction acts by targeting ceramide, a bioactive lipid involved in the disturbance of biochemical processes within the cell membrane including differentiation, proliferation, cell growth arrest, and apoptosis [4]. Therefore, the rational and safe usage of *C. variegatum* is absolutely important to gain benefit from its therapeutic value. Previously, the *in vitro* toxicity of the AECVL on non-competent or metabolic competent cell lines showed that this extract is neither genotoxic, nor mutagenic at non-toxic or moderately toxic concentrations [6]. This result suggested that the AECVL could be safely used at lower doses for medicinal purpose. To further ensure the safety of this extract, toxicity studies should be done also *in vivo* on animals. The laboratory animals considered closer to humans have been used for the *in vivo* evaluation of toxicity, which represents a key step in the safety evaluation as it may allow extrapolation of results to humans. Thus, it is recognized that a positive effect in an *in vivo* toxicity study on laboratory animals is indicative of a possible adverse effect in humans. Mice and rats are small rodents that are widely used in biomedical research since their genetic, biological and behavior characteristics closely resemble those of humans. The aim of the present study was to evaluate the acute and sub-chronic toxicity of the AECVL on rodents of both sexes.

Material and Methods

Preparation of the aqueous extract

Fresh leaves of *Codiaeum variegatum* (var. *mollucanum*) were collected in the morning in Mbankomo, a sub-division around Yaoundé, Cameroon. A voucher specimen of the plant material identified under the number 33570 HNC has been deposited at the National Herbarium of Cameroon. The leaves collected were thoroughly washed with tap water, rinsed with distilled water, dried at room temperature and grinded. From the powder obtained, 200 g from each batch was mixed with 2 l of distilled water for the preparation of the aqueous extract by decoction for 1 hour. After filtration with the Whatman No.1 filter paper, the filtrate collected was dried by lyophilization. The yield of the extract (24.20%, w/w) was calculated with respect to the initial weight of the dried plant powder.

Phytochemical screening of the aqueous extract

Phytochemical screening was carried out by the method described by Trease and Evans [7]. In brief, phytochemical composition of the AECVL was determined by analyzing the presence of some groups of compounds such as alkaloids, tannins, sterols, polyphenols, coumarins, leucocyanins, sugar, flavonoids, and terpenoids.

Experimental animals

Healthy adult *Wistar albino* mice (20–30 g; 9–11 weeks) and *Wistar albino* rats (170–210 g; 8–10 weeks) of both sexes were used respectively for the acute and the sub-chronic toxicity experiments. They were obtained from the Animal House of the Laboratory of Pharmacology and Toxicology (University of Yaoundé I, Cameroon) and were housed in polypropylene cages under normal laboratory conditions (12 hours light/dark cycle; 23 ± 2°C). Before the experiment, the animals were grouped for an acclimatization period of one week. All the animals were given food and tap water *ad libitum*, and the experiment was conducted according to international guidelines [8,9].

Acute toxicity study

Acute toxicity of the aqueous extract was evaluated on *Wistar albino* mice of both sexes, as per the World Health Organization guidelines [8]. Twenty-eight animals per sex were equally divided into seven groups of four animals each. The AECVL was dissolved in distilled water and administered orally with the aid of a blunt end needle and syringe. The control group received distilled water (10 ml Kg^{-1}) and the test groups received the AECVL at doses of 2, 4, 8, 12, 16, and 24 g Kg^{-1}. Mice were closely observed for the initial 4 hours after administration and clinical signs of toxicity such as aggressiveness, reaction to stimuli, locomotion, social interactions, aspects of feces and convulsions or coma were also observed. After this period, the animals were

supplied with food and water *ad libitum*. Dead animals were counted in each group within 48 hours following the administration of the AECVL, and the median lethal dose (LD_{50}) values were determined in both sexes. The animals were observed daily for the following 14 days and their body weight was registered every 2 days.

Sub-chronic toxicity study

Sub-chronic toxicity of the AECVL was evaluated on *Wistar albino* rats of both sexes, as per the Organization for Economic Co-operation and Development guidelines [9]. Twenty-four animals per sex were equally divided into four groups of six animals each. The control group received distilled water (10 mL Kg^{-1}), while the test groups received the AECVL at doses of 50, 100, and 200 mg Kg^{-1} body weight respectively for 28 consecutive days. These doses were chosen based on the approximation and extrapolation made on the efficient doses applied by traditional healers in the treatment of amoebic dysentery. Animals were observed for clinical signs of toxicity during the treatment period, and the body weights of animals were recorded every 2 days. At the end of the treatment, animals were fasted overnight, but allowed free access to water. These animals were anesthetized with ether and were sacrificed by cervical dislocation for the collection of blood samples in dry test tubes without anticoagulant. Vital organs such as heart, liver, lung, kidney, and spleen as well as genital organs such as prostate, seminal vesicles, testes, epididymis (for males), and ovaries, uterus (for females) were collected and the relative organ weight (weight of organ as proportion to the total body weight of each rat) was calculated and compared with the weight of the corresponding organ in the control group animals.

Preparation of serum samples and liver homogenates

The blood collected in dry test tubes was allowed to stand for complete clotting in ice for 1–2 hours. The clotted blood samples were centrifuged at 3,000 rpm for 15 minutes and serum samples were aspired off and conserved in aliquots at −20°C for the analysis of biochemical markers of renal and hepatic toxicity. The homogenate of the liver was prepared in a buffer solution (Tris 50 mM, KCl 150 mM; pH 7.4) at 20% (i.e., 20 g of the organ in 100 ml solution). In fact, the liver was cut into pieces with scissors and crushed in a mortar covered with ice. The homogenate was then centrifuged at 5,000 rpm for 30 minutes, and the supernatant was collected and conserved in aliquots at −20°C for the quantification of total protein.

Analysis of biochemical parameters

Total protein in blood serum samples was analyzed using the method of Biuret [10], while total protein in liver homogenate was analyzed using the Bradford method [11]. Creatinine was analyzed in blood serum samples using a kinetic method [12], and transaminases (Aspartate aminotransferase: AST; Alanine aminotransferase: ALT) were analyzed according to the method of Reitman and Frankel [13].

Statistical analysis

For each analyzed parameter, the data are expressed as mean ± standard deviation (SD) between different animals within each group. Comparisons between different groups were performed by the one-way analysis of variance. Significant difference between the control and experimental groups was assessed by Dunnett's test using the software *GraphPad Instat 3.0* (GraphPad software Inc., USA). The data were considered as significant when p value is less than 0.05.

Results

Chemical composition of the aqueous extract

The phytochemical screening of the AECVL reveals the presence of some groups of compounds such as polyphenols, tannins, sugars, and coumarins.

Acute toxicity

Oral administration of AECVL at doses up to 24 g Kg^{-1} induced no significant abnormal signs of toxicity. In fact, apart from the decrease in aggressiveness, locomotion and reaction to stimuli observed immediately after administration of the unique dose of AECVL, at doses greater than 16 g Kg^{-1}, all animals presented similar behavior in 4 hours postadministration and no mortality was registered in 48 hours after treatment. Figure 1 describes the body weight percentage variation per sex during the 14 days postadministration of the AECVL, and it is observed that all animals from both sexes gained weight during the study. An exception was noted in rats from groups that received the AECVL at doses greater than 16 g Kg^{-1}. In fact, the weight loss observed up to the 4th day would be due to the loss of appetite caused by the administration of high doses of extract. Overall, no

mortality was noted up to 14 days postadministration and LD_{50} of the AECVL was then estimated as greater than 24 g Kg^{-1} body weight.

Sub-chronic toxicity

Body and organ weights

As summarized in Figure 2, the body weight of the rats increases relatively during the study. When compared with the control groups, AECVL treatments did not cause significant change in the body weight of rats of both sexes. Similarly, the oral ingestion of AECVL over 28 days caused no significant changes in the weight of the studied organs (liver, kidneys, heart, lung, and spleen) regardless of whether the rat took the extract (test groups) or not (control groups) (Table 1). Likewise, the relative weights of genital organs from the treated rats of both sexes were not significantly affected during the study (Table 2).

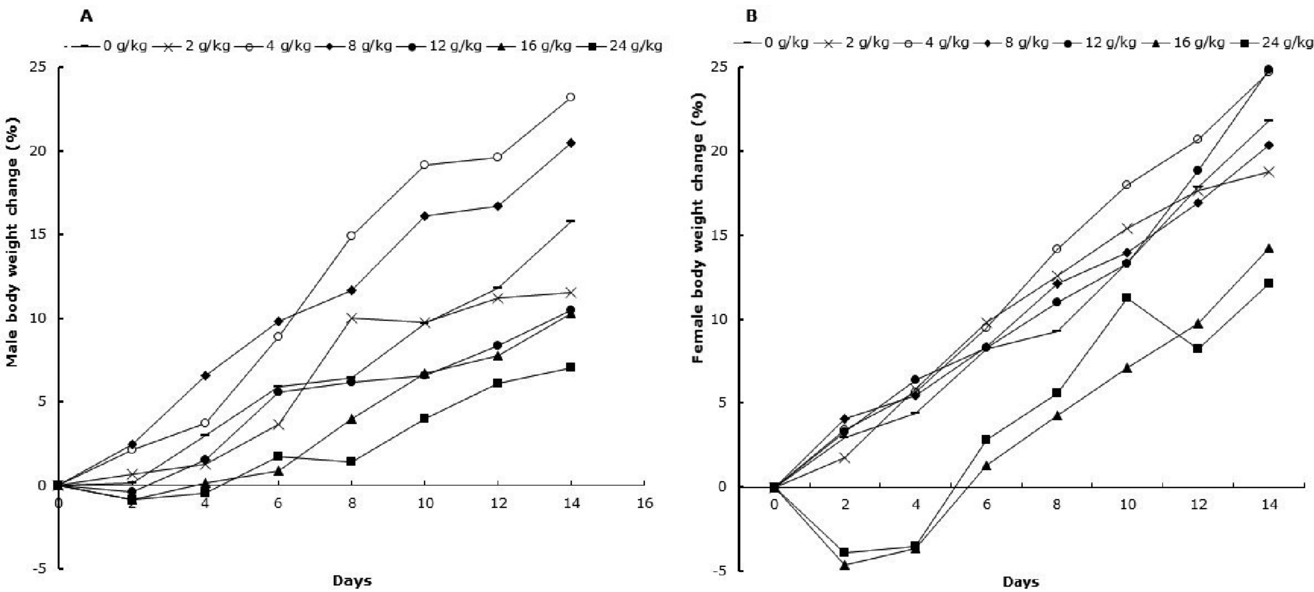

Figure 1. Percentage of body weight change of male (A) and female (B) mice during the 14 days after the administration of different single doses (0, 2, 4, 8, 12, 16, 24 g Kg^{-1}) of the aqueous extract of *Codiaeum variegatum* leaves. Each value represents the average between 4 animals per group.

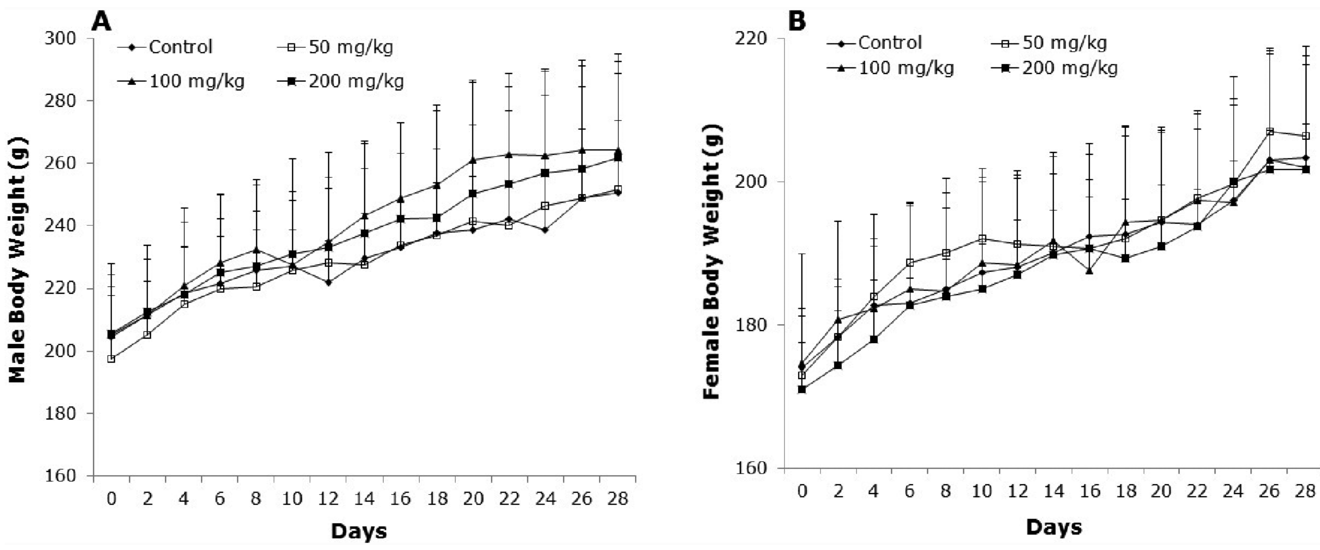

Figure 2. Body weight evolution of male (A) and female (B) rats during 28 consecutive days of oral administration of different repeated doses (0, 50, 100, 200 mg Kg^{-1}) of the aqueous extract of *C. variegatum* leaves. Each value represents the mean ± SD between six animals per group. (Dunnett test: * means significant difference compared to the control group; p ≤ 0.05).

Biochemical parameters of renal and hepatic toxicity

Quantification of biochemical parameters through the determination of total protein in blood serum samples and liver homogenate, the transaminases (AST, ALT) and creatinine levels, showed no significant difference as compared with the control groups of rats of both sexes (Table 3). A slight increase was observed in the level of transaminases after the treatment of male and female rats with AECVL. But, this increase remains statistically non-significant as compared to control groups. Notwithstanding, the calculation of AST/ALT ratio in all groups is less than 2, which is approximately to the normal value

Table 1. Relative weight of vital organs (g Kg^{-1} body weight) of male and female rats after administration of the aqueous extract of *Codiaeum variegatum* leaves at different doses (0, 50, 100, 200 mg Kg^{-1}) for consecutive 28 days.

Sex	Doses (mg.Kg^{-1})	Organ relative weight (g Kg^{-1} body weight)				
		Liver	Kidneys	Heart	Lungs	Spleen
Male	0	28.70 ± 1.94	6.61 ± 0.52	3.27 ± 0.27	5.89 ± 1.10	4.09 ± 1.35
	50	28.94 ± 0.80	6.54 ± 0.52	3.35 ± 0.35	5.81 ± 1.07	4.19 ± 0.77
	100	29.53 ± 1.21	6.48 ± 0.46	3.45 ± 0.20	5.90 ± 0.60	3.89 ± 0.66
	200	28.85 ± 1.52	6.51 ± 0.43	3.28 ± 0.36	5.95 ± 1.24	3.47 ± 0.73
Female	0	28.68 ± 0.90	6.25 ± 0.48	3.93 ± 0.13	5.87 ± 0.76	4.17 ± 0.91
	50	28.46 ± 1.63	6.34 ± 0.11	3.78 ± 0.22	5.54 ± 0.75	3.79 ± 0.94
	100	28.68 ± 1.31	6.37 ± 0.25	3.37 ± 0.28*	6.15 ± 0.34	4.52 ± 0.99
	200	29.78 ± 1.65	6.52 ± 0.29	3.53 ± 0.15	6.13 ± 1.16	4.90 ± 1.17

Each value represents the mean ± SD between six animals per group. (Dunnett test:* means significant difference compared to the control group, $p < 0.05$).

Table 2. Relative weight of genital organs (gKg^{-1} body weight) of male and female rats after administration of the aqueous extract of *Codiaeum variegatum* leaves at different doses (0, 50, 100, 200 mg Kg^{-1}) for consecutive 28 days.

Sex	Genital organs	Doses (mg Kg^{-1})			
		0	50	100	200
Male	Prostate	1.51 ± 0.50	1.39 ± 0.18	1.72 ± 0.49	1.48 ± 0.60
	Seminal vesicles	3.79 ± 1.29	3.82 ± 1.06	3.63 ± 1.25	4.77 ± 1.30
	Testes	11.23 ± 1.03	12.02 ± 1.79	11.25 ± 1.14	10.78 ± 1.15
	Epididymis	4.27 ± 0.60	4.34 ± 0.91	3.97 ± 0.32	3.79 ± 0.46
Female	Ovaries	0.69 ± 0.20	0.71 ± 0.17	0.86 ± 0.15	0.72 ± 0.12
	Uterus	2.88 ± 1.43	2.99 ± 0.84	2.41 ± 0.31	2.47 ± 0.56

Each value represents the mean ± SD between six animals per group. (Dunnett test:* means significant difference compared to the control group, $p < 0.05$).

Table 3. Biochemical markers of renal (serum creatinine) and hepatic (transaminases, total protein) toxicity of male and female rats after administration of the aqueous extract of *Codiaeum variegatum* leaves at different doses (0, 50, 100, 200 mg Kg^{-1}) for consecutive 28 days.

Sex	Doses (mg Kg^{-1})	Total serum protein (mg mL^{-1})	Total hepatic protein (mg 100 g^{-1})	ALT (UI L^{-1})	AST (UI L^{-1})	Creatinine (mg L^{-1})
Male	0	94.96 ± 4.95	3382 ± 286	56.25 ± 5.77	111.75 ± 7.07	8.35 ± 0.92
	50	91.88 ± 4.10	3393 ± 425	66.35 ± 11.83	120.08 ± 5.64	8.06 ± 0.90
	100	97.44 ± 15.21	3456 ± 221	68.65 ± 12.55	120.92 ± 7.28	8.63 ± 0.47
	200	115.48 ± 12.56	3461 ± 291	70.63 ± 10.29	139.15 ± 11.41	9.15 ± 0.49
Female	0	106.97 ± 10.27	3267 ± 382	42.81 ± 11.31	86.13 ± 9.15	9.23 ± 0.90
	50	99.79 ± 7.41	3276 ± 459	45.52 ± 10.50	83.52 ± 5.72	8.99 ± 1.74
	100	92.82 ± 10.53	3345 ± 170	53.96 ± 14.30	101.23 ± 9.15	10.32 ± 1.24
	200	107.38 ± 11.65	3317 ± 265	52.81 ± 12.79	115.50 ± 12.43	9.31 ± 0.82

Each value represents the mean ± SD between six animals per group. (Dunnett test:* means significant difference compared to the control group, $p < 0.05$). AST: aspartate aminotransferase; ALT: alanine aminotransferase.

of this ratio after drug administration. In fact, after drug administration, changes may be observed in AST and ALT levels in blood, but the AST/ALT ratio should not vary much and should be less than 2, in this case no damage happens to the liver. Therefore, an AST/ALT ratio of 2:1 or greater is suggestive to cell liver injury or other liver diseases.

Discussion

The present study constitutes a part of our ongoing project which intends to valorize the medicinal value of *Codiaeum variegatum* (var. *mollucanum*) in the treatment of intestinal amoebiasis. This infection is a real public health problem in developing countries. The drug of choice (metronidazole) used in the treatment of this disease is less efficient on some strains of the parasite [14] and toxic effects of this drug have been reported [15]. In view of this, medicinal plants have been recognised by the World Health Organization as potential alternatives in the treatment of diseases due to their composition with various primary and secondary metabolites. Therefore, this current investigation, in addition to our previous studies, is realized in order to support the safety profile of the AECVL. In this study, no significant adverse effect was observed in the acute toxicity study after administration of a single dose of the AECVL up to 24 g Kg^{-1} body weight. All animals treated with the AECVL survived beyond the 14 days observation period. The LD_{50} of AECVL was above 24 g Kg^{-1} body weight (b.w.). According to Delongeas et al., 1983 and Kennedy, Ferenz, and Burgess, 1986 [16,17], substances that present LD_{50} higher than 15 g Kg^{-1} by oral route is classified at the toxicity index class 6 and can be considered as relatively inoffensive. Therefore, it can be suggested that AECVL is non-toxic after acute oral administration.

In the sub-chronic toxicity, a relative increase was observed in the body weight, as well as in organ weights of male and female rats after administration of the AECVL for 28 days consecutively (Fig. 2). However, this increase was non-significantly different as compared to the corresponding body and organ weights for the control groups. Also, no significant changes were observed in the levels of the biochemical parameters (transaminases, creatinine, total serum, and liver proteins), speculating that there was no extract-induced toxic effects at the administered dose levels on the major organs involved in various vital functions, specifically, liver and kidney [18]. In fact, transaminases (AST and ALT) are important enzymes in assessing the liver function. ALT is specific to the liver while AST is associated to the liver and heart. All these two enzymes are mainly found in the cytoplasm of animal cells [19,20]. An increase in the level of these enzymes in the serum simply indicates permeability or cell rupture resulting in their secretion into the bloodstream [21]. The slight increase observed in the levels of these enzymes may simply or likely be associated with the drug administration. By the less, no significant difference in transaminase levels were observed across sex regardless of whether the studied rats were treated (test groups) with extracts or not (control groups). The non-significant difference in the levels of these two enzymes in the sub-chronic toxicity study indicates that there is no AECVL-induced toxicity at administered dose levels on the liver function and to some extent on the heart function. Creatinine is a biochemical parameter indicating an effect on the renal function [22,23]. An increase in the level of creatinine in the serum or urine is an indicator of kidney dysfunction or damage on the nephrons of the kidneys [18,24]. In the present study, there was no significant difference in creatinine levels between male and female rats of different groups, therefore, speculating that AECVL at administered dose levels are relatively safe on the renal function of the studied rats. Another important biochemical parameter is the total serum or liver proteins. A change in the quantity of this parameter is a sign of non-specific tissue damage or particularly liver toxicity [22]. Generally, endogenous proteins are implicated not only in the transport of xenobiotics into the bloodstream through the organs, but also their biotransformation in the liver for their activation, detoxification, or excretion [25,26]. No significant change was observed in the levels of total serum and liver protein suggesting that AECVL did not induce any toxic effect in the various organs of the treated rats compared to the organs in rats of the control groups. However, there is a need to perform histopathological analysis for each of the targeted organs.

Conclusions

Based on the aforementioned discussions, it can be concluded that, no observable adverse effect was noticed following the administration of the AECVL up to 24 g.Kg^{-1} b.w. in the acute toxicity study; and up to 200 mg.Kg^{-1} b.w. in the sub-chronic study on mice and rats of both sexes respectively. This speculated that the AECVL is relatively safe and

can be used at therapeutic doses without observable effects in traditional healthcare system for the treatment of amoebic dysentery.

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ORIGINAL RESEARCH

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Antitrypanosomal, antiplasmodial, and antibacterial activities of extracts from selected *Diospyros* and Annonaceae species

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ABSTRACT

Aim: To screen methanol extracts from root bark, leaves, and stem bark of selected plant species from the genus *Diospyros* and some Annonaceae species for antitrypanosomal, antiplasmodial, and antibacterial activities against selected test organisms.

Methods: Antitrypanosomal and antiplasmodial assays of methanol extracts from selected plant species were carried out in single concentration screens and in dose-response for active extracts. The minimum inhibitory concentration (MIC) values of selected plant extracts against selected bacterial strains were determined by microplate dilution method in sterile 96-well microtiter plates.

Results: In the dose-response antitrypanosomal assay, the most potent extracts tested exhibited activities against *Trypanosoma brucei brucei* (Lister 427 strain) with IC_{50} values ranging from 1.28 to 7.85 µg/ml, with methanol extract of *Diospyros verrucosa* stem bark being the most active with IC_{50} value of 1.28 µg/ml. In the dose-response antiplasmodial assay, three extracts exhibited activities against *Plasmodium falciparum* (strain 3D7) with IC_{50} values ranging from 4.55 to 24.22 µg/ml, with methanol extract of *Diospyros capricornuta* root bark being the most potent with IC_{50} value of 4.55 µg/ml. In the antibacterial assay, the investigated extracts exhibited a wide range of activities against *Staphylococcus aureus* [American Type Culture Collection (ATCC) strain 25923], *Bacillus cereus* (ATCC strain 11775), and *Escherichia coli* (ATCC strain 8740) with MIC values ranging from 0.00125 to 0.00625 mg/ml (more active), 0.125 to 0.500 mg/ml (moderately active), and 1.00 to 8.00 mg/ml (less active) while some extracts were inactive at the highest concentration tested of 16.00 mg/ml.

Conclusions: Methanol extracts obtained from root bark, leaves, and stem bark of selected plant species from the genus *Diospyros* and some Annonaceae species that showed good activities in antitrypanosomal, antiplasmodial, and antibacterial assays corroborate reported literature about the traditional medicinal uses of the members of genus *Diospyros* and some Annonaceae species.

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Antitrypanosomal; antiplasmodial; antibacterial

Introduction

Infectious diseases are the leading causes of death worldwide. About 14.9 million annual human deaths worldwide are caused by infectious diseases [1]. According to the World Health Organization (WHO), human African trypanosomiasis (HAT), malaria, and bacterial diseases are among the infectious diseases with the highest epidemics [2].

HAT, commonly known as sleeping sickness is a disease caused by two subspecies of extracellular protozoan parasites, namely *Trypanosoma brucei gambiense* and *T. b. rhodesiense*. The four drugs

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currently available for the treatment of human African sleeping sickness are pentamidine, suramin, melarsoprol, and eflornithine. Nifurtimox, another drug, that was introduced in the market in the 1960s for the treatment of Chagas disease (human American trypanosomiasis), is restricted to treatment of HAT in combination with other trypanocidal drugs for patients who do not respond to late stage medicines [3]. In 2009, nifurtimox–eflornithine combination therapy used for the treatment of late stage HAT, caused by *T. b. gambiense* infections, was included on the WHO essential medicines list. Despite the advancement in HAT treatment, the currently available drugs are unsatisfactory for various reasons including unacceptable toxicity, poor efficacy, undesirable route of administration, and drug resistance [4]. This inspires the need to carry out ethnopharmacological investigations towards identification of possible active plant extracts that may be investigated for the development of new antitrypanosomal pharmaceuticals.

Malaria is a disease caused by infection of red blood cells with protozoan parasites of the genus *Plasmodium* inoculated into the human host by the blood-feeding female *Anopheles* mosquitoes. Treatment of malaria is also affected by drug resistance. If different drugs with different mechanisms of resistance are used together, the emergence and spread of resistance can be limited [5]. As a result, combination therapy is used to reduce the development of drug resistance, and most countries with *P. falciparum* malaria have adopted artemisinin-based combination therapies (ACTs) as first-line medications. ACTs have been estimated to reduce malaria mortality in children aged 1–23 months by 99% and in children aged 24–59 months by 97% [6]. Despite the use of combination therapy, *Plasmodium falciparum* resistance to ACTs has been detected in five countries in the Greater Mekong sub-region. Drug resistance has been documented for all classes of antimalarial chemotherapies and is a major threat to malaria control efforts [5]. Thus, the discovery of antiplasmodial active plant extracts for potential drug development is important to increase the number of alternative medicines available.

The Gram-positive bacterium *Staphylococcus aureus* is the causative agent of skin inflammations, intestinal infections, and pneumonia. The emergence of strains of *S. aureus* resistant to some antibiotics such as methicillin has been documented [7,8]. *Bacillus cereus*, a Gram-positive bacterium that causes two types of gastrointestinal diseases (the diarrheal and the emetic syndromes) together with the Gram-negative bacterium, *Escherichia coli* which result in three clinical syndromes (namely diarrheal disease, urinary tract infections, and meningitis) is also resistant to available chemotherapies [9,10]. Thus, the development of antibacterial active plant extracts that can be used for the discovery of antibacterial drugs is also necessary.

Medicinal plants provide a reliable source of biologically active compounds; and thus, a search for extracts that are active against parasitic protozoans such as trypanosomes and plasmodia as well as pathogenic bacteria could aid the discovery of drugs. The genus *Diospyros* is known for various traditional medicinal uses including for the treatment of HAT, malaria, headache, diarrhea, dysentery, stomach ache, and inflammatory conditions [11–18]. According to interviewed natives during the field excursion, *Diospyros natalensis* is used as a herbal remedy for the treatment of fever and internal body pain [19]. Plant species in the family Annonaceae are also known for their uses as traditional medicines for the treatment of various diseases. *Greenwayodendron suaveolens* is used as a herbal remedy for the treatment of malaria and helminthiasis [20,21]. The genus *Uvariodendron* is used as a traditional medicine for the treatment of skin inflammation and liver disorders [22]. The root of *Uvaria tanzaniae* is used as a herbal remedy for the control of fever [23]. In this work, therefore, we report the *in vitro* antitrypanosomal, antiplasmodial, and antibacterial activities of extracts from selected *Diospyros* and some Annonaceae species.

Materials and Methods

Collection of plant materials

The root bark, leaves, and stem bark of *Diospyros* species selected for the study were collected in Tanzania as follows: *D. bussei* Gurke in June 2014 at Koloha-Kwakihande, Mkange village in Bagamoyo district. GPS location: S 06°03'24.0", E 038°36'21.6”; elevation 196 m. *Diospyros natalensis* (Harv.) Brenan in May 2014 at Manolo Forest Reserve in Lushoto District. GPS location: S 04°39'02.3", E 038°12'36.0". *Diospyros squarrosa* Klotzsch in May 2014 at Madala, Tuliani Village in Handeni District. GPS location: S 05°40'18.7", E 038°05'20.4"; elevation 595 m. *Diospyros verrucosa* Hiern in June 2014 at Gongo Village in Bagamoyo District. GPS location: S 06°09'57.8", E 038°37'33.1"; elevation 302 m. *D. capricornuta* F. White in June 2014 at Pugu forest reserve in Kisarawe District. GPS location: S 06°53'28.4", E 039° 05'56.3"; elevation 269 m.

Diospyros kabuyeana F. White in June 2014 at Pugu Forest Reserve in Kisarawe District. GPS location: S 06°54'26.2", E 039°05'51.1" (Fig. 1). The plant species were identified in the field and confirmed at the herbarium of the Department of Botany, University of Dar es Salaam where voucher specimens FMM 3663, FMM 3661, FMM 3660, FMM 3664, FMM 3667, and FMM 3669 of *D. bussei*, *D. natalensis*, *D. squarrosa*, *D. verrucosa*, *D. capricornuta*, and *D. kabuyeana*, respectively, are preserved.

The root bark, leaves, and stem bark of the species selected for the study from the family Annonaceae were collected in Tanzania as follows: *Greenwayodendron suaveolens* subs. *usambaricum* Verdc in October 2015 at River Mombo Forest, Kisiwani Village in Muheza District. GPS location: 37 M 0461716 Universal Transverse Mercator (UTM) 9434638; elevation 961 m. *Uvaria tanzaniae* Verdc in October 2015 at Fanusi in Kisiwani Village, Muheza District. GPS location: 37 M 0464474 UTM

Figure 1. Representatives of plant species collected (a) Diospyros bussei; (b) Diospyros capricornuta; (c) Diospyros kabuyeana; (d) Diospyros natalensis; (e) Diospyros squarrosa; and (f) Diospyros verrucosa.

9434330. *Uvariodendron usambarense* R.E.Fr. in October 2015 at River Mombo Forest, Kisiwani Village in Muheza District. GPS location: 37 M 0461716 UTM 9434638; elevation 961 m. The selected species were identified in the field and confirmed at the herbarium at the Department of Botany, University of Dar es Salaam where voucher specimens FMM 3708, FMM 3711, and FMM 3707 of *G. suaveolens* subs. *usambaricum*, *U. tanzaniae*, and *U. usambarense*, respectively, are preserved.

Extraction of plant materials

The air-dried and pulverized root bark, leaves, and stem bark of plant species selected for the study (20 g each) were each extracted using methanol at room temperature for 48 hours. Concentration of extracts was done by removal of solvent under reduced pressure using a rotary evaporator to afford the crude extracts for the biological assays. Methanol was used due to its polarity to mimic the use of water in preparation of decoctions of traditional medicines.

In vitro *antitrypanosomal assay*

The *in vitro* antitrypanosomal assay of the methanol extracts of selected plant species was carried out at the Centre for Chemico- and Biomedicinal Research, Rhodes University in South Africa in 2016. The method described by Hirumi and Hirumi [24] was used to culture parasites.

Trypanosoma brucei brucei trypomastigotes (Lister 427 strain) were cultured at 37°C in a 5% CO_2 incubator in Iscove's modified Dulbecco's medium containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 4 mM L-glutamine (Lonza). The medium was further supplemented with 10% fetal calf serum, penicillin/streptomycin sulfate (100 units/ml and 0.1 mg/ml, respectively), 1 mM hypoxanthine, and Hirumi's modified Iscove's medium 9 (1.5 mM cysteine, 1.25 mM pyruvic acid, 0.1 mM cytosine, 0.15 mM thymidine, 0.1 mM uracil, 0.05 mM bathocuproinedisulfonic acid, and 0.2 mM 2-mercaptoethanol).

To assess the antitrypanocidal activity in a single concentration screen, extracts were added to *in vitro* cultures of *T. b. brucei* placed in 96-well plates at a fixed concentration of 25 μg/ml in duplicate followed by incubation at 37°C for 48 hours. Residual parasite viability in the wells was determined by adding 20 μl resazurin solution (0.135 mg/ml in phosphate buffered saline) and incubating for an additional 2–4 hours. Reduction of resazurin to resorufin by viable parasites was assessed by measuring fluorescence (excitation 560 nm, emission 590 nm) in a SpectraMax M3 plate reader. Fluorescence readings were converted to percentage parasite viability relative to the average readings obtained from untreated control wells. Results were expressed as percentage parasite viability against extracts in concentration of 25 μg/ml.

Extracts that reduced parasite viability to <25% (inhibition > 75%) were considered for further testing in a dose-response assay. To determine the antitrypanocidal potency of active extracts, *in vitro* cultures of *T. b. brucei* were added to serial dilutions of extracts in 96-well plates and incubated for 48 hours. The 50% inhibitory concentration (IC_{50}) values were determined by plotting percentage viability *vs.* log [extract] and performing non-linear regression using GraphPad Prism (version 5.02) software. Pentamidine (an existing drug for the treatment of trypanosomiasis) was used as a positive control drug standard and yielded an IC_{50} value of 0.5 nM.

In vitro *antiplasmodial assay*

The *in vitro* antiplasmodial assay of methanol extracts was carried out at the Centre for Chemico- and Biomedicinal Research, Rhodes University in South Africa in 2016. The method described by Makler and Hinrichs [25] was used to determine antiplasmodial activities of methanol extracts in a single concentration screen and in dose-response for active extracts.

A *Plasmodium falciparum* chloroquine-sensitive strain (3D7) was cultured in Roswell Park Memorial Institute medium 1640 containing 25 mM HEPES and 2 mM L-glutamine (Lonza). The medium was further supplemented with 0.5% (w/v) Albumax II (Thermo Fisher Scientific), 22 mM glucose, 0.65 mM hypoxanthine, 0.05 mg/ml gentamicin, and 2%–4% (v/v) human erythrocytes. Cultures were maintained at 37°C under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

To assess antiplasmodial activity in a single concentration screen, extracts were added to parasite cultures (adjusted to 2% parasitaemia, 1% haematocrit) in 96-well plates at a fixed concentration of 25 μg/ml in duplicate followed by incubation at 37°C for 48 hours. Parasite lactate dehydrogenase enzyme activity in the individual wells was subsequently determined by removing 20 μl of the parasite cultures and mixing it with 125 μl colorimetric substrate solution containing 44 mM tris (hydroxymethyl) aminomethane (pH 9), 0.18 M L-lactic acid, 0.13 mM acetylpyridine adenine

dinucleotide, 0.39 mM nitrotetrazolium blue chloride, 0.048 mM phenazine ethosulfate, and 0.16% (v/v) Triton X-100. Color development was monitored by measuring absorbance at 620 nm in a SpectraMax M3 plate reader (Molecular Devices). Absorbance values were converted to percentage parasite viability relative to untreated control cultures after subtracting background absorbance readings obtained from wells containing erythrocytes alone (i.e., without parasites). Wells without extracts and without parasites, thus, acted as positive and negative control sets. Results were expressed as percentage parasite viability against extracts in concentration of 25 μg/ml.

Extracts that reduced parasite viability to <25% (inhibitions > 75%) were considered for further testing in a dose-response assay. To determine the antiplasmodial potency of active extracts, parasite cultures (adjusted to 2% parasitaemia, 1% haematocrit) were added to serial dilutions of extracts in 96-well plates in duplicate followed by incubation at 37°C for 48 hours. As described above, absorbance was measured at 620 nm and percentage parasite viability in extract-treated wells calculated relative to untreated control wells, after subtracting background absorbance readings obtained from non-parasitized control wells. The IC_{50} values were determined by plotting percentage viability *vs*. log [extract] and performing non-linear regression using GraphPad Prism (version 5.02) software. For comparative purposes, chloroquine (an antimalarial drug) was used as standard and produced an IC_{50} value of 2.5 nM.

In vitro *antibacterial assay*

In vitro antibacterial screening of methanol extracts of root bark, leaves, and stem bark from selected plants was carried out at the Department of Pharmacy and Pharmacology in the Faculty of Health Sciences, University of the Witwatersrand in 2016.

Solvents (acetone and dimethyl sulfoxide) were supplied by Merck (Darmstadt, Germany). Ciprofloxacin and *p*-iodonitrotetrazolium (INT) chloride were purchased from Sigma-Aldrich (Missouri, USA). Ninety-six well microtiter plates were supplied by AEC-Amersham (Johannesburg, South Africa). Tryptone Soya broth was obtained from Thermo Fisher Scientific (Waltham, USA). The three pathogens namely *Staphylococcus aureus* (ATCC strain 25923), *Bacillus cereus* (ATCC strain 11775), and *Escherichia coli* (ATCC strain 8740) were supplied by Davies Diagnostics (Johannesburg, South Africa).

Bacterial strains were cultured in Tryptone Soya broth media. Tryptone Soya broth (30 g) suspended in 1 L of distilled water was autoclaved at 121°C in 30 minutes. The mixture was left to cool to room temperature. The media (20 ml) were transferred into each of the sterile culturing test tubes which were then separately inoculated with *S. aureus*, *B. cereus*, and *E. coli*, respectively. Test tubes containing media (Tryptone Soya broth) and inoculum were incubated at 37°C overnight. The bacterial cultures were observed after 24 hours of growth; and thus, ready for antibacterial assays.

The minimum inhibitory concentration (MIC) values of selected plant extracts against the aforementioned bacterial strains were determined by microplate dilution method in sterile 96-well microtiter plates [26]. The initial concentrations of stock solutions of plant extracts and ciprofloxacin (positive control) were prepared to 32.00 and 0.01 mg/ml, respectively. Plant extracts were dissolved using either acetone or 50% dimethyl sulfoxide/water (when samples did not dissolve in acetone) and ciprofloxacin using sterile water.

Each bacterial culture obtained after 24 hours of incubation at 37°C was diluted in two subsequent dilutions. The first dilution was carried out in 1:10 followed by the second dilution in 1:100. The resulting culture after the second dilution was placed in each of serially diluted 96-well microtiter plates (100 μl/well) (containing extracts at various concentrations) for inoculation with respective bacterial strains. Inoculated microtiter plates were then incubated at 37°C for 24 hours.

To determine MIC values of extracts, 40 μl (200 μg/ml) of *p*-INT chloride solution was added into inoculated wells and plates were examined after 4 hours (guided by a column for positive control). The MIC value of each extract was read at the lowest concentration where a marked reduction in color formation (purple/pink) due to bacterial growth inhibition was noted.

Results and Discussion

In vitro *antitrypanosomal activity*

The *in vitro* antitrypanosomal activities of methanol extracts of root bark, leaves, and stem bark of selected plant species were obtained by screening extracts against *Trypanosoma brucei brucei* in a single concentration screen at 25 μg/ml. Results (Table 1) were obtained as percentage inhibition of the test organism. Fifteen of the twenty one extracts inhibited the growth of the parasite by greater

than 75% (Table 1), and were considered for the dose-response assay to determine IC_{50} values by serial dilutions.

In the dose-response assay, results were obtained as percentage viability of the test organism against logarithm of sample concentration (μg/ml) (Fig. 2). The IC_{50} values of tested samples are presented in Table 1. The tested extracts exhibited IC_{50} values ranging from 1.28 to 7.85 μg/ml. Extracts which showed high activities are *Diospyros verrucosa* stem bark (DVSM) methanol extract (IC_{50}: 1.28 μg/ml), *Diospyros capricornuta* root bark (DCRM) methanol extract (IC_{50}: 1.56 μg/ml), and *Uvaria tanzaniae* root bark (UTRM) methanol extract (IC_{50}: 2.12 μg/ml). Others were *Diospyros verrucosa* root bark (DVRM) methanol extract (IC_{50}: 2.23 μg/ml), *Diospyros natalensis* stem bark (DNSM) methanol extract (IC_{50}: 2.85 μg/ml), and *Diospyros verrucosa* leaves (DVLM) methanol extract (IC_{50}: 2.99 μg/ml). Most of these extracts from the genus *Diospyros* showed good activities compared to the literature data for *Diospyros mespiliformis* leaves which exhibited antitrypanosomal activity against *Trypanosoma brucei brucei* at the MIC value of 500 μg/ml [17]. These extracts, together with other samples tested in a dose-response antitrypanosomal assay, could potentially contain active constituents against *T. b. brucei*. Thus, these findings concur with ethnomedicinal uses of some members of the genus *Diospyros* for the treatment of HAT.

In vitro *antiplasmodial activity*

The antiplasmodial activities of methanol extracts of root bark and stem bark of the selected plant species were determined by screening extracts against a chloroquine sensitive strain of *Plasmodium falciparum* (3D7) at a single concentration of 25 μg/ml. Results (Table 2) were obtained as percentage inhibition of the test organism. In this case, only three extracts inhibited parasite growth by more than 75%, and were considered for dose-response assay to determine IC_{50} values by serial dilutions.

In dose-response antiplasmodial assay, results were obtained as percentage viability of the test organism against logarithm of sample concentration (μg/ml) (Fig. 3). The IC_{50} values of tested samples in dose-response are presented in Table 2. The studied extracts exhibited activities with IC_{50} values

Table 1. Antitrypanosomal activities of methanol extracts from root bark, leaves, and stem bark of selected plant species.

Sample	Extract	% Inhibition at 25 μg/ml ± SD	IC_{50} (μg/ml)
Diospyros bussei Gurke leaves (Ebenaceae)	DBLM	70.6 ± 7.3	NT
Diospyros bussei root bark	DBRM	65.7 ± 2.7	NT
Diospyros bussei stem bark	DBSM	66.0 ± 4.0	NT
Diospyros capricornuta F. White leaves (Ebenaceae)	DCLM	73.5 ± 5.3	NT
Diospyros capricornuta root bark	DCRM	81.6 ± 0.3	1.56
Diospyros capricornuta stem bark	DCSM	74.1 ± 7.1	NT
Diospyros kabuyeana F. White leaves (Ebenaceae)	DKLM	81.0 ± 0.5	3.32
Diospyros kabuyeana stem bark	DKSM	79.3 ± 1.7	NT
Diospyros natalensis (Harv.) Brenan leaves (Ebenaceae)	DNLM	82.6 ± 1.5	3.74
Diospyros natalensis root bark	DNRM	80.5 ± 0.3	3.02
Diospyros natalensis stem bark	DNSM	78.3 ± 0.6	2.85
Diospyros squarrosa Klotzsch root bark (Ebenaceae)	DSRM	83.2 ± 1.5	5.38
Diospyros verrucosa Hiern leaves (Ebenaceae)	DVLM	81.1 ± 0.4	2.99
Diospyros verrucosa root bark	DVRM	79.3 ± 0.9	2.23
Diospyros verrucosa stem bark	DVSM	78.3 ± 0.7	1.28
Greenwayodendron suaveolens subs. *usambaricum* Verdc root bark (Annonaceae)	GSRM	79.4 ± 4.8	7.85
Greenwayodendron suaveolens subs. *usambaricum* stem bark	GSSM	77.5 ± 1.4	3.54
Uvaria tanzaniae Verdc root bark (Annonaceae)	UTRM	83.5 ± 0.5	2.12
Uvariodendron usambarense R.E.Fr. Leaves (Annonaceae)	UULM	82.4 ± 0.1	4.71
Uvariodendron usambarense root bark	UURM	83.3 ± 0.1	3.45
Uvariodendron usambarense stem bark	UUSM	83.7 ± 0.1	4.08
Pentamidine (positive control), IC_{50}			0.000509 μM

SD = standard deviation.
Note: codes abbreviations; first letter (generic name), second letter (specific name), third letter (part of plant collected, L = leaves, S = stem bark, and R = root bark), the last letter "M" methanol (solvent used for extraction), NT = not tested.

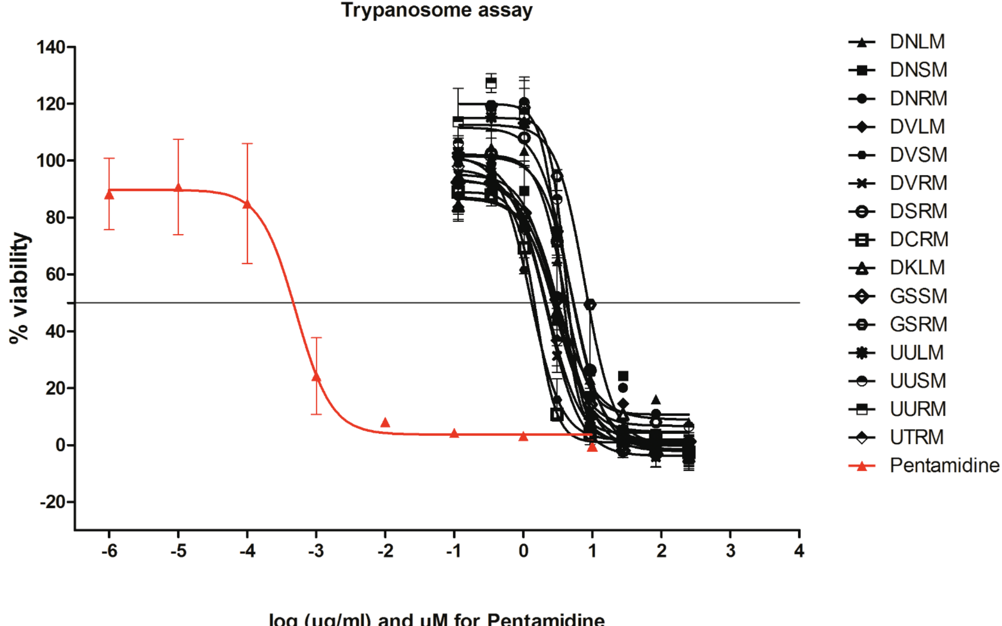

Figure 2. Dose-response antitrypanosomal activities of selected active methanol extracts from root bark, leaves, and stem bark of plant species investigated.

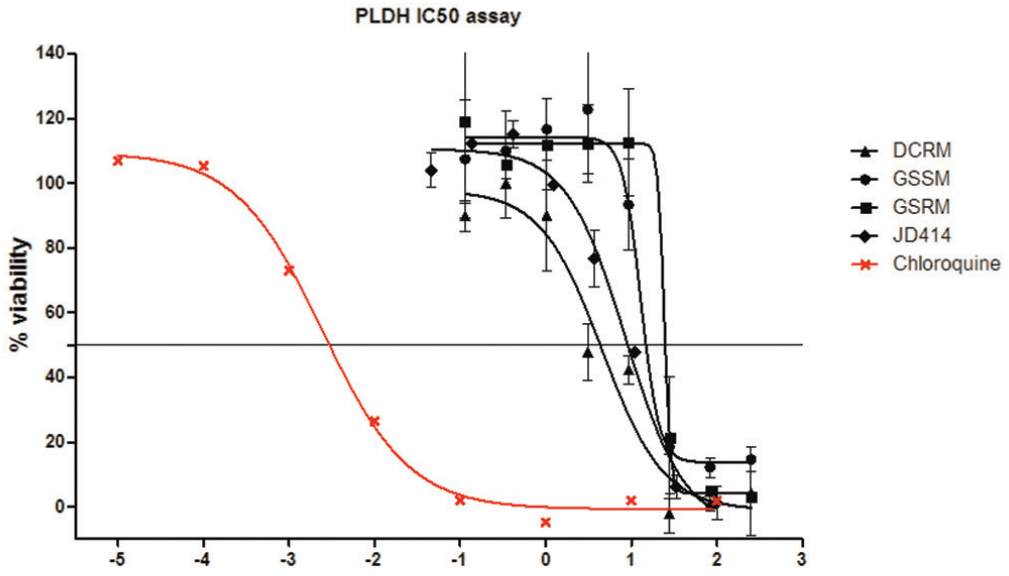

Figure 3. Dose-response antiplasmodial activities of selected active methanol extracts from stem bark and root bark of plant species studied.

Table 2. Antiplasmodial activities of methanol extracts from root bark and stem bark of selected plant species.

Sample	Extract	% Inhibition at 25 µg/ml ± SD	IC_{50} (µg/ml)
Diospyros capricornuta root bark	DCRM	85.6 ± 1.8	4.55
Greenwayodendron suaveolens subs. *usambaricum* root bark	GSRM	100.0 ± 3.2	24.22
Greenwayodendron suaveolens subs. *usambaricum* stem bark	GSSM	83.6 ± 5.7	12.89
Chloroquine (positive control), IC_{50}			0.002454 µM

SD = standard deviation.
Note: codes abbreviations; first letter (generic name), second letter (specific name), third letter (part of plant collected, S = stem bark, and R = root bark), the last letter "M" methanol (solvent used for extraction).

ranging from 4.55– to 24.22 μg/ml. Among the three samples tested in the dose-response assay, DCRM methanol extract exhibited the best activity with an IC_{50} value of 4.55 μg/ml. DCRM methanol extract exhibited good antiplasmodial activity compared to the literature data for *D*. *melanoxylon* which exhibited antiplasmodial activity against *Plasmodium falciparum* at IC_{50} value of 29 μg/ml [27]. Extracts investigated in the dose-response antiplasmodial assay could potentially contain lead compounds which are active against *Plasmodium falciparum*. Thus, the findings reported in this article concur with ethnobotanical uses of some members of the genus *Diospyros* and the family Annonaceae for the treatment of malaria.

In vitro *antibacterial activity*

Results for antibacterial assay were obtained as MIC values of the investigated samples in mg/ml per pathogen. The investigated extracts exhibited activities against the tested organisms with MIC values ranging from 0.00125 to 0.00625 mg/ml (more active), 0.125 to 0.500 mg/ml (moderately active), 1.00 to 8.00 mg/ml (less active), and some were inactive at the highest concentration tested of 16.00 mg/ml (Table 3).

UTRM methanol extract exhibited promising activities against *Staphylococcus aureus* and *Bacillus cereus* with MIC values of 0.00125 and <0.00625 mg/ml, respectively (Table 3). *Greenwayodendron suaveolens* subs. *usambaricum* root bark (GSRM) methanol extract and *Uvariodendron usambarense* stem bark (UUSM) methanol extract both exhibited potent activities against *B*. *cereus* with MIC values of <0.00625 mg/ml (Table 3). DVSM methanol extract and DVRM methanol extract both showed good activities against *Escherichia coli* with MIC values of <0.00625 mg/ml (Table 3). DVSM methanol extract and *Diospyros verrucosa* root bark methanol extract both exhibited good activities against *E*. *coli* compared to the literature data for *Diospyros melanoxylon* methanol bark extract which exhibited antibacterial activity against *E*. *coli* at MIC value of 3.0 mg/ml [28]. For samples which exhibited activities in MIC values of <0.00625 mg/ml, the amounts of the samples available during antibacterial assay were not enough to reach the end point.

Diospyros bussei leaves (DBLM) methanol extract, *D*. *bussei* stem bark (DBSM) methanol extract,

Table 3. Antibacterial activities of methanol extracts from root bark and stem bark of selected plant species.

Sample	Extract	MIC in mg/ml per pathogen (test organism)		
		Staphylococcus aureus **(ATCC 25923)**	***Bacillus cereus*** **(ATCC 11775)**	***Escherichia coli*** **(ATCC 8740)**
Diospyros bussei leaves	DBLM	8.00	2.00	0.125
Diospyros bussei root bark	DBRM	NA	NA	0.500
Diospyros bussei stem bark	DBSM	NA	2.00	0.125
Diospyros capricornuta leaves	DCLM	0.250	2.00	1.00
Diospyros capricornuta root bark	DCRM	4.00	0.125	1.00
Diospyros capricornuta stem bark	DCSM	2.00	4.00	1.00
Diospyros kabuyeana leaves	DKLM	8.00	4.00	0.125
Diospyros kabuyeana stem bark	DKSM	NA	1.00	0.125
Diospyros natalensis leaves	DNLM	0.250	1.00	0.500
Diospyros natalensis root bark	DNRM	NA	NA	1.00
Diospyros natalensis stem bark	DNSM	NA	NA	0.250
Diospyros squarrosa leaves	DSLM	NA	4.00	0.250
Diospyros squarrosa root bark	DSRM	1.00	4.00	NA
Diospyros squarrosa stem bark	DSSM	NA	NA	0.500
Diospyros verrucosa leaves	DVLM	1.00	2.00	0.500
Diospyros verrucosa root bark	DVRM	NA	0.500	<0.00625
D. verrucosa stem bark	DVSM	NA	0.500	<0.00625
Greenwayodendron suaveolens subs. *usambaricum* root bark	GSRM	1.00	<0.00625	NA
Uvaria tanzaniae root bark	UTRM	0.00125	<0.00625	NA
Uvariodendron usambarense leaves	UULM	8.00	0.500	0.500
Uvariodendron usambarense stem bark	UUSM	4.00	<0.00625	NA
Ciprofloxacin (positive control)	—	0.0025	0.00008	0.00063
50% Acetone/H_2O (negative control)	—	NA	NA	NA
50% DMSO/H_2O (negative control)	—	NA	NA	NA

Note: codes abbreviations; first letter (generic name), second letter (specific name), third letter (part of plant collected, L = leaves, S = stem bark, and R = root bark), the last letter "M" methanol (solvents used for extraction); NA = no activity.

Diospyros kabuyeana leaves (DKLM) methanol extract, and *D. kabuyeana* stem bark (DKSM) methanol extract exhibited reasonable activities against *Escherichia coli* with MIC values of 0.125 mg/ml. DCRM methanol extract and *Diospyros natalensis* leaves (DNLM) methanol extract showed moderate activities against *Bacillus cereus* and *Staphylococcus aureus* with MIC values of 0.125 and 0.250 mg/ml, respectively. DNSM methanol extract and *Diospyros squarrosa* leaves (DSLM) methanol extract both exhibited modest activities against *Escherichia coli* with MIC values of 0.250 mg/ml.

DVRM methanol extract, DVSM methanol extract, and *Uvariodendron usambarense* leaves (UULM) methanol extract exhibited modest activities against *Bacillus cereus* with MIC values of 0.500 mg/ml. *Diospyros bussei* root bark (DBRM) methanol extract, DNLM methanol extract, *Diospyros squarrosa* stem bark (DSSM) methanol extract, DVLM methanol extract, and UULM methanol extract showed moderate activities against *Escherichia coli* with MIC values of 0.500 mg/ml. Extracts which showed good antibacterial activities could potentially contain constituents which are active against respective bacterial strains. Thus, these results concur with ethnobotanical uses of some members of the genus *Diospyros* and the family Annonaceae for the treatment of bacterial diseases.

Conclusions

Methanol extracts investigated in *in vitro* antitrypanosomal, antiplasmodial, and antibacterial assays that showed good activities corroborate reported literature about the traditional medicinal uses of the genus *Diospyros* (Ebenaceae) and some Annonaceae species from which plant species investigated were selected for the study. Thus, the results provide a rational support for the use of the selected plant species in traditional medicine. The findings warrant further phytochemical investigations for potential lead compounds from plant extracts that showed good antitrypanosomal, antiplasmodial, and antibacterial results. The interesting plants for future antitrypanosomal investigations are *Diospyros capricornuta*, *D. kabuyeana*, *D. natalensis*, *D. squarrosa*, *D. verrucosa*, *Greenwayodendron suaveolens* subs. *usambaricum*, *Uvaria tanzaniae*, and *Uvariodendron usambarense*. The plants potential for future antiplasmodial investigations are *D. capricornuta* and *G. suaveolens* subs. *usambaricum*.

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ORIGINAL RESEARCH

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Therapeutic potential of *Achillea fragrantissima* extracts in amelioration of high-fat diet and low dose streptozotocin diabetic rats

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ABSTRACT

Aim: *Achillea fragrantissima* is a perennial herb used by Arabic Bedouin for the treatment of several pathologies. Type 2 diabetes mellitus (T2DM) is a metabolic disorder that is usually associated with hyperlipidemia into what is nowadays known as the metabolic syndrome epidemic. This study aim is to evaluate the effects of alcoholic and ethyl acetate extracts of *A. fragrantissima* in metabolically manipulated T2DM rats.

Materials and Methods: Induction of T2DM in rats was through high-fat diet for 12 consecutive weeks followed by a single injection of streptozotocin. The activities of both *A. fragrantissima* extracts were assessed in comparison with glibenclamide and fenofibrate as reference drugs.

Results: Elevated blood glucose levels, serum lipid profile, liver functions, and kidney functions witnessed a significant reduction by treatment with the extracts. Meanwhile, Oxidative stress markers (malondialdehyde, glutathione peroxidas, and superoxide dismutase), pro-inflammatory biomarkers (tumor necrosis factor-alpha and interleukin-6), and adhesion molecules (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) assessments recorded significant improvement after treatment with *A. fragrantissima* extracts. The results were also associated with the improvement in histopathological examination of pancreas, liver, and kidney tissues.

Conclusion: The current study suggests that *A. fragrantissima* extracts might be considered as a promising natural supplements for management of T2DM manifestations.

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Achillea fragrantissima; adhesion molecules; antioxidant, pro-inflammatory cytokines; T2DM; chemical composition

Introduction

Type 2 diabetes mellitus (T2DM) is usually associated with obesity leading to what is recently has been known as metabolic syndrome which is the clustering of interconnected biochemical, physiological, clinical, and metabolic factors manifested through hyperglycemia, central obesity, hypercholesterolemia, and elevated triglycerides (TG) due to processed food consumption [1]. It is estimated that 415 million (8.8%) of adults aged 20–79 years old are type 2 diabetic patients, while it is predicted that by 2040 about 642 million or 10% of adults aged 20–79 years old will have diabetes [2].

Achillea fragrantissima (Forssk.) Sch. Bip. is a member of the Asteraceae or Compositae family, Spermatophyta superdivision, Angiospermae division, Dicotyledoneae class. *Achillea fragrantissima* has many synonyms and common names in different languages such as lavender cotton in English and Qaysūm in Arabic, and it is a perennial herb with yellow petals flower. *Achillea* species has been known and used by the Arabic Bedouinas folk for

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treatment of respiratory diseases, skin diseases, gastro-intestinal disturbances, high blood pressure, stomach aches, hepatobiliary complaints, and as analgesic [3–6]. Moreover, *A. fragrantissima* extract demonstrated *in-vitro* α-glucosidase inhibitory effect [7].

Induction of T2DM through introduction of high fat and low dose of streptozotocin (STZ) is based on simulating the natural pathology stages of diabetes by directing the model through two main key stages which include induction of insulin resistance state and insulin insufficiency state. Insulin resistance state is induced by subjecting the rats to high-fat (high energy) diet based on the theory of characterization of obesity as a state of chronic low-grade inflammation [8] that can lead to propagation of insulin resistance state through body cells, which in turn leads to T2DM [9]. The second stage which is insulin insufficiency, that is developed by incompetency of β-cell in performing their function by manipulating with low dose of STZ after being exhausted by the high-fat diet (HFD) period [10].

Association of T2DM and hyperlipidemia is usually treated by prescribing both anti-diabetic drug and hypolipidemic medications, concurrently. Glibenclamide is a potent hypoglycemic sulfonylurea derivative for management of T2DM, while fenofibrate is a fibric acid derivative which is commonly prescribed as a lipid-lowering agent [11].

The main target of conducting this study was to assess the activity of alcoholic and ethyl acetate extracts of *A. fragrantissima* in the treatment of T2DM, hyperlipidemia, and other metabolic repercussions, which could shed light on developing a potential natural alternative to minimize the side effects and drug-drug interactions that evolve when multiple medications has been consumed simultaneously.

Materials and Methods

Chemicals

Analytical grade solvents were used for extraction and chromatographic separation purchased from Sigma-Aldrich (St Louis, MO), Merck (Darmstadt, Germany), BDH (Poole, UK), Riedel de Haën (Seelze, Germany), and Fluka (Buchs, Switzerland). STZ (PubChem CID: 29327) and cholesterol (PubChem CID: 5997) powders were purchased from Sigma-Aldrich Co. (St. Louis, MO). Glibenclamide was purchased from Sanofi-Aventis, Egypt. Fenofibrate was purchased from Minapharm under license of Fournier, Egypt, where crushed tablets were suspended in distilled water.

Plant material

The aerial parts of *Achillea fragrantissima* Sch. Bip. (family: Asteraceae) were collected from Sinai desert, Wadi Al Gady, Egypt during the flowering stage. Authentication of the plant was performed, where the voucher specimen was deposited in the herbarium of National Research Centre (NRC), Cairo (Egypt).

Preparation of extracts

Aerial parts of *A. fragrantissima* approximately (3.2 kg) were air-dried for a week until constant weight, and subsequently, powdered and exhaustively extracted using ethanol (80%) by using continuous extraction apparatus. The total ethanol extract (Alc. Ext) was evaporated under vacuum at 40°C to yield brown oily viscous residue, which was divided into two portions: the first portion was diluted with distilled water to a concentration of 100 mg/ml, while the second portion of the residue was partitioned with ethyl acetate to yield the ethyl acetate extract (Eth. Ac. Ext.), which was evaporated under reduced pressure to yield a residue and was diluted with distilled water into a concentration of 100 mg/ml extract.

Phytochemical investigation

Quantitative estimation of total polyphenolic and flavonoid contents

Total polyphenolic compounds were quantified by Folin–Ciocalteau method using gallic acid as standard [12,13]. While, total flavonoids content was estimated using rutin as reference [14].

Chromatographic techniques

Aliquot of 5 µl of Alc. Ext and Eth. Ac. Ext was applied, separately, to two thin-layer chromatography (TLC) with the available authentic samples of phenolic compounds. TLC was performed on silica gel GF_{254} pre-coated plates (Merck, Darmstadt, Germany). The chromatograms were visualized under UV light at 254 and 366 nm before and after exposure to ammonia vapor, as well as spraying with $AlCl_3$ or *p*-anisaldehyde/sulfuric acid reagent using the solvent systems: S_1, benzene/ethyl acetate (9:2, v/v) and S_2, hexane/ethyl acetate (1.5:8.5, v/v). Paper chromatography (descending) Whatman no. 1 column chromatography (CC) papers, using solvent systems (v/v): S_3, acetic acid/H_2O (1.5:8.5, v/v) and S4, *n*-butanol/acetic acid/H_2O (4:1:5, upper layer) were applied. Spray reagents used were: R1,

sulphuric acid/methanol (30%), followed by heating at 105°C for 1–2 min for terpenes and R2, 1 g powder of $AlCl_3$ in 100 ml of ethanol for flavonoids. CC was carried out using silica gel (Si) 60 (E. Merck) and Sephadex LH-20 (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

Extraction and isolation

In order to identify the active components in the effective fraction, part of the ethylacetate extract (14 g) was preliminary subjected to a polyamide column (150 g, 120 × 5 cm) using a step gradient of H_2O/MeOH (100:0–0:100 v/v) for elution to give 44 fractions. The eluted fractions were grouped into four main fractions (Fc I–Fc IV) based on paper chromatography (PC) examination. They were collected, monitored by TLC (solvent systems S1 and S2) and Comp-PC (solvent systems S3 and S4) using Whatman no. 1 paper sheets. Fraction I (Fc. I, 150 mg) was subjected to repeated column chromatography on microcrystalline cellulose using *n*-BuOH/*iso*-propanol/H2O [butanol:isopropanol: water ratio (BIW), 4:1:5 v/v/v, top layer] as an eluent, followed by repeated cellulose column chromatography for each major subfraction using MeOH/BIW (50%) to give pure samples of 1 (18 mg) and 2 (20 mg). Fraction II (300 mg) was chromatographed on a Sephadex column chromatography (10% aqueous MeOH as an eluent) to give pure sample of 3 (31 mg). Fc III (150 mg) was separated on a Sephadex column eluted with MeOH to give pure sample of 4 (9 mg). Fc IV (0.8 g) was subjected to column chromatography (2 × 9 × 40 cm, 150 g) using Si 60 and elution with benzene/ethyl acetate (9:1) provided compound 5 (15 mg) after crystallization from chloroform. Subfraction Fc IV-2 was applied on Si CC (100 × 2 cm, 30 g) and elution was made with toluene/ethyl acetate (7:3) and the yellow precipitate obtained was subjected to Sephadex LH-20 (ethanol) affording compound 6 (28 mg) and 7.

Animals

This study was performed on 70 adult male albino of the Wistar strain rats weighing 160 ± 10 g, supplied from the animal house of the NRC (Dokki, Giza, Egypt). The study was approved by the Medical Research Ethics Committee of the NRC approval number (12-017) following the Guide for the Care and Use of Laboratory Animals_(Eighth Edition, 2011, published by The National Academies Press, 2101 Constitution Ave. NW, Washington, DC 20055) [15]. Animals were kept for 2 weeks to accommodate to laboratory conditions and were allowed *ad libitum* to chow and water.

Induction of T2DM and experimental design

Induction of type 2 diabetes was performed through feeding on high-fat chow, which was produced by addition of (lard fat to standard normal chow to raise the ratio of total fat to 20%) [16] as well as oral administration of cholesterol at a dose of 30 mg/0.3 ml olive oil/kg of animal body weight (BW) five times a week for the whole experiment period [17]. Table 1 shows constituents of the normal chow and HFD chow.

After 12 weeks of dietary manipulation, rats were subjected to a single intra-peritoneal STZ injection (40 mg/kg BW, dissolved in 0.01 M citrate buffer, pH 4.5) [16], after which rats were given 5% glucose solution 2 hours following STZ injection to avoid hypoglycaemic shock.

Animals were divided into seven groups (10 rats each). Group I served as normal control and the animals were fed with standard normal chow and received a single intra-peritoneal of 0.01 M citrate buffer (pH 4.5). Groups II (Normal/Alc. Ext) and III (Normal/Eth. Ac Ext), separately, received orally normal chow and received a single intra-peritoneal of 0.01 M citrate buffer, pH 4.5 and given daily oral dose of Alc. Ext and Eth. Ac Ext of *A. fragrantissima* [500 mg/kg animal weight (AW)], respectively daily for three consecutive weeks [18]. Group IV (HFD-STZ control group) received high-fat chow and received a single intra-peritoneal STZ injection (40 mg/kg BW, dissolved in 0.01 M citrate buffer, pH 4.5) and did not receive any treatment. Groups V (HFD-STZ/Alc. Ext) and VI (HFD-STZ/Eth. Ac. Ext) were the diabetic rats that treated with daily oral dose (500 mg/kg AW) of Alc. Ext and Eth. Ac. Ext, respectively for three consecutive weeks [18]. Group VII (HFD-STZ/reference drugs) received a daily oral dose of glibenclamide (10 mg/kg BW) [18]. along with fenofibrate (50 mg/kg BW) [19].

Table 1. The constituents of the normal chow and HFD chow.

Constituents	Regular chow (%)	High-fat chow (%)
Carbohydrates	72.2	62.4
Fats	3.4	20.2
Proteins	19.8	13.2
Cellulose	3.6	3.2
Vitamins and minerals	0.5	0.5
Salts	0.5	0.5

Sample preparation

Three weeks of the respective diet, the animals were fasted overnight, each rat was anaesthetized with diethyl ether and its blood samples were collected. The serum was prepared by centrifugation and used for biochemical investigation. The rats were then sacrificed by cervical dislocation, the livers rapidly removed, washed in saline, dried on filter paper, weighed, and homogenized in 50 mM phosphate buffer, pH 7.4 using an Ultra-Turrax homogenizer where resultant homogenate (20% w/v) then centrifuged at 3,000 rpm for 15 minutes at 4°C. The resulted supernatant was stored at −80°C for further investigation.

Biochemical investigations

Estimation of BW, blood glucose levels, and liver glycogen content

A weekly body weighting using digital scale, as well as, determination of serum fasting blood glucose levels using enzymatic colorimetric Biodiagnostic kits, Catalog no. GL1320 [20] using rats' tail vein by a portable glucometer (Accu-Chek Active, Roche Diagnostics Ltd., Mannheim, Germany) periodically at day 0, 3, 7, 14, and 21 of diabetes induction were performed. Liver glycogen content was estimated according to the method of Carroll et al. [21] where one gram of liver tissue was boiled in 5 ml 30% potassium hydroxide for 15 minutes, then addition of 5 ml of absolute ethyl alcohol and centrifuged at 3,000 rpm for 10 minutes, discarded the supernatant and dissolved the precipitate in 5 ml distilled water, then, developing color using anthrone reagent (0.05% anthrone, 1% thiourea, and 72% per volume H_2S0_4) measuring absorbance at 610 nm.

Estimation of lipid profiles parameters

Lipid profile assessments were conducted through estimation of serum total cholesterol (TC), low-density lipoprotein (LDL-C), high-density lipoprotein (HDL-C), TG, total lipids (TL), and phospholipids (PL) according to the method of Richmond [22], Wieland and Seidel [23], Burstein et al. [24] and Lopez-Virella et al. [25], Fossati and Prencipe [26], Zöllner and Kirsch [27], Zilversmit et al. [28] and Connerty et al. [29], respectively using kits supplied from Biodiagnostics (Egypt).

Estimation of hepatic functions markers

Investigation of serum aspartate and alanine aminotransferase (AST and ALT), alkaline phosphatase (ALP), and total bilirubin were proceeded according to the method of Reitman and Frankel [30], Bowers et al. [31], and Walters and Gerarde [32], respectively using kits provided from Biodiagnostics (Egypt). Whereas, γ-Glutamyltransferase (GGT) was estimated kinetically according to the method of Persijn and van der Slik [33,34] using kits provided from the Egyptian Company of Biotechnology, Spectrum, Egypt.

Estimation of kidney functions markers

Serum urea was determined colorimetrically according to the method of Fawcett and Scott [35] using kits purchased from Biodiagnostics (Egypt). While, serum creatinine was determined by kinetic analysis according to the method of Bartels et al. [36] using kits purchased from Biodiagnostics, Egypt.

Estimation of oxidative stress markers

The oxidative stress markers hepatic malondialdehyde (MDA), hepatic superoxide dismutase (SOD), and hepatic glutathione peroxidase (GPx) were estimated according to the method of Satoh [37], Nishikimi et al. [38], Paglia and Valentine [39], respectively using kits purchased from Biodiagnostics (Egypt).

Estimation of pro-inflammatory markers

Serum tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels were estimated by an enzyme-linked immunoassay (ELISA) kit purchased from Quantikine®, R & D systems (MN, USA).

Estimation of serum adhesion molecules

Serum soluble intercellular adhesion molecule-1 (sICAM-1) and vascular cell adhesion molecule-1 (sVCAM-1) levels were measured by ELISA kit purchased from Quantikine®, R and D systems (MN, USA) and EIAab® (USA), respectively.

Histopathological investigation

Liver and kidney samples were preserved in 10% formalin, while pancreas samples were preserved in Bouin's solution. Samples were molded into paraffin bees wax tissue blocks and cut into 4-μm thickness sections and stained by hematoxylin and eosin stain for routine examination through the light electric microscope, as well as, Masson'strichrome stain for detection of fibrosis and collagen [40].

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Results were analyzed statistically by one-way analysis of variance (ANOVA) followed by post-hoc

Tukey's procedure for comparison of therapeutic group's means. Differences were considered significant at $P < 0.05$ using SPSS (SPSS for windows 7, version 20, Chicago, IL) software.

Results

Phytochemical results

Quantitative estimation of total polyphenolic and flavonoid contents

Total polyphenolics content (expressed as milligram of gallic acid equivalent per milligram of plant extract) was higher for Eth. Ac. Ext (25.73 ± 0.39) compared to Alc. Ext (17.52 ± 0.19). Similar observations were made with total flavonoids content (mg/g extract), in rutin equivalents and found to be 1.09 ± 0.02 for Eth. Ac. Ext and 0.79 ± 0.01 for the Alc. Ext.

Phytochemical identification of the ethyl acetate extract fractions

Seven phenolic compounds were isolated from the Eth. Ac. Ext of *A. fragrantissima*. Three were the flavonoid glycosides apigenin-6-*C*-glucoside, quercetin-3-*O*-galactoside, and apigenin-7-glucoside [41]. Four flavonoid aglycones, luteolin, jaceidin, and scutellarein 6,7-dimethyl ether [41], along with a triterpene, taraxerol acetate, were identified. The compounds were identified on the basis of spectral data (UV, ^{1}H-, and ^{13}C-NMR) and the analytical data were in agreement with those reported in the literature.

Biochemical results

Weight monitoring assessment

After 12 weeks of dietary manipulation (HFD and oral cholesterol), rats showed a significant BW increase when compared to normally fed control group with an increase in weight by 85% of their initial weight, while rats feeding on standard chow only increased by 14.32% of their initial weight. Meanwhile, introduction of Alc. Ext and Eth. Ac. Ext of *A. fragrantissima* along with HFD caused significant reduction when compared with diabetic control feeding on the same high-fat chow mix as shown in Table 2.

Fasting blood glucose (FBG) and liver glucogen content assessment

Table 3 represents FBG levels, where HFD-STZ diabetic rats demonstrated a significant increase in FBG reaching 549.74% when compared to normal control group. Treatment with Alc. Ext and Eth. Ac. Ext of *A. fragrantissima* and combination of glibenclamide and fenofibrate for 1 week caused significant decrease by 36.62%, 30.24%, and 32.66%, respectively when compared to untreated diabetic rats. While, treatment for 2 weeks by Alc. Ext and Eth. Ac. Ext of *A. fragrantissima* as well as combination of glibenclamide and fenofibrate exhibited significant decrease in blood glucose level by percentage of improvement 75.06%, 40.59%, and 31.62%, respectively when compared to untreated diabetic group. Wheras, after three weeks of treatment by Alc. Ext and Eth. Ac. Ext, as well as the combination of (glibenclamide and fenofibrate) caused significant reductions in fasting blood glucose level by 78.02%, 68.84%, and 70.69%, respectively when compared to untreated diabetic rats. Furthermore, treatment by Alc. Ext and Eth. Ac. Ext caused FBG levels to reach normal value range.

Regarding liver glycogen content, HFD-STZ diabetic rats exhibited a significant decrease in liver glycogen content reaching 51% when compared with normal control. Treatment with Alc. Ext and Eth. Ac. Ext caused significant increase in liver

Table 2. Effect of alcoholic and ethyl acetate extracts of *A. fragrantissima* on BW of HFD-STZ diabetic rats and different therapeutic groups.

Groups	Initial wt.	HFD 12 weeks	Week of treatment		
			The first	The second	The third
Normal control	158.7 ± 3.302	183.25 ± 10.59^{b}	189.38 ± 4.44^{b}	188.12 ± 7.84^{b}	188.13 ± 7.85^{b}
Normal/Alc. Ext	161.30 ± 3.093	183.00 ± 18.85^{b}	181.00 ± 11.30^{b}	186.13 ± 25.73^{b}	187.13 ± 25.73^{b}
Normal/Eth. Ac. Ext	160.90 ± 3.247	183.00 ± 7.01^{b}	187.17 ± 5.60^{b}	185.00 ± 11.34^{b}	182.0 ± 11.34^{b}
HFD-STZ control	159.60 ± 1.265	292.13 ± 38.57^{a}	290.13 ± 38.57^{a}	291.13 ± 38.56^{a}	296.13 ± 38.57^{a}
HFD-STZ/Alc. Ext	160.40 ± 2.951	299.67 ± 7.79^{a}	190.00 ± 40.2^{b}	184.34 ± 38.7^{b}	180.34 ± 38.7^{b}
HFD-STZ/Eth. Ac. Ext	159.45 ± 2.252	290.71 ± 30.77^{a}	226.57 ± 20.88$^{a/b}$	196.861 ± 26.1^{b}	196.87 ± 26.07^{b}
HFD-STZ/Reference drugs	159.78 ± 3.153	293.43 ± 17.57^{a}	202.43 ± 4.58^{b}	197.43 ± 5.01^{b}	195.43 ± 5.01^{b}

Values are expressed as mean ± SD, (n = 10). P-Value < 0.05, Groups superscripted by (a) symbol are significantly different from normal control group, and those superscripted by (b) are significantly different from HFD-STZ control group.

Table 3. Effect of alcoholic and ethyl acetate extracts of *A. Fragrantissima* on fasting blood glucose of HFD-STZ diabetic rats and different therapeutic groups.

Groups	72 hours after STZ injection	Week of treatment		
		The first	The second	The third
Normal control	68.29 ± 2.55[b]	62.29 ± 3.22[b]	67.43 ± 1.94[b]	76.29 ± 3.51[b]
Normal/Alc.Ext	67.15 ± 4.08[b]	69.15 ± 4.08[b]	67.14 ± 5.81[b]	84.57 ± 6.61[b]
Normal/Eth.Ac Ext	59.86 ± 2.62[b]	65.86 ± 4.27[b]	63.86 ± 4.59[b]	68.86 ± 2.84[b]
HFD-STZ control	443.71 ± 18.65[a]	396.7 ± 18.35[a]	410.71 ± 15.18[a]	395.73 ± 16.34[a]
HFD-STZ/Alc. Ext	432.0 ± 25.22[a]	251.43 ± 16.4[a/b]	102.43 ± 14.11[b]	87.00 ± 4.46[b]
HFD-STZ/Eth. Ac. Ext	465.29 ± 7.63[a]	276.72 ± 21.9[a/b]	244.0 ± 17.05[a/b]	123.29 ± 17.67[a/b]
HFD-STZ/Reference drugs	444.57 ± 18.58[a]	267.15 ± 30.18[a/b]	280.86 ± 7.17[a/b]	116.0 ± 5.33[b]

Values are expressed as mean ± SD, (*n* = 10). *P*-Value < 0.05 ,Groups superscripted by (a) symbol are significantly different from normal control group, and those superscripted by (b) are significantly different from HFD-STZ control group.

Table 4. Effect of alcoholic and ethyl acetate extracts of *A. fragrantissima* on liver glycogen content of HFD-STZ diabetic rats and different therapeutic groups.

Groups	Glycogen (mg/g tissue)
Normal control	8.76 ± 0.45[b]
Normal/Alc. Ext	7.54 ± 1.35[b]
Normal/Eth. Ac. Ext	7.95 ± 1.06[b]
HFD-STZ control	4.27 ± 0.14[a]
HFD-STZ/Alc. Ext	6.18 ± 0.37[a/b]
HFD-STZ/Eth. Ac. Ext	6.19 ± 0.13[a/b]
HFD-STZ/Reference drugs	5.35 ± 1.22[a]

Values are expressed as mean ± SD, (*n* = 10). *P*-Value < 0.05,Groups superscripted by (a) symbol are significantly different from normal control group, and those superscripted by (b) are significantly different from HFD-STZ control group.

glycogen content with 44.76% and 44.96%, respectively when compared with untreated diabetic group. Meanwhile, combination of glibenclamide and fenofibrate caused insignificant increase in liver glycogen content as illustrated in Table 4.

Lipid profile assessment

Serum lipids profile investigation as shown in Table 5 revealed that HFD-STZ diabetic group exhibited significant elevation in serum TC, LDL-C, TG, TL, PL reaching 169.22%, 761.09%, 239.00%, 177.35%, and 157.53%, respectively and significant reduction by a value of 49.97% in serum HDL-C when compared to the normal control group. Treatment of HFD-STZ diabetic rats with Alc. Ext and Eth. Ac. Ext caused significant decrease in values of serum TC, LDL-C, TG, TL, and PL by 50.13%, 63.78%, 63.37%, 50.32%, and 50.00%, respectively for Alc. Ext; 47.10%, 63.61%, 64.67%, 52.03%, and 46.81%, respectively for Eth. Ac. Ext and when compared to untreated diabetic group. Concerning HDL, treatment by Eth. Ac Ext caused significant increase by the value 42.43%; while treatment with alcoholic extract of *A. fragrantissima* and reference drug combination (glibenclamide and fenofibrate) caused insignificant increase in serum HDL when compared to untreated diabetic group.

Liver functions assessment

Regarding liver functions assessment, as shown in Table 6, it is shown that HFD-STZ induced diabetic rats exhibited significant elevation in hepatic functions parameters ALT, AST, ALP, GGT, and total bilirubin reaching to 171.48%, 76.69%, 145.22%, 435.75%, and 65.62%, respectively when compared to the normal control levels. Treatment with Alc. Ext and Eth. Ac. Ext deduced significant reduction in ALT,

Table 5. Effect of alcoholic and ethyl acetate extracts of *A. fragrantissima*on lipid profile parameters of HFD-STZ diabetic rats and different therapeutic groups.

Groups	TC (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)	TG (mg/dl)	TL (mg/dl)	PL (mg/dl)
Normal control	88.10 ± 5.01[b]	12.31 ± 1.89[b]	75.38 ± 6.3[b]	31.38 ± 6.79[b]	209.13 ± 18[b]	91.25 ± 5.72[b]
Normal/Alc. Ext	87.13 ± 1.33[b]	17.90 ± 1.18[b]	64.75 ± 6.15[b]	24.67 ± 1.79[b]	195.03 ± 12.3[b]	86.67 ± 4.08[b]
Normal/Eth. Ac. Ext	85.50 ± 1.52[b]	18.96 ± 2.49[b]	62.59 ± 3.20[b]	24.67 ± 1.79[b]	195.03 ± 12.3[b]	85.07 ± 3.08[b]
HFD-STZ control	237.19 ± 4.76[a]	106. 0 ± 9.86[a]	37.72 ± 2.55[a]	106.38 ± 6.36[a]	580 ± 49.14[a]	235.0 ± 11.95[a]
HFD-STZ/Alc. Ext	118.28 ± 11.8[a/b]	62.06 ± 5.73[a/b]	43.40 ± 2.0[a]	38.97 ± 8.69[b]	288.13 ± 24[a/b]	117.5 ± 3.89[a/b]
HFD-STZ/Eth. Ac. Ext	125.47 ± 14.7[a/b]	62.35 ± 3.99[a/b]	53.52 ± 2.53[a/b]	37.59 ± 6.29[b]	278.3 ± 14.3[a/b]	125.00 ± 4.56[a/b]
HFD-STZ/Reference drugs	94.69 ± 11.97[b]	39.92 ± 4.02[a/b]	46.05 ± 2.32[a]	60.90 ± 6.93[a/b]	265.63 ± 22.1[a/b]	100.0 ± 5.98[b]

Values are expressed as mean ± SD, (*n* = 10). *P*-Value < 0.05,Groups superscripted by (a) symbol are significantly different from normal control group, and those superscripted by (b) are significantly different from HFD-STZ control group.

Table 6. Effect of alcoholic and ethyl acetate extracts of *A. fragrantissima* on liver functions of HFD-STZ diabetic rats and different therapeutic groups.

	AST (U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)	Total bilirubin (mg/dl)
Normal control	35.10 ± 1.99[b]	34.71 ± 3.65[b]	32.81 ± 2.62[b]	8.97 ± 1.21[b]	0.63 ± 0.07[b]
Normal/Alc. Ext	42.40 ± 4.02[b]	41.54 ± 2.85[b]	32.11 ± 6.0[b]	12.84 ± 2.3[b]	0.63 ± 0.069[b]
Normal/Eth. Ac. Ext	41.28 ± 9.1[b]	37.64 ± 1.39[b]	33.91 ± 2.21[b]	12.1 ± 2.12[b]	0.65 ± 0.03[b]
HFD-STZ control	95.27 ± 7.89[a]	61.34 ± 5.37[a]	80.46 ± 4.09[a]	48.05 ± 7.07[a]	1.05 ± 0.17[a]
HFD-STZ/Alc. Ext	65.46 ± 1.99[a/b]	49.33 ± 5.89[a/b]	41.74 ± 3.28[a/b]	28.44 ± 2.47[a/b]	0.73 ± 0.15[b]
HFD-STZ/Eth. Ac. Ext	77.82 ± 1.92[a/b]	50.95 ± 6.20[a/b]	43.73 ± 2.40[a/b]	31.85 ± 3.095[a/b]	0.80 ± 0.04[a/b]
HFD-STZ/Reference drugs	76.14 ± 2.95[a/b]	54.53 ± 1.96[a/b]	47.32 ± 2.79[a/b]	27.50 ± 6.62[a/b]	0.84 ± 0.11[a/b]

Values are expressed as mean ± SD, (n = 10). P-Value < 0.05, Groups superscripted by (a) symbol are significantly different from normal control group, and those superscripted by (b) are significantly different from HFD-STZ control group.

AST, ALP, GGT, and total bilirubin values by 31.28%, 19.51%, 48.13%, 40.83%, and 30.33%, respectively for treatment with Alc. Ext and 18.30%, 16.86%, 45.65%, 33.74%, 24.00%, and 21.63%, respectively for Eth. Ac. Ext group when compared with untreated diabetic group.

Kidney functions assessment

HFD-STZ induced diabetic rats suffered a significant elevation in serum urea and creatinine levels as shown in Table 7 which reached 182.24% and 124.25% respectively, when compared with normal negative control. Treatment with Alc. Ext and Eth. Ac. Ext caused significant decrease in serum urea and creatinine values, this decrease reached 48.34% and 32.85%, respectively for Alc. Ext and 48.34% and 45.76%, respectively for Eth. Ac. Ext when compared with untreated diabetic control group.

Oxidative stress assessment

Untreated diabetic rats suffered a significant increase in hepatic MDA this increase reached a percentage of 202.08%. However, a significant reduction in both hepatic GPx and SOD occurred by percentage of 28.26% and 80.76%, respectively when compared with normal control. Treatment of HFD-STZ induced diabetic rats with Alc. Ext and Eth. Ac. Ext caused a significant decrease in MDA by the value of 47.50% and 44.41%, respectively as shown in Figure 2. Furthermore, as demonstrated upon treatment, Alc. Ext and Eth. Ac. Ext shows significant elevation in GPx levels occurred by value of 21.07% and 32.2%, respectively and a significant increase in SOD levels by 156.87% and 135.30%, respectively when compared to untreated diabetic group as shown in Figures 1–3.

Pro-inflammatory markers assessment

HFD-STZ diabetic control rats illustrated significant increase in serum TNF-α and IL-6; with a percentage 86.77% and 34.18%, respectively when compared with normal control values. Treatment of HFD-STZ diabetic by Alc. Ext and Eth. Ac. Ext caused

Table 7. Effect of alcoholic and ethyl acetate extracts of *A. fragrantissima* on kidney functions of HFD-STZ diabetic rats and different therapeutic groups.

Groups	Serum urea (mg/dl)	Serum creatinine (mg/dl)
Normal control	35.09 ± 3.7[b]	0.54 ± 0.03[b]
Normal/Alc. Ext	36.52 ± 1.62[b]	0.53 ± 0.03[b]
Normal/Eth. Ac. Ext	36.26 ± 2.52[b]	0.50 ± 0.07[b]
HFD-STZ control	99.12 ± 3.45[a]	1.21 ± 0.34[a]
HFD-STZ/Alc. Ext	51.21 ± 4.16[a/b]	0.81 ± 0.07[a/b]
HFD-STZ/Eth. Ac. Ext	51.75 ± 4.45[a/b]	0.66 ± 0.10[b]
HFD-STZ/Reference drugs	48.03 ± 6.74[a/b]	0.63 ± 0.07[b]

Values are expressed as mean ± SD, (n = 10). P-Value < 0.05 is considered significant using one way ANOVA with post-hoc Tukey's procedure. Groups denoted by (a) symbol are significantly different from normal control group, while those denoted by (b) are significantly different from HFD-STZ control group.

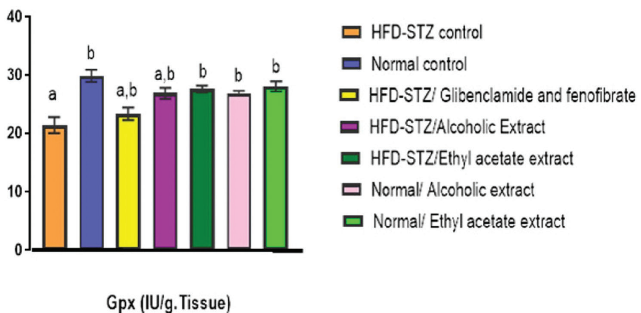

Figure 1. Therapeutic effects of *A. fragrantissima* extracts on Glutathione peroxidase levels. Values are expressed as Mean ± SD, (n = 10). P-value < 0.05 is considered significant using using one way ANOVA with post hoc tukey's procedure. Groups denoted by (a) symbol are significantly different from normal control group, while those denoted by (b) are significantly different from HFD-STZ control group.

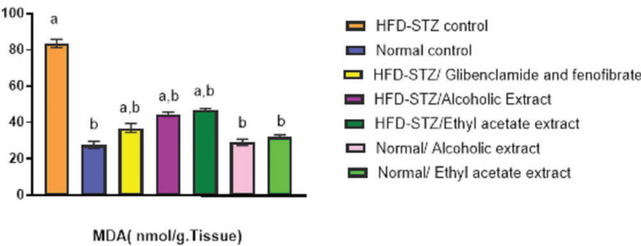

Figure 2. Therapeutic effects of *A. fragrantissima* extracts on Malondialdehyde levels. Values are expressed as Mean ± SD, (n = 10). P-value < 0.05 is considered significant using using one way ANOVA with post hoc tukey's procedure. Groups denoted by (a) symbol are significantly different from normal control group, while those denoted by (b) are significantly different from HFD-STZ control group.

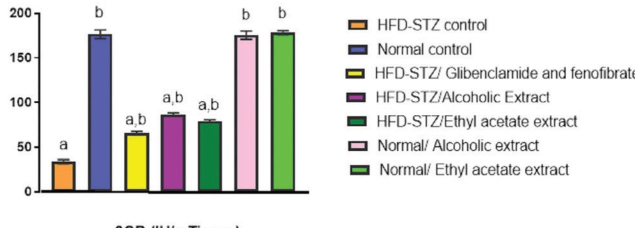

Figure 3. Therapeutic effects of *A. fragrantissima* extracts on superoxide dismutase levels. Values are expressed as Mean ± SD, (n = 10). P-value < 0.05 is considered significant using using one way ANOVA with post hoc tukey's procedure. Groups denoted by (a) symbol are significantly different from normal control group, while those denoted by (b) are significantly different from HFD-STZ control group.

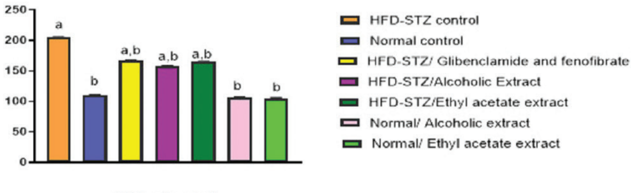

Figure 4. Therapeutic effects of *A. fragrantissima* extracts on Tumor necrosis factor alpha levels. Values are expressed as Mean ± SD, (n = 10). P-Value < 0.05 is considered significant using using one way ANOVA with post hoc tukey's procedure. Groups denoted by (a) symbol are significantly different from normal control group, while those denoted by (b) are significantly different from HFD-STZ control group.

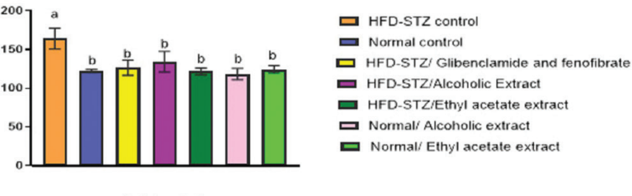

Figure 5. Therapeutic effects of *A. fragrantissima* extracts on IL 6 levels. Values are expressed as Mean ± SD, (n = 10). P-Value < 0.05 is considered significant using using one way ANOVA with post hoc tukey's procedure. Groups denoted by (a) symbol are significantly different from normal control group, while those denoted by (b) are significantly different from HFD-STZ control group.

significant reduction in serum TNF-α and IL-6, with percent change of 23.32% and 17.66%, respectively for alcoholic extract and 19.66% and 25.80%, respectively for Eth. Ac. Ext when compared to HFD-STZ induced diabetic control rat as recorded in Figures 4 and 5.

Atherosclerosis indicators assessment

HFD-STZ diabetic control group rats suffered a significant increase in sICAM-1 and sVCAM-1 as demonstrated in Figures 6 and 7, this increase reached 205.09%, 133.96%, and 36.63%, respectively when compared with normal control levels. Eth. Ac. Ext. treatment caused a significant reduction in sICAM-1 and sVCAM-1 levels by a percentage of 43.37% and 19.71%, respectively, while treatment by alcoholic extract of *A. fragrantissiman* recorded a significant reduction in VCAM-1 by 5.26%, while an insignificant decrease in sICAM-1 when compared with untreated HFD-STZ induced diabetic control.

Histopathological results

Pancreatic sections of normally fed non-diabetic rats when given Alc. Ext and Eth. Ac. Ext showed no histopathological alteration as recorded in Figure 8B and C in comparison to normal control group that showed no histological alteration and the normal histological structure of the islets of Langerhans cells as the endocrine portion as well as the acini of the exocrine portion as shown in Figure 8A. Meanwhile, HFD-STZ induced diabetic rat's pancreas sections showed degeneration and atrophy in the islets of Langerhans cells (Fig. 8D). However, HFD-STZ diabetic rats treated by alcoholic extract of *A. fragrantissima* demonstrated atrophy in the islets of Langerhans cells (Fig. 8E). Fortunately, HFD-STZ diabetic rats treated by Eth. Ac. Ext of *A. fragrantissima* showed some improvement, as it was found that the islets of Langerhans cells showed moderate degeneration and atrophy with congestion in the blood vessels (Fig. 8F). While, pancreas sections of HFD-STZ induced diabetic rats treated by the drug

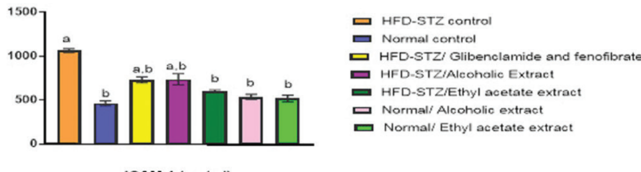

Figure 6. Therapeutic effects of *A. fragrantissima* extracts on Intercellular Adhesion Molecule 1 levels. Values are expressed as Mean ± SD, (n = 10). P-value < 0.05 is considered significant using using one way ANOVA with post hoc tukey's procedure. Groups denoted by (a) symbol are significantly different from normal control group, while those denoted by (b) are significantly different from HFD-STZ control group.

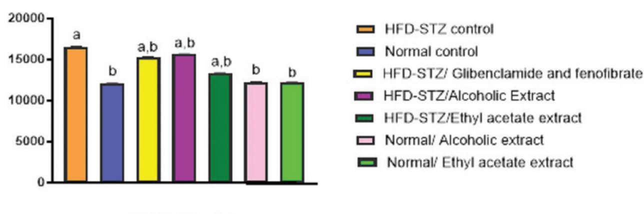

Figure 7. Therapeutic effects of *A. fragrantissima* extracts on vascular cell adhesion molecule 1 levels. Values are expressed as Mean ± SD, (n = 10). P-Value < 0.05 is considered significant using using one way ANOVA with post hoc tukey's procedure. Groups denoted by (a) symbol are significantly different from normal control group, while those denoted by (b) are significantly different from HFD-STZ control group.

Figure 8. Effect of *A. fragrantissima* extracts on pancreatic tissue histology in HFD-STZ induced diabetic rats and other therapeutic group. (A) Normal control, (B) normal/alcoholic extract, (C) normal/ethyl acetate extract, (D) HFD-STZ induced diabetic, (E) HFD-STZ/alcoholic extract, (F) HFD-STZ/ethyl acetate extract, and (G) HFD-STZ/glibenclamide and fenofibrate; Where each group denoted with a letter only for (H & E) stained slides and those stained with Masson's trichome are denoted with a letter accompanied with (*), scale bar = 200 μm.

combination of fenofibrate and glibenclamide showed atrophy in the islets of Langerhans cells (Fig. 8G).

Liver sections in normal control group showed no histological alteration and normal histological structure of the central vein and surrounding hepatocytes were recorded in (H & E) stained slides as shown in Figure 9A and Masson'strichome stained slides (Fig. 9A*); as well as, liver sections of normally fed non-diabetic rats given Alc. Ext and Eth. Ac. Ext showed no histological alterations as recorded in Figure 9B and B* and Figure 9C and C*. HFD-STZ diabetic rats liver sections demonstrated sever dilatation and congestion in the central vein as well as the portal veins associated with degeneration in the hepatocytes surrounding and adjacent the first one, while the inflammatory cells infiltration was detected in the portal area (Fig. 9D); while in the slides stained with Masson'strichome stained increased collagen depositions as it developed mild fibrosis in the portal area (Fig. 9D*). Treatment of diabetic rats by alcoholic extract of *A. fragrantissima* deduced slight improvement in liver sections, where it less congestion in the portal veins associated with less oedema in the portal area (Fig. 9E and E*). While treatment of diabetic rats by ethyl extract of *A. fragrantissima* caused a noticeable improvement in liver sections was recorded in Figure 9F, where less dilatation and congestion of the portal veins and less deposition of collagen as shown in Figure 9F*. Treatment by drug combination of glibenclamide and fenofibrate caused diminished improvement in liver sections, where there was congestion in the central and portal veins associated with oedema and mild fibrosis in the portal area (Fig. 9G and G*).

HFD-STZ induced diabetic kidney sections demonstrated degeneration in the lining epithelium in some of cortical tubules and necrosis with congestion in the glomeruli. Focal hemorrhage was noticed in between the degenerated tubules at the corticomedullary portion Figure 10D. HFD-STZ induced diabetic rats treated by alcoholic extract of *A. fragrantissima* deduced minor improvement in kidney sections, where it still showed degenerative change and necrosis in the lining epithelium of some few tubules at the cortex Figure 10E. While treatment of HFD-STZ induced diabetic rats by ethyl extract of *A. fragrantissima* demonstrated improvement in kidney sections, where no histopathological alteration was detected when compared to normal control sections as in Figure 10F. Kidney sections of HFD-STZ induced diabetic rats treated by drug combination of glibenclamide and fenofibrate showed congestion in the cortical blood vessels and glomeruli Figure 10G.

Discussion

Obesity and hyperlipidemia are considered to be one of the epidemics due to processed foods consumption and non-active life style [42]. The current study simulated metabolic syndrome through association between hyperlipidemic state accompanied with hyperglycemia (T2DM) propagated due to dietary manipulation through HFD followed by moderate destruction of β-cells by inflammation due to low dose of STZ, which was in agreement with the findings of Hwang et al. [43]. After treatment for three weeks with Alc. Ext and Eth. Ac. Ext of *A. fragrantissima,* FBG levels were significantly reduced and even surpassed the hypoglycemic effect recorded after treatment by glibenclamide. Even though, it was recorded that fenofibrate enhances glibenclamide's hypoglycemic effect due to inhibition of cytochrome *p*-450 isoforms responsible for the metabolism of glibenclamide [11].

A present data indicated that the Eth. Ac. Ext has more activity against oxidative stress parameters than the Alc. Ext. The activity may be related to the presence of various classes of phenolic compounds of flavones and flavonols classes that were present in the two extracts, but in different amounts. In our present work, three flavones derivatives luteolin, apigenin-6-C-glucoside, and cosmosiin (apigenin 7-glucoside) were isolated and identified from the Eth. Ac Ext of *A. fragrantissima*. Numerous reports showed that apigenin and other flavones exhibited different properties, such as anti-inflammatory, anti-oxidative, and antihepatotoxic properties and the hypolipidemic and antidiabetic activities of these flavones are likely due to their properties [44]. Structure–activity relationship study has revealed that the specific structure of flavones with the presence of the 3′- and 4′-OH groups in the B-ring and a double bond between C-2 and C-3 are important factors for their recognition and binding by glycogen phosphorylase (GP). Flavones inhibited dephosphorylated GP, and they could have the potential to contribute to the protection or improvement of control of T2DM [13]. Quercetin was found to inhibit hepatic cholesterol biosynthesis *in vitro* and to have a hypocholesterolemic effect *in vivo* [44].

The susceptibility to free radical attack and harmful effect on liver function might be done with the excessive storage of fat in the liver and the diabetic

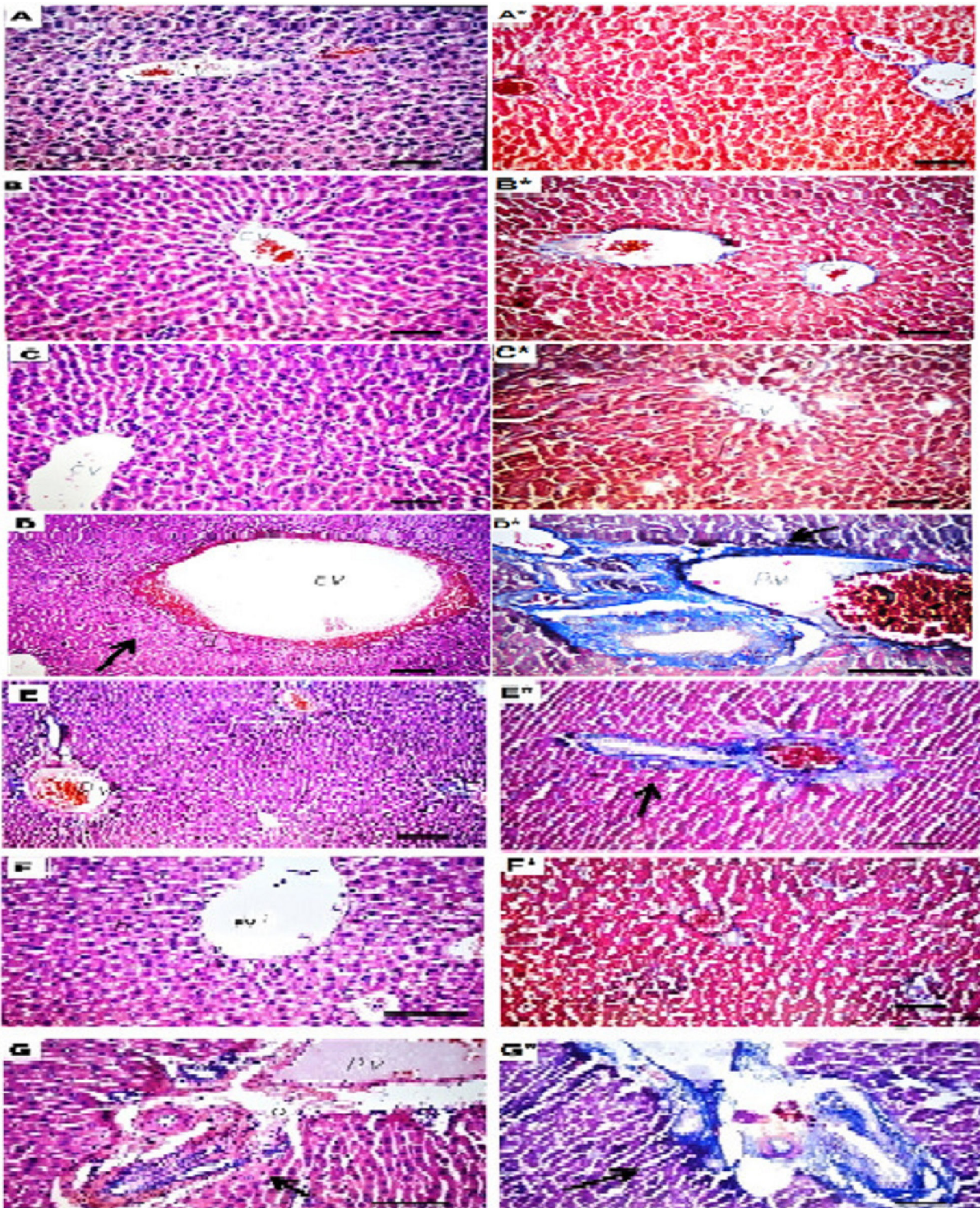

Figure 9. Effect of *A. fragrantissima* extracts on hepatic tissue histology in HFD-STZ induced diabetic rats and other therapeutic group. (A) Normal control, (B) normal/alcoholic extract, (C) normal/ethyl acetate extract, (D) HFD-STZ induced diabetic, (E) HFD-STZ/alcoholic extract, (F) HFD-STZ/ethyl acetate extract, and (G) HFD-STZ/glibenclamide and fenofibrate; Where each group denoted with a letter only for (H & E) stained slides and those stained with Masson's trichome are denoted with a letter accompanied with (*), scale bar = 200 µm.

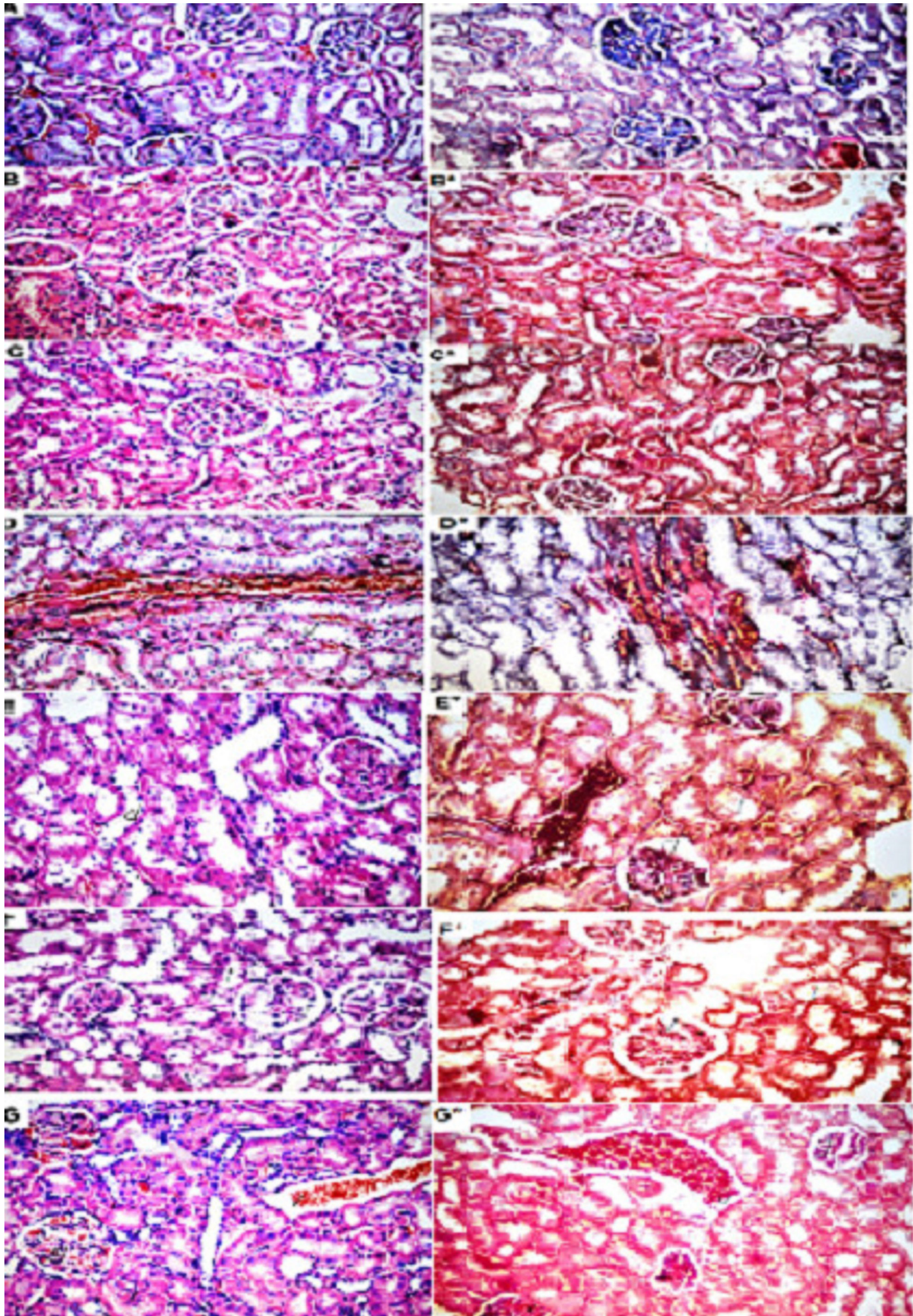

Figure 10. Effect of *A. fragrantissima* extracts on kidney tissue histology in HFD-STZ induced diabetic rats and other therapeutic group. (A) Normal control, (B) normal/alcoholic extract, (C) normal/ethyl acetate extract, (D) HFD-STZ induced diabetic, (E) HFD-STZ/alcoholic extract, (F) HFD-STZ/ethyl acetate extract, and (G) HFD-STZ/glibenclamide and fenofibrate; Where each group denoted with a letter only for (H & E) stained slides and those stained with Masson's trichome are denoted with a letter accompanied with (*), scale bar = 200 μm.

state. Treatment with the Eth. Ac. Ext of *A. fragrantissima*, containing the flavones aglycone luteolin and the glycosides apigenin-6-C-glucoside and cosmosiin, may prevent oxidative damage by detoxifying reactive oxygen species (ROS); thus, reducing hyperlipidemia [45] with a concomitant decrease in ALT, AST, and ALP enzyme activities. The decreased ALT and AST enzyme activities in serum, as a result of the treatment with the Alc. Ext and Eth. Ac. Ext, might be ascribed to the ability of phenolic compounds to maintain membrane integrity extract. Other flavonoids were also reported, such as afroside, cirsimartin, chrysoplenol, and cirsiliol [3]. *A. fragrantissima* tannin content reaches 8% such as resorcin, phloroglucin, methyl phloroglucin, and pyrocatechol. High percentage of tannins may have reduced lipid peroxidation by acting as antioxidants, and hence, aiding the endogenous antioxidant enzymes involved in the scavenging inactivation of the ROS or redox metal ions before lipid peroxidation takes place.

Also, *A. fragrantissima* showed a significant α-glucosidase inhibitory activity due to the presence of phenolic compounds which were previously isolated from *Achillea* species. This activity may related to these compounds that have the potential of reducing the postprandial glucose and insulin peaks to reach normoglycaemia and delay the absorption of ingested carbohydrates [7], as this pathway may be differed from the regular mechanisms of acarbose and miglitol α-glucosidase inhibitors [46].

A significant reduction in liver glycogen content level was showed in STZ-induced diabetic rats. These results were in consistence with the findings of Mandour [18]. However, treatment by Alc. Ext and Eth. Ac. Ext caused increase in liver glycogen content suggesting increased gluconeogenesis and a decreased glycogenolysis [47]. Structure–activity relationship study has revealed that the present flavones posses special structure concerning the presence of the 3′- and 4′-OH groups in the B-ring and a double bond between C-2 and C-3, which was an important factor for their recognition, binding, and inhibition to GP [48].

In the present study, dietary manipulation caused significant increase in BW associated with hyperlipidemia which was harmony with the findings of Shalaby et al. [12]. Extracts of *A. fragrantissima* caused a significant reduction in BW as well as serum lipid profile parameters (TC, LDL-C, TG, TL, and PL). Additionally, treatment by Eth. Ac. Ext caused significant increase in HDL-C values, this could be attributed to the quercetin which was found to inhibit hepatic cholesterol biosynthesis *in vitro* and to have a hypocholesterolemic effect *in vivo* [44].

Approximately, 70%–80% of T2DM patients suffer from non-alcoholic fatty liver [49], which accounts for 13% probability of hepatocellular carcinoma (HCC) [49]. In the present study, significant increase in ALT, AST, ALP, GGT, and total bilirubin in HFD-STZ diabetic control, while treatment with Alc. Ext and Eth. Ac. Ext caused significant decrease when compared to untreated diabetic rats; this reduction might be ascribed to the ability of phenolic compounds to maintain membrane integrity as well as flavonoids such as afroside, cirsimartin, chrysoplenol, and cirsiliol [50].

Diabetic nephropathy also known as diabetic kidney disease (DKD) is one of the most serious complication of diabetes [51]. Regarding kidney clearance performance and functions in the present work, an elevation in serum urea and creatinine levels was recorded in serum of untreated diabetic group. Treatment with Alc. Ext and Eth. Ac. Ext of *A. fragrantissima* caused significant reduction in serum urea and creatinine levels. This results were in accordance with the results of Mandour [18] who studied the acute and long-term safety evaluation of *A. fragrantissima* consumption.

Meanwhile, concerning oxidative stress state, results of the present study elucidated that the induction of diabetes by HFD and low dose of STZ caused a significant elevation in hepatic MDA. These results were in agreement with the findings of Zheng et al. [52] and Mohamed et al. [53]. A significant decrease in hepatic GPx and SOD suggesting an increase in the surge of ROS produced by CYP2E1 which is an imperative cause of insulin resistance in diabetes and related non-alcoholic fatty liver [54]. Additionally, treatment with Alc. Ext and Eth. Ac. Ext induced significant reduction in hepatic MDA and significant elevation in GPx and SOD levels, predicting the retrieval of oxidative equilibrium state which can be correlated with the essential oil of *Achillea* species, as it can suppress the inflammatory responses of lipopolysaccharides (LPS)-stimulated RAW 264.7 macrophages. This improvement including decreased levels of cellular nitric oxide (NO) and superoxide anion production, lipid peroxidation, and glutathione (GSH) concentration as existing monoterpenes are the most representative metabolites [50].

Insulin resistance and adipose tissue hyperplasia are considered as inflammatory status which

is accompanied with increased pro-inflammatory mediators and cytokines [9]. In the current study, a significant elevation in serum TNF-α and IL-6 levels was recorded in untreated HFD-STZ diabetic group. Meanwhile, treatment with Alc. Ext and Eth. Ac. Ext of *A. fragrantissima* reverted these changes claiming that this plant extract possess an anti-inflammatory activity which have been attributed to its flavonoid content as well as highly oxygenated terpenoids [6,7]. These results came in agreement with another study reported other *Achillea* species has showed anti-inflammatory activities as they inhibit lipopolysaccharide-induced NO production in RAW264.7 macrophage cells [55].

Cardiovascular complications are known to be lethal consequences of T2DM [56]. In the current work, untreated diabetic group exhibited increased adhesion molecule attributed to increased phospholipase activity due to increased vasoconstrictive eicosanoids. These results were consistent with Varga et al. [57]. However, treatment of HFD-STZ induced diabetic rats by Alc. Ext and Eth. Ac. Ext caused reduction in sICAM-1 and sVCAM-1 values, suggesting that this plant extracts could have counteracted to some extent the cardiovascular repercussion evolved due to metabolic disorder.

Regarding histopathological examination, HFD-STZ diabetic pancreatic tissue sections showed degeneration and atrophy in the islets of Langerhans cells and β-cells accompanied with congestion in the blood vessels. These observations were in agreement with the findings of Ding et al. [58]. Meanwhile, treatment with alcoholic extract of *A. fragrantissima* and combination drugs of glibenclamide and fenofibrate caused moderate improvement, while treatment with Eth. Ac. Ext caused remarkable amelioration. Moreover, examination of liver tissue showed that HFD-STZ untreated diabetic group suffered severe dilatation and congestion of portal and central vein as well as showing mild fibrosis in the portal area which was clearly observed in slides stained by Masson'strichome accompanied with loss of the hepatic lobular architecture. These findings were in agreement with the results of Shalaby et al. [48]. Furthermore, treatment by *A. fragrantissima* Alc. Ext and Eth. Ac. Ext caused reverting of some of these changes. Also, the histopathological examination of untreated diabetic kidney tissue showed degeneration in lining epithelium in some of cortical tubules and necrosis with congestion in the glomeruli accompanied with hemorrhage in the corticomedullary portion. This observation was in harmony with observations of Suman et al. [59]. However, treatment with alcoholic and ethyl acetate extracts of *A. fragrantissima* showed minor degeneration in epithelium of some cortical tubules in alcoholic extract group and even a nearly similar to normal renal architecture in Eth. Ac. Ext treated rats.

Conclusion

Results of current study suggest that Alc. Ext and Eth. Ac. Ext of *A. fragrantissima* showed a very promising anti-diabetic, hypolipidemic, anti-inflammatory, and antioxidant activity. Both extracts reduced and may even diminished most of the changes deduced through simulation of T2DM and metabolic syndrome pathological condition which exhibited altered blood glucose level, serum lipid profile, liver, and kidney functions parameters as well as inflammatory, oxidative stress, and vasoconstriction biomarkers. These findings were in agreement with the results of histopathological examinations of pancreas, liver, and kidney tissues. These findings have confirmed the traditional uses of the plant under investigation. However, further clinical investigations should be carried out to encourage using *A. fragrantissima* as a candidate nutraceutical for ameliorating metabolic disturbances associated with diabetes.

Funding

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A comparative study on micronutrients and antinutrients of leaf extracts of *Pterocarpus soyauxii and Pterocarpus santalinoides*

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ABSTRACT

Aim: The micronutrients and antinutrients of leaf extracts of *Pterocarpus soyauxii* and *Pterocarpus santaiinoides* were investigated.
Methods: The leaf extracts were air-dried for 7 days and pulverized using a mechanical homogenizer, and then passed through a 0.5 mm size mesh sieve. They were subjected to atomic absorption spectrophotometry, standard titrations, and quantitative assay for micronutrients, vitamins, and antinutrients determinations, respectively.
Results: The micronutrients assay revealed the presence of calcium (Ca^{2+}), potassium (K^+), copper (Cu^{2+}), zinc (Zn^{2+}), iron (Fe^{2+}), magnesium (Mg^{2+}), manganese (Mn^{2+}), and phosphorous (P) in the two plant samples. The highest micro element found was potassium (K^+) and least was copper (Cu^{2+}) with 882.00 mg/100 g and 0.31 mg/100 g in *Pterocarpus santalinoides* respectively, while *Pterocarpus soyauxii* has (K^+) with 736.00 mg/100 g and (Cu^{2+}) with 0.48 mg/100 g. Statistical differences ($p < 0.05$) were observed in Ca^{2+}, K^+, Cu^{2+}, Fe^{2+}, and Mn^{2+} amount, while non-significant differences were found in Zn^{2+}, Mg^{2+}, and P when comparatively tested. The results obtained revealed the presence of two fat-soluble vitamins (vitamins A and E) and two water-soluble vitamins (B_2 and C). Vitamin C content was found to be higher in *Pterocarpus santalinoides* (21.09 mg/100 g) and lower in *Pterocarpus soyauxii* (14.61 mg/100 g), while least vitamin content recorded was vitamin E with 0.02 mg/100 g and 0.03 mg/100 g in *Pterocarpus soyauxii* and *Pterocarpus santalinoides*, respectively. Other quantified antinutrients were alkaloids, oxalates, and cyanogenic glycosides, which ranges from 0.07% to 2.50%. Significant difference ($p <$ 0.05) was observed in oxalate, alkaloid, and cyanogenic glycosides amount of the two specimens.
Conclusion: The result showed that the leaf extracts contain appreciable number of micronutrients, vitamins, and low level of antinutrients and could contribute to the recommended dietary allowance of the body.

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Introduction

The significance of nutrition in public health problems has been a subject of intense renewed interest and has also widened the horizon of the roles of nutrients in food [1,2]. Nutrition is the science of food and its relationship to health. The nutritive value of food defines what a food is made of and its impact on the body as it relates to cholesterol, fat, salt, and sugar intake. Nutrients are categorized as either macronutrients (needed in relatively large amounts) or micronutrients (needed in small quantities). Macronutrients include carbohydrates, fats, fiber, proteins, and water, while micronutrients are minerals and vitamins [3].

Vegetables are fresh edible portion of herbaceous plants, whose part or parts are eaten as supporting food or main dishes. They may be aromatic, bitter

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or tasteless [4], generally succulent and consumed as a side dish with starchy staples [5]. The green leaves of vegetables are rich in retinol (vitamin A), vitamin B complex, folic acid, vitamin C, and mineral elements such as calcium, iron, zinc, potassium, copper, magnesium, and phosphorus [6–10]. In the world today, there are several reports on the nutritional benefits of vegetable species; one of these includes *Pterocarpus soyauxii and Pterocarpus santalinoides* [9–11].

Pterocarpus soyauxii and *Pterocarpus santaiinoides* are called “Oha” and “Nturukpa”, respectively, in Igbo land (Figure 1). They belong to the genus, *Pterocarpus*, which is tropically and sub-tropically distributed and belong to the family Leguminosae. There are about 60 species of the genus *Pterocarpus*, of which 20 of these are found in African countries such as Nigeria, Cameroon, Sierra Leone, and Equatorial Guinea. *Pterocarpus* is coined from two Greek words “Pteran” meaning wing and “Karpos” meaning fruit, thereby it has a meaning winged fruit [11].

Pterocarpus soyauxii (oha) is a tree about 27–34 m tall, girth up to 3.3 m with undivided stem. It has a reddish grey-bark which detaches in flakes, slash white and exuding a red gum. Its leaves are compound with 11–13 leaflets, alternate, and lateral leaflets with veins which are crowded, but disappear before leaf margins. Its fruits are obliquely orbicular, compressed in dehiscent pods, 6.9 cm with numerous prick thorns, and it is monoecious [11]. The plant is native to west tropical Africa and occurs in mixed deciduous and evergreen forests. It requires much light and moist soil to grow properly [11].

On the other hand, *Pterocarpus santalinoides* tree grows 9–12 m tall with a trunk up to 1 m in diameter and flaky bark. The leaves are pinnate, 10–20 m long with leaflets. The flowers are orange-yellow produced in panicles. The fruit is a pod, 3.5–6.0 cm long with a wing extending three-quarters around the margin. *Pterocarpus santalinoides* is monoecious, and has flowering from December to March. It could be found in river banks, usually on sandy and moist soils [11].

The leaves of *Pterocarpus santalinoides* contain alkaloids, flavonoids, tannins, saponins, and phenols which are known to have antimicrobial activity. The presence of flavonoids also indicates the medicinal value of *Pterocarpus santalinoides* because they are antioxidants and free radical scavengers which prevent oxidative stress, and also, have strong anticancer activity protecting the cell against all stages of carcinogenesis [12,13].

The aim of the present study is to compare the micronutrients, vitamins, and antinutrients of *Pterocarpus soyauxii* and *Pterocarpus santalinoides*.

Materials and Methods

Collection and preparation of samples

Fresh leaves of *Pterocarpus soyauxii* and *Pterocarpus santalinoides* were harvested fresh from the farm located at Afugiri, Umuahia North Local Government, Abia State, Nigeria and were identified at the Department of Plant Science and Biotechnology of the Abia State University. The leaves of the different samples were handpicked, rinsed with tap water, and then with deionized water. These samples were air-dried for 7 days. The dried samples were pulverized using a mechanical homogenizer, and then passed through a 0.5 mm size mesh sieve. Each of the samples was stored in an air-tight plastic container until analysis and properly labeled.

Determination of micronutrients composition

Exactly, 1 g of the sample was weighed and predigested for a short period of time (75°C for 10 minutes) in 69% HNO_3 and 30% H_2O_2 (w/v: 10 ml) and later heated at 120°C. The digested solutions were filtered using Whatman filter paper No. 1 and diluted to 50 ml with deionized water. The concentrations of the micronutrients in the digested solutions were determined using an atomic absorption spectrophotometer [14].

Vitamin contents

Standard spectrophotometric methods of Association of Official Analytical Chemists [15] and Okwu [16] were used in the determination of Vitamins A, B_2, and E, while Vitamin C was determined using a titrimetric method [16].

Antinutrient estimation

Alkaloid content was quantitatively determined by the methods of Harborne [17] and cyanogenic glycosides were determined using the methods of Obadoni and Ochuko [18].

Oxalate determination

The titration method as described by Day and Underwood [19] was used for oxalate estimation. One gram of sample was weighed into 100 ml conical flask. Seventy-five milliliter of 3 M H_2SO_4 was added and stirred for 1 hour with a magnetic stirrer. This was filtered using a Whatman filter paper

No. 1. Twenty-five milliliter of the filtrate was then taken and titrated while hot against 0.05 M KMnO4 solution until a faint pink color persisted for at least 30 seconds [1]. The titer value was recorded.

Equation:

$$C_2O_4^{2-} + 8H^+ + MnO4 \rightleftharpoons 2CO_2 + 4H_2o + Mn^{2+}$$

The ion ratio is 1:1

Using the formula

$M_aV_a = M_bV_b$

Where,

M_a = molarity of oxalate solution

V_a = volume of oxalate solution

M_b = molarity of potassium permanganate solution

V_b = volume of potassium permanganate solution

The relationship of the M_a can be given as:

g/l = molarity × molar mass

$$\% \text{ oxalate} = \frac{X}{1} \times \frac{20}{1000} \times \frac{100}{20} \times \frac{100}{2}$$

Where,

X = weight of oxalate obtained by multiplying molar mass and

molarity of oxalate

20_a = volume of extract aliquot taken for analysis

1000 = reference volume for molar concentration

100_a = total volume of extract

20_b = volume of extract aliquot taken for analysis

100_b = scaling factor to convert to percentage

5 = weight of sample taken for analysis

Molar mass of oxalate is given as 90 g/mol (12 × 2 + 16 × 4 × 2)

Statistical analysis of data

The experimental data were analyzed for statistical significance by *t*-test using the GraphPad Prism® statistics software package, version 7.03. All determinations were done in triplicate. Mean and standard deviations (SD) were calculated using Microsoft Excel program and the probability tested was at 95% level of significance, so as to establish research hypothesis.

Table 1. Micronutrient composition of *Pterocarpus soyauxii* and *Pterocarpus santalinoides*.

	Samples (mg/100 g)	
Parameters	***Pterocarpus soyauxii***	***Pterocarpus santalinoides***
Ca^{2+}	243.00 ± 1.00[a]	257.00 ± 1.00[b]
K^+	736.00 ± 1.58[a]	882.00 ± 3.00[b]
Cu^{2+}	0.48 ± 0.03[a]	0.38 ± 0.01[b]
Zn^{2+}	3.44 ± 0.02[a]	3.61 ± 0.05[a]
Fe^{2+}	22.16 ± 0.01[a]	19.63 ± 0.03[b]
Mg^{2+}	256.00 ± 3.00[a]	286.00 ± 2.00[a]
Mn^{2+}	3.65 ± 0.02[a]	3.15 ± 0.03[b]
P	327.00 ± 2.00[a]	348.00 ± 4.00[a]

Values are mean ± SD for triplicate determination. Values in the same row bearing the same letter of the alphabet are not statistical difference ($p < 0.05$).

Results

Micronutrient composition of Pterocarpus soyauxii *and* Pterocarpus santalinoides

Table 1 showed the results obtained from the micronutrient contents of *Pterocarpus soyauxii* and *Pterocarpus santalinoides*. Calcium (Ca^{2+}), potassium (K^+), copper (Cu^{2+}), zinc (Zn^{2+}), Iron (Fe^{2+}), magnesium (Mg^{2+}), manganese (Mn^{2+}), and phosphorous (P) were appreciably present in the two samples. However, significant differences were observed in Ca^{2+}, K^+, Cu^{2+}, Fe^{2+}, and Mn^{2+}, while non-significant difference was found in Zn^{2+}, Mg^{2+}, and P when compared to each other ($p < 0.05$).

Vitamin content of Pterocarpus soyauxii *and* Pterocarpus santalinoides

Vitamin Screening of *Pterocarpus soyauxii* and *Pterocarpus santalinoides* was shown in Table 2. Two fat-soluble vitamins (Vitamins A and E) and two water-soluble vitamins (B_2 and C) were evaluated. Vitamin C content was found more in *Pterocarpus santalinoides* (21.09 mg/100 g) and less in *Pterocarpus soyauxii* (14.61 mg/100 g),

Table 2. Vitamin content of *Pterocarpus soyauxii* and *Pterocarpus santalinoides*.

		Samples (mg/100 g)	
Parameters	**Types**	***Pterocarpus soyauxii***	***Pterocarpus santalinoides***
Vitamin A	Fat Soluble	2.75 ± 0.01[a]	2.91 ± 0.01[a]
Vitamin B_2	Water Soluble	0.10 ± 0.03[a]	0.19 ± 0.03[a]
Vitamin C	Water Soluble	14.61 ± 0.04[a]	21.09 ± 0.03[b]
Vitamin E	Fat Soluble	0.02 ± 0.00[a]	0.03 ± 0.00[a]

Values are mean ± SD for triplicate determination. Values in the same row bearing the same letter of the alphabet are not statistical difference ($p < 0.05$).

Table 3. Antinutrient content of *Pterocarpus soyauxii* and *Pterocarpus santalinoides*.

	Samples (%)	
Antinutrients	***Pterocarpus soyauxii***	***Pterocarpus santalinoides***
Alkaloids	2.06 ± 0.12[a]	2.50 ± 0.20[a]
Oxalates	1.69 ± 0.01[a]	0.46 ± 0.02[b]
Cyanogenic glycosides	0.07 ± 0.00[a]	0.09 ± 0.00[a]

Values are mean ± SD for triplicate determination. Values in the same row bearing the same letter of the alphabet are not statistical difference ($p < 0.05$).

while the least vitamin content was Vitamin E with (0.02 mg/100 g and 0.03 mg/100 g) in *Pterocarpus soyauxii* and *Pterocarpus santalinoides*, respectively.

Antinutrient content of Pterocarpus soyauxii *and* Pterocarpus santalinoides

The antinutrient screening of *Pterocarpus soyauxii* and *Pterocarpus santalinoides was* expressed in percentage (Table 3). From the result, alkaloids, oxalates, and cyanogenic glycosides were quantified. There was a significant difference on oxalate at ($p < 0.05$), while there were no significant differences observed in alkaloids and cyanogenic glycosides parameter when comparatively evaluated.

Discussion

The mineral, vitamin, and antinutrient compositions of leaf extracts of *Pterocarpus soyauxii* and *Pterocarpus santalinoides* were evaluated using standard analytical procedures.

Micronutrients are comprised of vitamins and minerals, which are required in small quantities to ensure normal metabolism, growth, and physical well-being. The chief nutritive significance of green leafy vegetables is their richness in minerals and vitamins [3]. Minerals are also required for basic body functions such as heartbeat, muscle contractions, movement, growth, and regulatory processes [20]. Studies on the micronutrient composition of *Pterocarpus soyauxii* and *Pterocarpus santalinoides* as shown in (Table 1) revealed appreciable amounts of Calcium (Ca^{2+}), potassium (K^+), copper (Cu^{2+}), zinc (Zn^{2+}), Iron (Fe^{2+}), magnesium (Mg^{2+}), manganese (Mn^{2+}), and phosphorous (P) in the tested samples.

The values obtained showed that the calcium content was greater in *P. santalinoides* (257.00 ± 1.00 mg/l00 g) than *P. soyauxii* (243.00 ± 1.00 mg/l00 g). However, these values are higher than the values reported for *Amaranthus hybridus* L. (44.15 mg/l00 g) Akubugwo et al. [21] and lower than the recommended dietary allowance (RDA) for calcium (l000 mg/day) [22].

The potassium content of *P. soyauxii* and *P. santalinoides* had (736.0 ± 1.58 mg/100 g) and (882.0 ± 3.00 mg/100 g), respectively. These quantified amounts were found higher than those reported for other leafy vegetables studied in Nigeria, whose ranges are (405.03–301.01 mg/100 g) [23]. Potassium helps to regulate heart beat and muscle functioning [24].

Copper contributes in hemoglobin formation, red blood cells in iron and energy metabolism [25]. It also helps in elastin formation, a vital skin protein that helps to keep skin healthy and flexible, also helps in collagen formation, another vital protein for building bones, muscles, and connective tissues. It is needed to maintain healthy nerves and joints. It is not necessary to supplement this mineral as excessive copper intake can lead to toxicity. Toxic levels of copper can cause vomiting, joints and muscle pain, and with extreme overdosing it can even be fatal [2,26]. The copper contents were (0.48 ± 0.01 mg/100) and (0.38 ± 0.01 mg/100 g) for *P. soyauxii* and *P. santallnoides*, respectively. These values are slightly discordant with those obtained from other leafy vegetables studied in Nigeria (0.51–0.67 mg/100 g) [23]. The RDA of copper is

(a) (b)

Figure 1. (A) *Pterocarpus soyauxii* and (B) *Pterocarpus santalinoides.*

0.9 mg/day [25] which indicates that the vegetables studied, when taken in adequate amounts can meet this RDA of copper.

Zinc supports healthy immune system, helps in wound healing, synthesis of DNA and maintenance of sense of taste. It also helps in normal growth and development during pregnancy, childhood, and adolescence. It is essential for growth and sexual development in man [27] and important in cell proliferation and protein synthesis [28]. The result of the zinc content of the studied vegetables, *P. soyauxii* (3.44 ± 0.02 mg/100 g) and *P. santalinoides* (3.61 ± 0.05 mg/100 g) is compared well with 3.88 mg/100 g reported for *Amaranthus hybridus* L. [21]. The investigated vegetables are rich in zinc, because they met the RDA of zinc for children (1–3 years), 3.0 mg/day [22].

Iron plays numerous biochemical roles in the body, including oxygen binding in hemoglobin and acting as an important catalytic centre in many enzymes such as the cytochrome oxidase [29]. Iron content of *P. soyauxii* (22.16 ± 0.01 mg/100 g) and *P. santalinoides* (19.63 ± 0.03 mg/100 g) was higher than the RDA for iron (8 mg/day) [25] indicating that the studied vegetable could be recommended in diets for reducing anemia, which affects over one million people worldwide [30].

Magnesium is known to prevent cardiomyopathy, muscle degeneration, growth retardation, alopecia, dermatitis, immunologic dysfunction, gonadal atrophy, impaired spermatogenesis, congenital malfunctions, and bleeding disorders [31]. The present study showed magnesium contents (256.0 ± 3.00 mg/100 g) for *P. soyauxii* and (286.0 ± 2.00 mg/100 g), which were higher than the RDA of Mg (240 mg/day) showing that the studied vegetables are rich in magnesium.

Manganese content of *P. soyauxii* (3.65 ± 0.02 mg/100 g) and *P. santalinoides* (3.15 ± 0.03 mg/100 g) is compared well to the report from some other leafy vegetables in Nigeria (3.91 mg/100 g) [23] and higher than the RDA for manganese (1.8 mg/day) showing that *P. soyauxii* and *P. santalinoides* are rich sources of manganese, which is a component of several metalloenzymes e.g., superoxidase dismutase.

The phosphorous content are (327.00 ± 2.00 mg/100 g) and (348.0 ± 4.0 mg/l00 g) for *P. soyauxii* and *P. santalinoides*, respectively, which were lower than the RDA for phosphorous (800 mg/day). Therefore, its adequate intake can yield the RDA of 800 mg. The soil of the area from which these vegetable are derived can be enriched with calcium and phosphorous fertilizer to enhance their content in these studied vegetables. Phosphorous has been implicated in growth and maintenance of bones, teeth, and muscles [32].

Vitamins are an essential organic nutrient, most of which are not synthesized, or only in insufficient amounts in the body and are mainly obtained through food. When their intake is inadequate, vitamin deficiency disorders are the consequences. Vitamins are vital to health and need to be considered when determining nutrition security. They also serve as biocatalysts as well as precursors to various body factors and cofactors. They are classified based on their solubility to aqueous and lipid solvents; water-soluble and fat-soluble [33] and have specific health benefits. For example, consumption of vegetables rich in vitamin A will promote good sight and boost immune system [34]. The vitamin screening of *Pterocarpus soyauxii* and *Pterocarpus santalinoides* was shown in Table 2. Two fat-soluble vitamins (Vitamins A and E) and two water-soluble vitamins (B_2 and C) were evaluated. Vitamin C content was found higher in *Pterocarpus santalinoides* (21.09 mg/100 g) and less in *Pterocarpus soyauxii* (14.61 mg/100 g) which serves as a biological antioxidant in aqueous environment, while the least vitamin content was recorded in Vitamin E with (0.02 mg/100 g and 0.03 mg/100 g) in *Pterocarpus soyauxii* and *Pterocarpus santalinoides*, respectively, when compared to other examined vitamins. Despite the least recorded value of vitamin E, it still has a crucial role to play when absorbed in the body as a signaling molecule and also helps in the inhibition of platelet aggregation [35]. The tested plant samples are good sources of B complex and can be used in the maintenance of good health in humans and animals when consumed appreciably [36].

The antinutrient content of the studied vegetables indicated the alkaloid contents as 2.06% ± 0.12% and 2.50% ± 0.20% for *P. soyauxii* and *P. santalinoides*, respectively. Pure isolated plant alkaloids are used as medicinal agents for analgesic, antispasmodic, and bactericidal effects [37,38]. As shown in the results (Table 3), *P. santalinoides* has higher alkaloid content than *P. soyauxii* and this may be attributed to its efficacy in soup preparation for nursing mothers immediately after leaving the labor room instead of *P. soyauxii.*

Oxalate content of *Pterocarpus soyauxii* (1.69% ± 0.11%) and *Pterocarpus santalinoides* (0.45% ± 0.22%) are higher than those reported for the leafy vegetables studied in Nigeria (0.27% and 0.38%), respectively [39], but lower than the critical value

of oxalate consumption (2.0%–5.0%) [40]. This indicates that the oxalate level of the tested plant may not cause kidney disease, which is associated with the consumption of diet, high in oxalate when consumed adequately [41].

Cyanogenic glycosides content of *Pterocarpus soyauxii* (0.07% ± 0.00%) and *Pterocarpus santalinoides* (0.09% ± 0.00%) were lower than those reported for the same vegetables as (0.34% and 0.42%), respectively [39] and also, lower than the critical level, 200 mg/kg. Toxicity of cyanogenic glycoside shows symptoms of diarrhea, vomiting, and heart failure in human. Its level in leafy vegetables can be reduced during processing such as soaking, boiling or frying [42].

Conclusion

It can be inferred from the present study that leaf extracts of *Pterocarpus soyauxii* (Oha) and *Pterocarpus santalinoides* (Nturukpa) harvested from Afugiri, Umuahia North L.G.A Abia State, Nigeria, contain an appreciable amount of vitamins and mineral elements, and low level of antinutrients. Although, the antinutrient contents of these vegetables are low, they should be removed to improve their nutritional quality by soaking and washing. Therefore, these plants could serve as supplementary diet supplying the body with micronutrients, hence, contributing to the alleviation of micronutrient deficiencies, if consumed in sufficient amount.

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REVIEW ARTICLE

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Anti-hypertensive plants of rural Pakistan: Current use and future potential

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ABSTRACT

Aim/Background: Hypertension is surging around the world and negatively affecting lives and livelihoods along its way. This plight is felt especially hard by the world's rural population in developing nations, where many barriers to medical care exist. These barriers can exhibit themselves as monetary or distance barriers. In Pakistan, where barriers to medical care caused by the remoteness and financial hardship are strong, people have drawn on traditional medicinal plants to treat this plight of hypertension.
Materials and Methods: In this review, we assess the interface between hypertension and plant-based medicine among a population with limited health care access. We queried the scientific databases of Google Scholar, PubMed, Scopus, and Web of Science for the search terms: "medicinal plants of Pakistan, ethnobotany of Pakistan, and anti-hypertension plants of Pakistan".
Results: This search revealed about 114 species that are used to combat hypertension in Pakistan. It also showed how the majority of species are harvested from sustainable aerial plant parts or leaves.
Conclusion: The promotion of cultivation and validation studies may prove promising for these remedies that people in need of health care turn to.

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Introduction

Hypertension deteriorates patient health, and it is a dangerous risk factor for death [1–3]. Currently, hypertension affects over a quarter (26.4%) of the global adult population, and it is projected to increase [4,5]. This prevailing risk factor of hypertension led to 12.8% of deaths worldwide in 2004, and a daunting 92 million disability-adjusted life years and 7.6 million premature deaths in 2001 [6,7]. A major cause of uncontrolled hypertension is poor patient compliance. The World Health Organization (WHO) estimates that 50–70% of the hypertensive patient population does not take their anti-hypertensive medication as prescribed [8]. Hypertension, especially uncontrolled hypertension, severely elevates the risk of cardiovascular disease, ischemic heart disease, stroke, and kidney failure [9–12]. The decline in health and longevity, the potential of medical emergencies, and the projected increase in hypertension show the burden of hypertension in the world.

In Pakistan, hypertension is a significant health problem that has taken root. Over 28% (male 28.8% and female 28.0%) of adults over the age of 25 have hypertension [13]. This has increased since the early 1990s, when the percentage of hypertension in rural areas was 16.2% and that in urban areas was 21.6% [14]. Overall, Pakistan is an unfortunate world leader in the number of people who have hypertension. Interestingly, it is also a country where there is a strong presence and use of ethnobotany—especially regarding medicinal plants

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[15]. In all, Pakistan serves as an excellent region of the world, to study the interface of hypertension and plant-based remedies.

Medicinal plants as part of traditional remedies play an intricate part of human health care. Molecular evidence shows that even Neanderthals made use of medicinal plants [16–18]. Modern humans also have treated, and continue to treat, diseases with medicinal plants in the form of traditional medicine. Although percentages of traditional medicine use around the globe are difficult to pinpoint, estimates show that the majority of the population in developing nations consistently rely on traditional medicine and a large percentage, just under 50%, of the population in many developed nations, have incorporated traditional medicine into their personal health care [7,19,20].

The development of many synthetic drugs has been originated from plant compounds. Seventy-five percent of the drugs have been approved for infectious diseases between 1981 and 2002 that are originated from natural sources [21]. Additionally, some estimates show that about 25% of modern medicines has originated from tropical forest plants, and out of these, 74% were derived from plants with traditional herbal medicine use [22]. It is worth noting that out of the estimated 3,50,000 plant species on earth [23,24], about 50,000 species are used for medicinal purposes [25]. Thus, searching for new, active compounds in plants can be seen as a productive strategy for drug development [26]. Interestingly, herbal remedies are often believed to be safer than synthetic drugs [27,28]. Further, price often hinders the availability of synthetic drugs [29]. Thus, patients, including many low-income patients, may seek out and use local and traditional treatments.

In Pakistan, a large majority of the population seeks and relies on traditional medicine such as herbal medicine [30]. Significantly, out of the 6,000 flowering plants in the country, 400–600 plants are considered medicinally important [31]. Of these, many are traded both locally and on national and international markets [32].

A number of studies on medicinal plants used in the management of hypertension in different parts of the world exist [1,12,33–43], as well as in Pakistan [44–52]. In this review, we aim to analyze, identify, and describe the species and plant parts harvested for the treatment of hypertension throughout Pakistan. We do this to reveal the interplay between populations with hypertension, who, at the same time, experience monetary barriers to medical treatment.

Material and Methods

We conducted a systematic search of the literature regarding anti-hypertensive medicinal plant species in Pakistan. For this, we queried the scientific databases of Google Scholar, PubMed, Scopus, and Web of Science for the search terms: medicinal plants of Pakistan, ethnobotany of Pakistan, and "anti-hypertensive plants of Pakistan". For extra rigor, we also queried these same terms in a general search engine, Google. All pertinent articles up to December 2017 were reviewed.

In this review, we follow current taxonomy and naming of species as found in the International Plant Names Index (www.ipni.org) and The Plant List (www.theplantlist.org). We arranged and categorized species by botanical family, growth form, status, and locality.

Result

Quantitative review of ethnomedicinal plants used for hypertension

Our review is based on the publications found on Web of Science, Google Scholar, Scopus, and PubMed up to 2017 with the term "medicinal plants of Pakistan, "ethnobotany of Pakistan" or "anti-hypertensive plant of Pakistan" in the title", which resulted in 902 studies in the past 27 years (**Table 1**).

Table 1. Database with accompanying range of years and search terms.

Database	Search term	Range of year	Total articles	Selected articles
Web of Science	"Anti-hypertensive plants in Pakistan"	1990–2018	221	12
	"Ethnobotany of Pakistan"	2000–2018		25
Google Scholar	"Anti-hypertensive plants of Pakistan"	1990–2017	415	30
Scopus	"Anti-hypertensive plants of Pakistan"	1990–2018	156	8
	"Ethnobotany of Pakistan"	2000–2018		25
PubMed	"Anti-hypertensive plants of Pakistan"	2016–2017	110	7
	"Ethnobotany of Pakistan"			17
Google		1990–2016		16

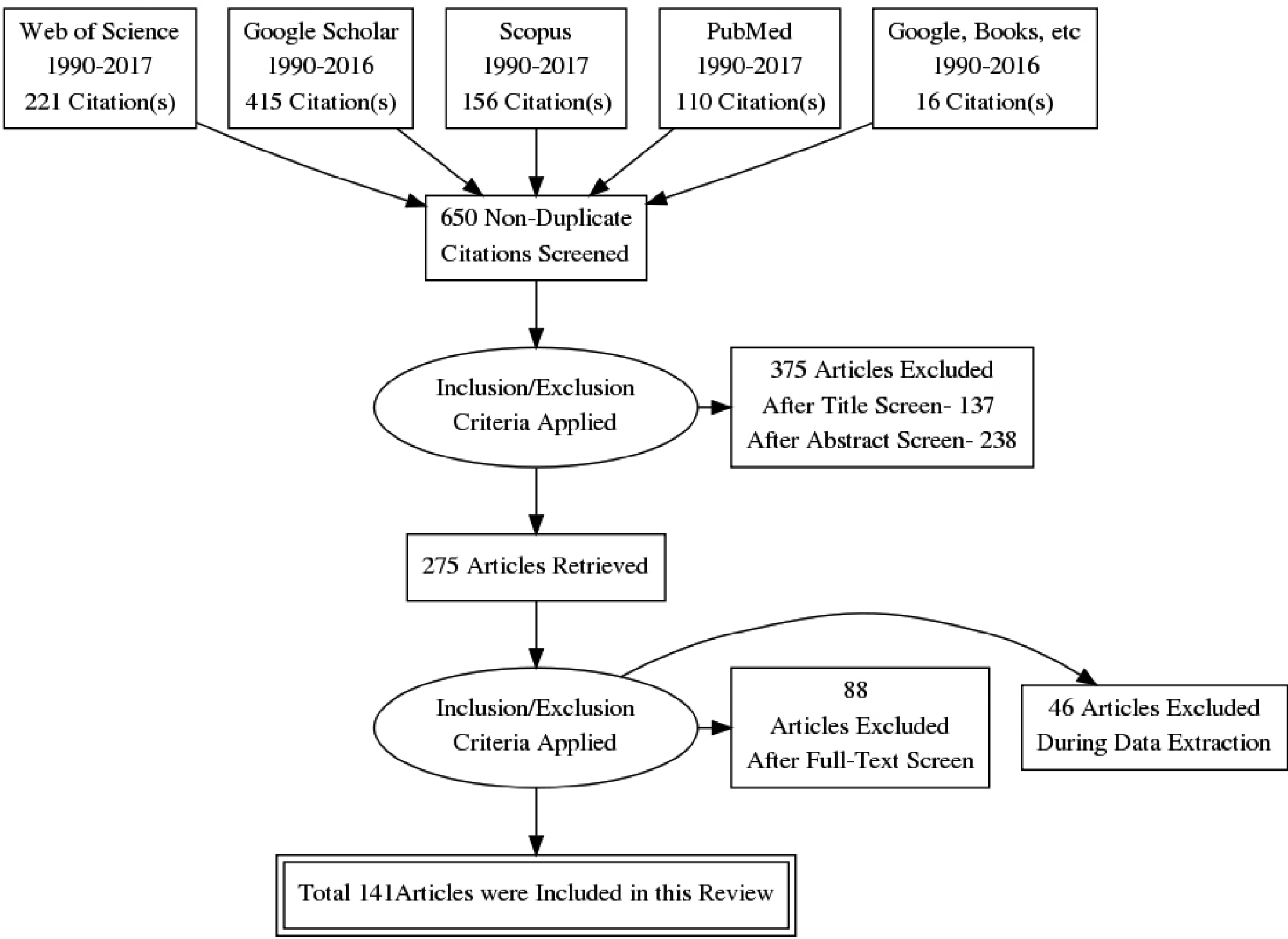

Figure 1. A flow of information through the different phases of a systematic review.

We omitted published studies outside the focus of our aim. After reading the abstracts and keywords of published articles; by focusing only on articles pertaining to hypertensive activities, medicinal plants used to treat hypertension, or ethno-botanical studies which show the species was against hypertension. This reduced the sample articles to 141 publications. Most articles were selected from Scopus; followed by Google Scholar, and Web of Science. Figure 1 represents the different phases of our methodology for our review article.

The spatial distribution of medicinal plants use for hypertension in Pakistani regions shows that the northern regions of Pakistan like Malakand Division (Dir, Swat, Chitral, Kohistan, and Buner), Hazra Division (Abbatabad, Mansehra, and Battagram), Azad Kashmir, and Gilgit-Baltistan have the most studies (Fig. 2). We suspect that this is due to the rich floristic diversity in the northern regions of the country.

Taxonomic diversity of medicinal plant

In this review, we identified 114 medicinal plant species belonging to 97 genera, and 55 botanical families that are used to treat hypertension in Pakistan (Table 2). Additionally, the frequency of citation, growth form, status, part used, and location are given (Table 2). The most dominant botanical families with more cited species included Lamiaceae (14 species), followed by Asteraceae (7 species), Apiaceae (6 species), Fabaceae (5 species), Amaryllidaceae (4 species), Myrtaceae and Brassicaceae (4 species), Poaceae (3), Solanaceae (3 species), Linaceae, Amaranthaceae, Rosaceae, Papaveraceae, Ranunculaceae, Asclepiadaceae, Chenopodiaceae, Oleaceae, Verbenaceae, Zingiberaceae, Zygophyllaceae, Scrophulariaceae, and Euphorbiaceae (2 species), while the rest of the families have one cited species each (Fig. 3).

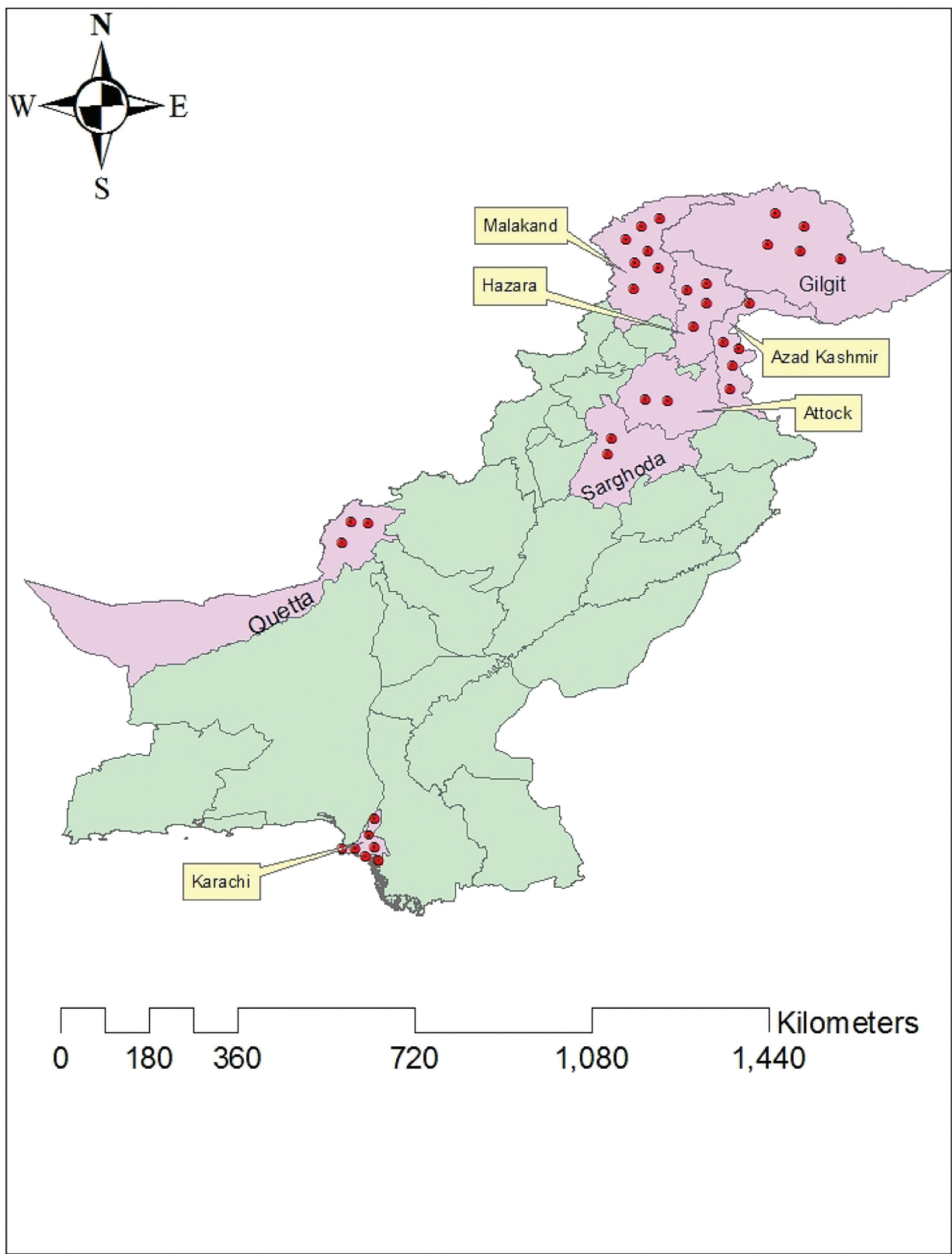

Figure 2. Geographical distribution of case studies of Pakistan in the review, red dots shows the number of anti-hypertensive case studies per division.

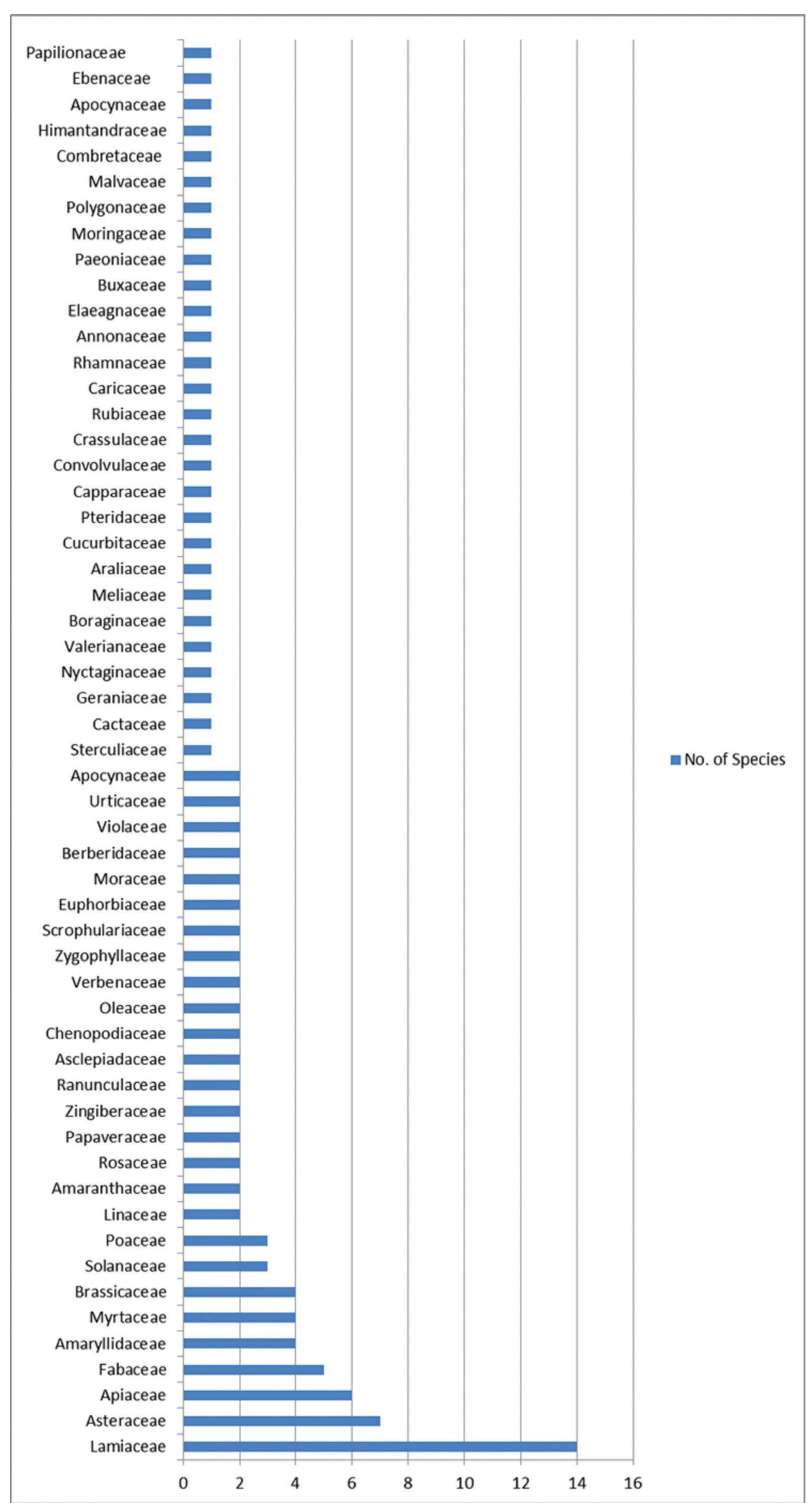

Figure 3. Number of plant species in across families.

Table 2. Ethnomedicinal plants used to relieve hypertension in Pakistan.

Family	Plant name	Growth form	Status	Part used[a]	Study area[b]	References
Lamiaceae	*Salvia bucharica* Popov	Herb		Lv, Fl	Kalat and Khuzdar, Baluchistan	[53]
	Otostegia limbata Benth. ex Hook.f.	Shrub	Wild	R, Lv	Dir, KPK	[52]
					Allai Valley	[54]
	Origanum vulgare L.	Herb	Wild	AP	Dir, KPK	[52]
	Ocimum basilicum L.	Herb	Cultivated	Lv	Dir, KPK	[52]
					Swat, KPK	[55]
	Lavandula stoechas L.	Herb	Wild	AP	Karachi, Pakistan	[56]
	Mentha viridis (L.) L.	Herb	Wild Cultivated	AP	Dir, KPK	[52]
	Mentha longifolia (L.) L.	Herb	Wild	AP		[109]
	Isodon rugosus (Wall. ex Benth.) Codd	Shrub	Wild	Lv	Bugrote valleys, Gilgit	[57]
				Lv	Toli Peer, Azad Kashmir	[114]
	Teucrium stocksianum Boiss.	Herb	Wild	WP	Dir, KPK	[52]
	Lamium album L.	Herb	Wild	YS, Lv	Swat, KPK	[58]
	Ajuga bracteosa Wall. ex Benth.	Herb	Wild	WP	Dir, KPK,	[52]
					Swat, KPK	[59]
					Leepa Valley, Azad Kashmir	[60]
				Lv	Kotli, Azad Kashmir	[115]
					Swat, KPK	[55]
					Swat, KPK	[61]
	Ajuga parviflora Benth.	Herb	Wild	Lv	Swat, KPK	[55]
	Mentha longifolia (L.) L.	Herb	Wild	WP	Dir, KPK	[52]
					Mastung, Baluchistan	[62]
	Nepeta erecta (Royle ex Benth.) Benth.	Herb	Wild	Fl	Toli Peer, Azad Kashmir	[114]
Asteraceae	*Inula royleana* DC.	Herb	Wild	R	Batagram, KPK	[63]
	Artemisia annua L.	Shrub	Wild	Lv	Dir, KPK	[52]
	Achillea millefolium L.	Herb	Wild	AP	Karachi, Sindh	[64]
	Cichorium intybus L.	Herb	Wild	Se, AP	Dir, KPK	[52]
	Artemisia vulgaris L.	Shrub	Wild	AP	Dir, KPK	[52]
				Lv	Dir, KPK	[135]
	Artemisia parviflora Roxb. ex D.Don	Shrub	Wild	Se, Lv	Chitral, KPK	[65]
	Sonchus asper (L.) Hill	Herb	Wild	AP	Sargodha, Punjab	[111]
Apiaceae	*Daucus carota* L.	Herb	Cultivated	AP	Karachi, Sindh	[66]
	Carum copticum (L.) Benth. & Hook.f. ex C.B.Clarke	Herb	Wild	PE	Karachi, Sindh	[45]
	Ammi visnaga (L.) Lam.	Herb	Wild	WP	Various study sites from	[67]
	Apium graveolens L.	Herb	Cultivated Wild	Se	Pakistan	[67]
	Coriandrum sativum L.	Herb	Cultivated	Fr	Dir, KPK	[52]
					Swat, KPK	[55]
					Karachi, Sindh	[50]
	Foeniculum vulgare Mill.	Herb	Cultivated Wild	Fr	Dir, KPK	[52]
Fabaceae	*Cassia absus* Sesse & Moc.	Shrub	Wild	Se, Lv	Gujrat, Punjab	[68]
	Medicago polymorpha L.	Herb	Wild	AP	District Tank, KPK	[69]
	Medicago sativa L.	Herb	Wild	Lv	Ghizer district, Gilgit-Baltistan	[70]
	Castanospermum australe A.Cunn. & C.Fraser	Tree	Cultivated	PE	Karachi, Sindh	[71]
	Melilotus officinalis (L.) Pall.	Herb	Wild	Fl, Lv	Ghizer district, Gilgit-Baltistan	[72]
Amaryllidaceae	*Allium jacquemontii* Kunth	Herb	Wild	Bu	Dir, KPK	[52]
	Allium sativum L.	Herb	Cultivated	Bu	Narowal, Punjab	[73]
					District Tank, KPK	[69]
					Chitral, KPK	[74]
					Swat, KPK	[56]
					Dir, KPK	[75]
				Bu	Talagang, Punjab	[136]

(Continued)

Table 2. Ethnomedicinal plants used to relieve hypertension in Pakistan. (Continued)

Family	Plant name	Growth form	Status	Part used[a]	Study area[b]	References
	Allium cepa L.	Herb	Cultivated	Bu	Attock, Punjab	[76]
	Narcissus tazetta L.	Herb	Wild	Bu	Dir, KPK	[52]
Myrtaceae	*Myrtus communis* L.	Shrub	Wild	Lv	Dir, KPK	[52]
	Psidium guajava L.	Shrub	Cultivated	Lv	Narowal,Punjab	[73]
	Eucalyptus camaldulensis Dehnh.	Tree	Cultivated	Lv	Dir, KPK	[52]
	Eucalyptus globulus Labill.	Tree	Cultivated	Lv	Dir, KPK	[52]
Brassicaceae	*Sisymbrium brassiciforme* C.A. Mey.	Herb	Wild	Se	Dir, KPK	[52]
	Raphanus sativus L.	Herb	Cultivated	Se	Karachi, Sindh	[44]
	Eruca sativa Mill.	Herb	Cultivated	WP	Abbottabad, KPK	[77]
	Nasturtium officinale R. Br.	Herb	Wild	WP	Chitral, KPK	[116]
Solanaceae	*Solanum nigrum* L.	Herb	Wild	Lv	Dir, KPK	[52]
				Lv	Dir, KPK	[135]
	Withania somnifera (L.) Dunal	Herb	Wild	R, Lv	Dir, KPK	[52]
	Capsicum annuum L.	Herb	Cultivated	Fr	Swat, KPK	[56]
Poaceae	*Arundo donax* L.	Herb	Wild	R	Bahawalnagar, Punjab	[102]
	Andropogon muricatus Retz.	Herb	Wild	Ap	Karachi, Sindh	[105]
	Desmostachya bipinnata (L.) Stapf	Herb	Wild	RT	Toli Peer, Azad Kashmir	[114]
Linaceae	*Linum usitatissimum* L.	Herb	Cultivated	Se	Chitral, KPK	[65]
	Linum perenne L.	Herb	Wild	Se	Chitral, KPK	[78]
Amaranthaceae	*Achyranthes bidentata* Blume	Herb	Wild	AP	Islamabad, Pakistan.	[79]
	Achyranthes aspera L.	Herb	Wild	Lv, Sm	D. G. Khan, Punjab	[80]
Rosaceae	*Rosa brunonii* Lindl.	Shrub	Wild	Fr	Dir, KPK	[52]
	Rosa indica L.	Shrub	Cultivated	Fl	Mastung, Baluchistan	[63]
Papaveraceae	*Papaver somniferum* L.	Herb	Wild Cultivated	Fruit	Mastung, Baluchistan	[63]
	Fumaria indica (Hausskn.) Pugsley	Herb	Wild	AP	Dir, KPK	[52]
					Swat, KPK	[55]
				AP	Dir, KPK	[135]
Zingiberaceae	*Zingiber officinale* Roscoe	Herb	Cultivated	Rz	Karachi, Sindh	[81]
	Elettsaria cardamomum (L.) Maton.	Herb	Cultivated	Fr	Karachi, Sindh	[49]
Violaceae	*Viola canescens* Wall.	Herb	Wild	AP	Dir, KPK	[52]
	V. odorata L.	Herb	Wild	AP	Karachi, Sindh	[103]
Berberidaceae	*Berberis lycium* Royle	Shrub	Wild	Rz	Dir, KPK	[52]
				Rz	Kotli, Azad Kashmir	[115]
	Berberis vulgaris Royle	Shrub	Wild	Rz	Chitral, KPK	[116]
Moraceae	*Morus nigra* L.	Tree	Wild	Fr	Abbottabad, KPK	[77]
	Ficus carica L.	Shrub	Wild	Fr	Sargodha, Punjab	[117]
Ranunculaceae	*Nigella sativa* L.	Herb	Wild Cultivated	Se	Karachi, Sindh	[82]
	Clematis orientalis L.	Shrub	Wild	R	Azad Kashmir, Pakistan	[83]
Asclepiadaceae	*Caralluma tuberculata* N.E.Br.	Herb	Wild	AP	Dir, KPK	[52]
					Waziristan agency, Pakistan	[84]
					Mastung, Baluchistan	[62]
					Kalat and Khuzdar, Baluchistan	[53]
	Calotropis procera (Aiton) Dryand.	Shrub	Wild	WP	Islamabad, Pakistan.	[85]
Chenopodiaceae	*Chenopodium botrys* L.	Herb	Wild	AP	Dir, KPK	[52]
	Chenopodium ambrosiodes L.	Herb	Wild	Lv	Dir, KPK	[52]
Oleaceae	*Olea europaea* L.	Tree	Cultivated	FR	Karachi, Sindh	[46]
	Olea ferruginea Royle	Tree	Wild	Lv	Dir, KPK	[52]
Verbenaceae	*Phyla nodiflora* (L.) Greene	Herb	Wild	Lv	Jhelum, Punjab	[86]
	Verbena officinalis L.	Herb	Wild	Lv	Dir, KPK	[52]
Zygophyllaceae	*Peganum harmala* L.	Herb	Wild	PE	Karachi, Sindh	[87]
	Tribulus terrestris L.	Herb	Wild	Lv	Dir, KPK	[52]
Scrophulariaceae	*Scrophularia deserti* Delile	Herb	Wild	R	Mansehra, KPK	[88]
	Pedicularis punctata Decne.	Herb	Wild	Fl	Deosai, Gilgit-Baltistan	[89]

(Continued)

Table 2. Ethnomedicinal plants used to relieve hypertension in Pakistan. (Continued)

Family	Plant name	Growth form	Status	Part used[a]	Study area[b]	References
Euphorbiaceae	*Euphorbia thymifolia* L.	Herb	Wild	WP	Thar Desert, Sindh	[90]
	Euphorbia hirta L.	Herb	Wild	WP	Dir, KPK	[52]
Sterculiaceae	*Firmiana simplex* (L.) W.Wight	Tree	Wild	Gum, Se	Thar Desert, Sindh	[90]
Cactaceae	*Opuntia dillenii* (Ker Gawl.) Haw.	Herb	Wild	Sm	Dir, KPK	[52]
Geraniaceae	*Geranium wallichianum* D.Don ex Sweet	Shrub	Wild	Rz	Waziristan agency, Pakistan	[84]
					Chitral, KPK	[78]
					Mohmand Agency, Pakistan	[141]
Nyctaginaceae	*Boerhavia diffusa* L.	Herb	Wild	AP	Azad Kashmir, Pakistan	[91]
Valerianaceae	*Valeriana wallichii* DC.	Herb	Wild	Rz	Karachi, Sindh	[47]
Boraginaceae	*Onosma hispida* Wall. ex G. Don	Herb	Wild	AP	Dir, KPK	[52]
Meliaceae	*Melia azedarach* L.	Tree	Cultivated Wild	Fr	Batagram, KPK	[53]
Araliaceae	*Hedera nepalensis* K.Koch	Shrub	Wild	Lv	Dir, KPK	[52]
					Malakand, KPK	[92]
					Swat, KPK	[93]
Cucurbitaceae	*Memordica charantia* L.	Herb	Cultivated	Fr	Malakand, KPK	[92]
Pteridaceae	*Adiantum capillus-veneris* L.	Herb	Wild	WP	Allai Valley	[55]
Convolvulaceae	*Cuscuta reflexa* Roxb.	Herb	Wild	WP	Dir, KPK	[52]
					Karachi, Sindh	[94]
Capparaceae	*Capparis cartilaginea* Decne.	Shrub	Wild	PE	Karachi, Sindh	[95]
Crassulaceae	*Bryophyllum pinnatum* (Lam.) Oken	Herb	Wild	Le	Azad Kashmir, Pakistan	[96]
Rubiaceae	*Morinda citrifolia* L.	Tree	Cultivated	R	Karachi, Sindh	[51]
Caricaceae	*Carica papaya* L.	Tree	Cultivated	Se, Fr		[97]
Rhamnaceae	*Ziziphus oxyphylla* Edgew.	Shrub	Wild	R	Dir, KPK	[52]
					Swat, KPK	[61]
					Poonch Valley, Azad Kashmir	[98]
Annonaceae	*Polyalthia longifolia* (Sonn.) Thwaites	Tree	Cultivated	Sm, R	Lodhran,Gujrat, Punjab	[99] [100,108]
Elaeagnaceae	*Hippophae tibetana* Schltdl.	Shrub	Wild	Lv, Fr	Gilgit-Baltistan	[71]
Buxaceae	*Sarcococca saligna* Müll.Arg.	Shrub	Wild	Lv, R	Dir, KPK	[52]
				Lv	Chiral, KPK	[116]
Paeoniaceae	*Paeonia emodi* Royle	Herb	Wild	Rz	Dir, KPK	[52]
Moringaceae	*Moringa oleifera* Lam.	Tree	Cultivated	Gum	Thar Desert, Sindh	[90,104]
Polygonaceae	*Polygonum hydropiper* L.	Herb	Wild	Lv	Gilgit, Gilgit-Baltistan	[101]
Malvaceae	*Malva neglecta* Wallr.	Herb	Wild	WP	Mastung, Balochistan	[63]
Urticaceae	*Forrskalea tenacissima* L.	Herb	Wild	WP	Dir, KPK	[106]
Apocynaceae	Ervatamia coronaria (Jacq.) Stapf	Herb	Wild	LV	Karachi, Sindh	[112]
	Gymnema sylvestre (Retz.) R.Br. ex Sm.	Shrub	Wild	LV	Sarghoda, Punjab	[118]
Combretaceae	Terminalia actinophylla Mart.	Tree	Wild	Fr	Karachi, Sindh	[107]
Orchidaceae	Orchis mascula (L.) L.	Herb	Wild	Plant extract	Karachi, Sindh	[113]
Ebenaceae	Diospyros kaki L.f.	Tree	Wild	LV	R Sawabi, KPK	[119]
Leguminosae	Trigonella foenum-graecum L.	Herb	Wild	Plant extract	Karachi, Sindh	[120]

Parts used[a]: Leaves = Lv, Seed = Se, Fruit = Fr, Stem = Sm, Aerial part = AP, Whole plant = WP, Bulb = Bu, Flower = Fl, Rhizome = Rz, Root = R, Plant extract = PE, Study area[b]: KPK = Khyber Pakhtunkhwa.

Life form and status of medicinal plants treating hypertension

In this review, we categorized medicinal species as herb, shrub, or tree life forms (Fig. 4). Herbaceous plants dominated the life form of medicinal species used in remedies. The predominant use of herbaceous plants by local patients may be due to their availability and ease of collection [121].

This review identified 71% wild and 29% cultivated medicinal species**.** Almost all tree plants used to treat hypertension were cultivated, except *Olea ferruginea* Royle and *Firmiana simplex* (L.) W.Wight.

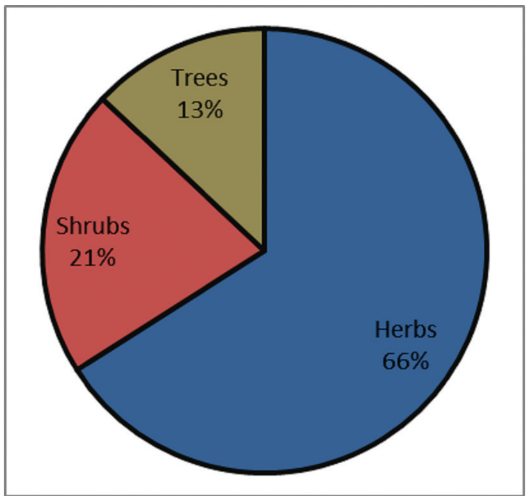

Figure 4. Life form of medicinal plants for the treatment of hypertension.

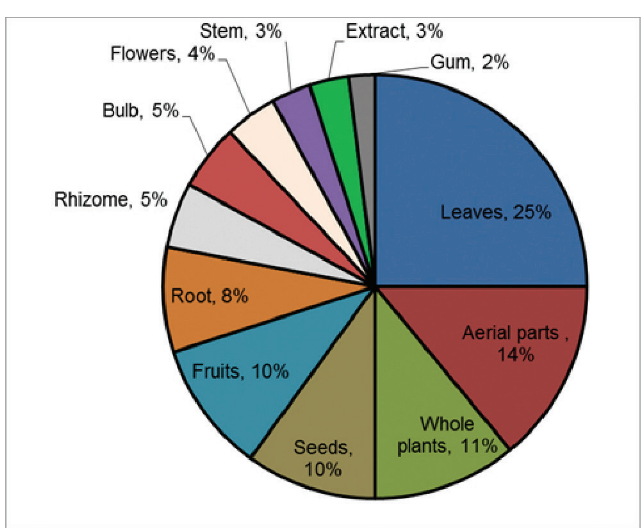

Figure 5. Percentage of plant parts used for herbal preparations to treat hypertension in Pakistan.

Some herbs present in the wild habitat can be cultivated, such as *Mentha viridis* (L.) L., *Papaver somniferum* L., *Nigella sativa* L., *Foeniculum vulgare* Mill., and *Apium graveolens* L.

Medicinal plant parts

Leaves along with aerial plant parts constituted the majority of usage specified. The whole plant, seeds, fruits, roots, and bulbs trailed with lesser use frequency (Fig. 5). This is promising since some research suggests that the use of aerial plant parts and leaves is sustainable with a minimal environmental impact and harm to plant populations [122].

Discussion

Hypertension is a significant public health problem, with a global prevalence of 40.8% and a control rate of 32.3%. Moreover, it is an increasingly common public health problem in low-income developing countries [123]. In the case of Pakistan, the prevalence of hypertension was found to be 19.1% [124].

Most of the local rural inhabitants in Pakistan use medicinal plants for curing hypertension, due to easy availability in these areas, while in addition the allopathic drug shops are scarce, and out of reach for the rural poor. In Pakistan, there are almost 50,000 herbalists scattered in the whole country. They are running their clinics both in rural and urban areas by using medicinal plants with minimal processing form [125].

The review reports the highest number of anti-hypertensive plants (63%) from Northern Pakistan—the Pan-Himalaya region (Figure 2). The majority of studies were reported from Northern Pakistan because of the many traditional practices and rich floral diversity. Understanding and applying the indigenous knowledge of mountain communities for biodiversity resource management is one of the key elements for achieving sustainable development [126]. The local people of Pakistan use at least 114 medicinal plants for the management of hypertension. The majority of the Pakistani population lives in rural villages [127]. Also, approximately 75% of that population relies on traditional medicines to treat ailments [128]. Much of the population lives at a great distance from medical facilities and often cannot afford the high price of health care [129,130]. Correspondingly, many of these people turn to herbal remedies for their health care needs.

In this review, we focused on ethnobotanical and ethnopharmacological uses of medicinal plants for hypertension treatment. Some of these plants have active ingredients and documented pharmacological anti-hypertensive effects and mechanism. These plants are: *Lavandula stoechas, Daucus carota, Carum copticum, Coriandrum sativum, Castanospermum australe, Allium sativum, Raphanus sativus, Eruca sativa, Zingiber officinale, Elettsaria cardamomum, Nigella sativa, Olea europaea, Peganum harmala, Valeriana wallichii, Cuscuta reflexa, Capparis cartilaginea, Carica papaya, Mentha longifolia, Sonchus asper, Moringa oleifera, Andropogon muricatus, Viola odorata, Forrskalea tenacissima, Terminalia bellerica, Polyalthia longifolia var. pendula, Galbulimima baccata,* and *Orchis mascula* represented in Table 2.

Medicinal herbs play a very important role in the development and discovery of drugs. They are a

leading basis for the development of pharmaceuticals [131]. Often, synthetic drugs are very expensive and a large population cannot pay for these medicines.

According to a report in 2011, in the United States, the total costs associated with hypertension were $46 billion in health care services, missed days of work, and medications [132]. In Cameroon, it was reported by Nole et al. [133] that in cases of non-complicated arterial hypertension the diabetic patients', with arterial hypertension, monthly cost of treatment was 60,500 Central African CFA franc Fcfa (1 US $ = 528 Fcfa), and certain pharmaceutical products and their cost like Actrapid HM (14,510 Fcfa), Lodoz (10,415 Fcfa) Insulatard HM (14,525 Fcfa), and Hexen 50 (13,860 Fcfa), etc., are rare in rural zones or expensive for patients. At the same time, using these anti-hypertensive drugs can cause numerous adverse effects and reactions, including orthostatic hypotension, dizziness, headache, and also decreased sexual function [134].

In China, the study of Chinese herbal formulas, for the management of hypertension, is an important area of research within integrative medicine and Traditional Chinese Medicine [134]. It was reported that approximately 80% population of Morocco and 21% population of Nigeria use alternative and complementary medicine in the management and treatment of hypertension [37,137]. In 2003, it was reported that about 63.9% of hypertensive patients use traditional herbal medicine in India [138]. Attempts by the low-income groups in rural areas of developing countries to control hypertension and its complications, when faced with scarce economic resources, often include adopting herbal remedies [139]. In many parts of the world, people use ethnomedicinal plants for the treatment of high blood pressure, because of its easy availability, less expensive, and also, due to lack of other health facilities. Such plants like Catharanthus roseus, Allium cepa, Momordica charantia, Azadirachta indica, Ocimum spp., Zanthoxylum spp. [133], Scutellaria spp., Achyranthis spp., Eucommia spp., Scrophularia spp., Epimedium spp., Cassia spp., Allium sativum, [134], and Mentha spp., Artemisia spp., Ajuga bracteosa, Ocimum basilicum, Foeniculum vulgare, Berberis lycium [52] are easily available in the wild as well as in cultivated habitats and make an attractive choice for the treatment of hypertension.

Hypertension affects both developed and underdeveloped countries. Barriers to health care combined with a strong tradition of medicinal plant use make Pakistan an excellent model for research on ethnomedicine. According to the National Health Survey of Pakistan, nearly 18% of all adults and 33% of all adults above 45 years old in Pakistan suffer from hypertension. In another study, it was reported that 18% of people in Pakistan were affected from hypertension with every third person over the age of 40 becoming susceptible to an extensive range of diseases [130–140].

Limitations, Conclusion, and Future Perspective

In this review, we reviewed ethno-botanical reports of species used for hypertension in Pakistan. These types of data are obtained from the authors conducting studies in the field, while interviewing community members such as elders of indigenous communities. These reports provide the parts of the plants used, mode of preparation of herbal medicines, and other traditional practices. In most cases, there are no experimental comparisons reported on these plants. So, potential future experiments or clinical studies are inferred from examining traditional collective knowledge of plant use. Having said that, this review brings to light the 30 (26% of resulting species) species which have been tested in animal models. While these articles delve into the mode and sometimes for the mechanism of action of these species, the phytochemistry is very limited. It is yet to be seen that what active compounds are present in these species that provide the rationale for age-old ancestral use for controlling hypertension.

The inexpensive nature of herbal medicine allows the low-income households access to health care. This is reflected in studies that show that a higher percentage of people in developing countries compared to developed countries rely on traditional medicine [19]. This is especially true in Pakistan, where a high percentage of the population lives in remote rural settings and access to health care is often cost prohibitive [126,129,130]. Given the alarming nature of hypertension trends around the globe, especially in developing nations, Pakistan served as an excellent model to investigate the interface between anti-hypertensive medicinal plants and the humans who depend on them. In this review, we identified 114 medicinal plant species that were used by people across Pakistan for the management of hypertension. The promotion and planting of these commonly accepted species may provide a benefit to people who cannot afford and/or may not be compliant with synthetic medications. Ethno-botanical studies of medicinal plant species used to treat hypertension in various regions of Pakistan suggest that people actively seek alternative treatments for

hypertension. Therefore, well-planned mechanistic studies on these species should be carried out in future work, along with studies on safety and toxicity.

Conflict of interest

The authors declare that there are no conflicts of interest.

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ORIGINAL ARTICLE

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Maternal toxicity and post-implantation assessments in rats gestationally exposed to *Polyscias fruticosa* leaf extract

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ABSTRACT

Background: *Polyscias fruticosa* (L.) Harms is used as a folk medicine across Afro-Asian regions of the world. Safety of *P. fruticosa* use in pregnancy remains completely unknown despite its extensive usage.
Objective: The study assessed maternal and post-implantation loss in pregnant rats gestationally exposed to *P. fruticosa* leaf extract (PFE).
Methods: Healthy female Wistar rats were co-habited (female:male; 6:2) with healthy fertile males. Confirmed pregnant rats were randomly re-assigned to normal saline (control, 5 ml/kg *po*), folic acid (5 mg/kg *po*), and PFE (100, 200, and 500 mg/kg *po*) and treated once daily for 15 gestational days. Maternal toxicity was assessed by cage-side observations, feed consumption, weight loss, relative organ/bodyweight ratio, biochemical assessment of liver and kidney function, morbidity, and death. Embryotoxicity was assessed by gross embryo assessment, embryo resorption, and brain/spinal cord histology.
Results: Two dams died from PFE (500 mg/kg) group. Feeding decreased across all groups as gestation progressed. No weight loss across all groups. Aspartate transaminase increased in PFE (200 and 500 mg/kg) relative to control. Alkaline phosphatase decreased ($P \leq 0.05$) in PFE compared to control. Direct bilirubin increased in PFE groups relative to control. Urea decreased in PFE groups relative to control. Creatinine levels decreased in PFE (100 mg/kg) relative to control but increased in PFE (500 mg) compared to control. Post-implantation loss increased in PFE (500 mg/kg) relative to control.
Conclusion: Gestational exposure to PFE (>100 mg/kg) produced risk of post-implantation loss and renal injury in dams; therefore, PFE should be avoided in pregnancy.

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Introduction

Use of herbal products, especially herbal medicines has increased immensely [1–3]. In poorer tropical regions of the world, such as in some parts of Africa and Asia, herbal medicine continues to be the main source of primary health care for most populations [4–6], while in the developed world it is used as a complement to conventional medical care. Many reports show that sensitive populations such as pregnant mothers use herbal products in one way or the other to manage their pregnancies [7–9] with most of them having the belief that herbal products are safe alternatives of the conventional medical care [10]. For example, a survey revealed that well over 52% of expectant mothers used herbal products as a complement to other health care to manage their pregnancies [7,8,11]. In a study involving 374 herbal medicine practitioners, 74.3% were of

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the view that herbal medicines do not have adverse effects because it is organic, while 24.1% admitted that herbal medicines may produce adverse effects and that some of their clients reported adverse outcomes [10]. It is in response to these health threats from the use of herbal products that the World Health Organization has strongly recommended inclusion of herbal medicines in existing national and regional pharmacovigilance systems [10]. Further experts in the herbal product industry have advised users of herbal products to exercise maximum caution [12,13], since improper use of herbal products just like any other drug could pose serious health risk. Despite all these efforts to regulate and reduce herb-related adverse events, evidence still shows that hand-in-hand increased use of herbal products is accompanied with high incidence of herb-related adverse events [14,15], especially among sensitive groups. For example, use of some herbal products has been identified with reproductive toxicity [16–18] and embryo toxicity [19]. It was reported that a number of herbs used as part of Chinese herbal medicine to treat threatened abortions had no safety data with respect to maternal, reproductive, and developmental toxicity [13,20]. Hitherto herbs thought to be safe are now identified with developmental toxicity in rats [14,21]. A recent report showed that two-thirds of emergency cases in the US were attributed to the use of herbal products [22].

Many factors underlie the problem of herb-related adverse events in addition to the general belief that it is organic in nature and therefore safe [10]. Other factors include the high potential of herbal products to be self-medicated [5,8], especially in some African countries, where traditional and cultural beliefs strongly influence its use [4]; the seeming lack of research data on herbal products to inform proper regulation and lack of safety data on most commonly used herbal products [7,23].

Scientific evaluation of herbal medicines in the light of their ethnobotanical context of use in Africa and in Ghana in particular has improved substantially. Nonetheless, these improvements have overly focused on efficacy assessments to the neglect of safety. One of such important herbal plants with poor safety assessment is *Polyscias fruticosa* (L.) Harms (Syn. *Panax fruticosa* L., *Nothopanax fruticosa* Miq.; family: Araliaceae). *Polyscias fruticosa* is put to many ethnobotanical uses across Afro-Asian regions of the world. In Ghana, *Polyscias fruticosa* leaf preparation is drunk to treat inflammatory and upper respiratory disorders among all class of people. To scientifically confirm or otherwise, it was shown that ethanol leaf extract of *P. fruticosa* exhibited anti-inflammatory, muco-suppressant, anti-tussive, and anti-asthma properties [24,25]. Aside Ghana, various crude preparations of *P. fruticosa* are used in Asia as tonic, anti-inflammatory, anti-toxin, anti-bacteria as well as a spice and a digestion agent [26]. As an example in Vietnam, apart from been eaten as salad, it is also used as a tonic to treat inflammation, ischemia, and to improve brain vasculature [27]. Also, a root extract of *P. fruticosa* is documented as a diuretic, febrifuge, treatment for dysentery, neuralgia, and rheumatic aches [28]. Ecologically, *Polyscias fruticosa* (L.) is widely distributed across Eastern Asia, Tropical islands of the Pacific Region [26] as well as in some African countries including Ghana [24,25]. Phytochemical fingerprints of *P. fruticosa* have been elaborated. Notably two oleanoic acid, saponins and polyacetylenes, were respectively isolated from leaves [29] and roots of *P. fruticosa* [26]. Also, three bisdesmosidic saponins were isolated from leaves of *P. fruticosa*, of which the first saponins was shown to exert inhibitory effect on α-amylase and α-glucosidase activity, and was thus speculated to be useful for diabetes treatment [27].

Although many studies have evaluated the pharmacological properties of *Polyscias fruticosa* in the light of various ethnobotanical context of use [24,25,27], these studies overly focused on efficacy of *P. fruticosa* but not its safety, especially in reproduction. It still remains to be established the safety of *P. fruticosa* with respect to maternal and embryo-fetal toxicity. This apparent research gap came to light upon a thorough PubMed and Google Scholar searches. To bridge this research gap and to provide rationale for follow-up studies on *P. fruticosa* as a potential herbal drug, this study investigated maternal and embryo-fetal toxicity in rats after gestational exposure of rats to crude *P. fruticosa* leaf extract (PFE).

Materials and Methods

Collection, identification, and authentication of medicinal plant

Leaves of *Polyscias fruticosa* were collected from the botanical gardens of Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. Leaves were identified and authenticated at the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi, Ghana. A voucher specimen (KNUST/

HM/13/W010) [25,30] was deposited at KNUST herbarium for future reference.

Preparation of Polyscias fruticosa *leaf extract*

Fresh *Polyscias fruticosa* leaves (2.5639 kg) were washed with clean water, shade-dried completely, and pulverised into powder (1.8444 kg). The powder was soaked in absolute ethanol (4.8 L) in a volumetric flask for 72 hours, and then filtered. Ethanol was retrieved from the dark-green filtrate in a rotary evaporator (B'U'CHI Olibath B-485) at 50°C. The residue was dried completely in a desiccator containing activated silica gel. The crude extract yielded 62.979 g, given a percentage yield of 3.414%. The final crude extract obtained, was named PFE and was referred to as PFE throughout the study. Subsequently, PFE was fractionated into ethyl acetate, petroleum ether, and ethanol fractions. Thin layer chromatography (TLC) was conducted on each fraction to determine component spots and retardation factor by following a previous method [31] with some modifications. The ethanol fraction was used in all experiments. Subsequently, the ethanol fraction was analysed by using a gas chromatography–mass spectrometry (GC–MS) technique under the following operating conditions: [Injector-1177 (splitless mode); Injector temperature-250°C; Injection volume-2 µl; Carrier Gas––Helium at Constant flow of 1.0 ml/minute; Analytical Column––Hewlett Packard-5 30 m, 0.25 mm, 0.25 µm; MS Acquisition range 30–650 m/z; Ionization mode–electronic ionisation; and column oven temperature (250°C), rate (10°C/minute), hold (7), and total (30)].

Acquisition of experimental animals and husbandry

Healthy 8 weeks old, female (150–180 g) and male (120–200 g) Wistar rats were purchased from the animal breeding unit of Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana. To afford adequate acclimatization, experimental animals were kept at the animal house of the School of Biological Sciences, University of Cape Coast for 2 weeks before the start of experiments. All experimental animals were maintained under ambient conditions of temperature, relative humidity, and 12 hours light/dark cycle, and housed in sanitized aluminium cages (20 × 15 × 8 cm) with base dressing of sawdust as bedding. They were given access to standard pellet diet (Grower Mash, Essaar, Ghana) and water *ad libitum*; however, these conditions were varied where necessary to meet specific experimental requirements. Experimental animals were handled in strict compliance with the guide for the Care and Humane Use of Laboratory Animals (National Research Council, 1996) as well as specific institutional and national guidelines on the humane use and handling of experimental animals in scientific experimentation.

Co-habitation and confirmation of pregnancy

Healthy dams without confirmed stage of oestrous cycle were co-habited with confirmed fertile male rats at a female:male ratio of 6:2. Post-coitus confirmation of pregnancy was done by detection of a vaginal copulatory plug as previously described [4,32]. All confirmed pregnant dams were randomly re-assigned to one of the five treatment groups. The day of detection of vaginal copulatory plug was considered day 1 of pregnancy.

Group allocation and bodyweight measurements

Experimental animals were randomly assigned to one of the five groups {control [normal saline (NS), 5 ml/kg], folic acid (5 mg/kg), PFE (100, 200, and 500 mg/kg, respectively)}. Each group had four dams except PFE (200 mg/kg) which had five dams. Bodyweight of dams were measured before treatment started and also at gestational days (GDs) 5, 10, and 15. Dams in each group were uniquely labelled.

Experimental design and dose administration

Dams in each group were dosed by oral gavage. Dosing was done once every morning at 8.30 am from GD 1–14 of gestation. Dams in the respective groups were treated as shown below:

Control group (5 ml/kg of NS *po*) once daily + free access to food and water

PFE (100 mg/kg *po*) once daily + free access to food and water

PFE (200 mg/kg *po*) once daily + free access to food and water

PFE (500 mg/kg *po*) once daily + free access to food and water

Folic acid (5 mg/kg *po*) once daily + free access to food and water

Measurement of food consumption

Feed for dams in each group was quantified before, and then re-quantified 24 hours later to determine average food consumption per day/group. Average

Table 1. TLC analyses of PFE.

Ethanol extract					
Solvent system	**Ratio**	**Resolution**	**Number of spots**	**Rf**	***Spots colour**
Ethanol:Ethyl acetate	1:5	Good	4	0.95	Light green
				0.73	Light yellow
				0.32	Light brown
				0.12	Light brown
		Solvent distance = 7.7 cm			
Ethanol:Ethyl acetate	1:3	Not well resolved	—	—	—
Ethanol:Ethyl acetate	1:1	Not well resolved	—	—	—

*Rf = retardation factor. Under ultra violet light (254 nm).

food consumption for each group at 5, 10, and 15 GDs was determined and compared statistically.

Cage-side observations

Dams in each group were inspected twice (morning and afternoon) daily for changes in skin, fur, eyes, mucous membrane, piloerection, aversion to handling and breathing as previously described [4,33]. Only behavioural and signs that occurred consistently in the morning and afternoon were considered to be related to treatment; and therefore, could be of toxicological significance.

Determination of mortality and morbidity among dams

Dead and moribund dams were determined for each treatment group at the end of treatment. Morbidity was defined to mean any animal that displayed a spectrum of physical or behavioural signs that impaired mobility, feeding, and response to touch.

Biochemical analysis

After anaesthesia followed by surgical opening of each dam as previously described [33], blood samples were collected from the inferior vena cava, processed, and analysed for liver and kidney function determinants as previously described [33].

Determination of relative organ weights

Heart, liver, and kidney (both right and left) were isolated from each dam, weighed, and inspected for any gross lesions of toxicological relevance. Organ to bodyweight ratios were determined for dams in each group and compared.

Post-implantation assessment

At GD 15, dams were euthanized by intravenous injection of sodium pentobarbital (40 mg/kg), caesarean-sectioned, gravid uterus isolated, weighed, surgically opened, and contents examined as previously reported [33]. Total implantation sites, number of embryos, and number of resorptions were determined for animals in each group. Post-implantation loss (the difference between total implantation sites and number of embryos) and % post-implantation loss (number of resorptions/total implantation sites × 100) were determined for each group. Embryos were removed, counted, and weighed. Embryos were closely inspected for external abnormalities as previously described [34,35].

Histological assessment of embryo-fetal brain and spinal cord

At GD 15, dams in each group were weighed, anesthetised, sacrificed, and caesarean-sectioned. Gravid uteri were isolated and weighed. Foetuses were harvested, inspected grossly, and fixed in Bouin's solution followed by physical examinations as previously reported [33]. Representative brain and spinal cord samples from each group were collected fixed, sectioned, followed by hematoxylin and eosin staining as previously described [36]. Three independent histo-pathologists microscopically studied the histological sections and final description of histological sections was by consensus.

Statistical analysis

Statistical analysis on data was done by using Graph Pad Prism Version 6 for Windows (Graph Pad Software, San Diego, CA). Some of the data were presented in tables as mean ± standard deviation (SD). Mean comparison between groups was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. $P \leq 0.05$ was considered statistically significant in all analyzes.

Table 2. GC–MS analysis of PFE showing target compounds.

Peak name	RT	Quan ions	Area	Amount/Rf
Trans-, alpha, -Bergamoten	14,793	119.0	5,967	5,967 counts
1 H-3a, 7-Methanoazulene	16,039	107.1	3,598	3,598 counts
Hematoporphyrin ix	16,313	202.9	1,189	1,189 counts
3,7,11,15-Tetramethyl-2-	18,638	67.0	7,960	7,960 counts
5-Nonadeeen-1-ol	18,891	67.0	1,619	1,619 counts
3,7,11,15-Tetramethyl-2-	19,079	67.0	2,306	2,306 counts
Falcarinol	20,625	91.2	4,6378	46,378 counts
3,7,11,15-Tetramethyl-2-	21,340	41.2	8,305	8,305 counts

Rf = retardation factor; RT = retention time.

Results

Phytochemical analyzes of PFE

Saponins, alkaloids, cyanogenic glycosides, and sterols were identified in PFE. TLC analysis showed four spots (Table 1). GC–MS profiling of PFE showed 12 peaks out of which 8 were identical to library compounds (Table 2).

Effect of treatments on maternal food consumption

Across all treatment groups, food consumption declined from GD 1 to 15. Although there were differences in food consumption among the groups; however, the differences were not statistically significant ($P \geq 0.05$) (Fig. 1).

Effect of treatments on maternal bodyweight gain

Although PFE (200 mg/kg)-treated dams had a higher net maternal bodyweight gain compared to control dams (Fig. 2); however, the mean difference was not statistically significant ($P \geq 0.05$). PFE (100 and 500 mg/kg) had lower maternal net bodyweight gain compared to control dams but the mean difference was not statistically significant ($P \geq 0.05$). Comparatively, folic-acid treated dams had the highest net maternal bodyweight gain compared to control and PFE groups; and the mean difference was statistically significant ($P \leq 0.05$) (Fig. 2). Also, there were no significant ($P \geq 0.05$) differences between mean organ (liver, heart, and kidney) weights at GD 15 between control dams and treatment groups (PFE and folic acid). Across all groups, right kidney weights were higher than left kidney weights. Although there were differences between the relative organ weights of control and treatment groups (PFE and folic acid), these differences were not statistically significant ($P \geq 0.05$) (Table 3).

Effect of treatments on behaviour and physical activity of dams

There were no treatment-related changes in physical activity among control dams and treatment

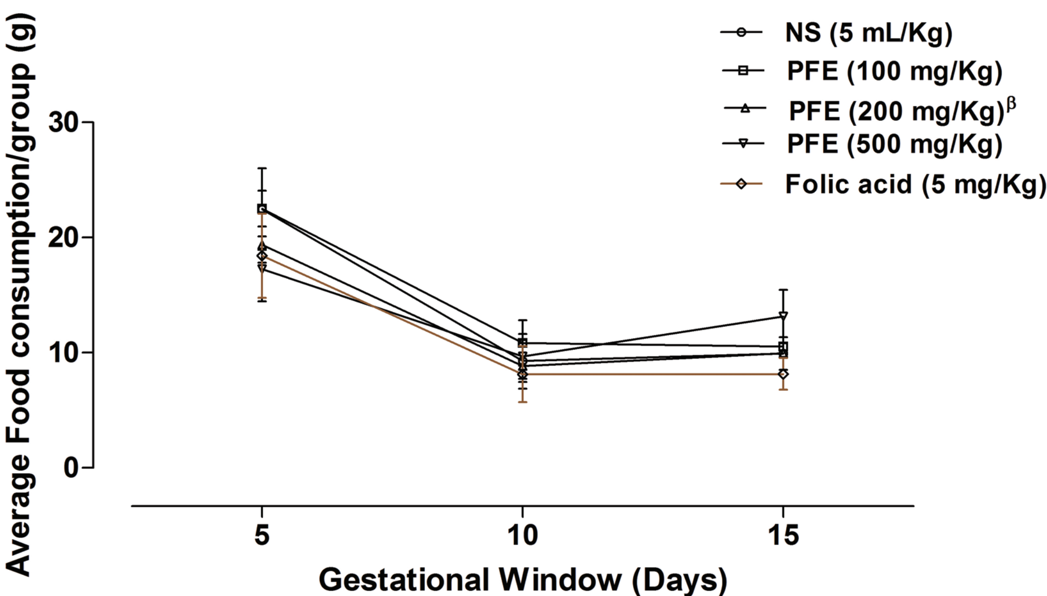

Figure 1. Effect of treatments on food consumption at GDs 5, 10, and 15. Each point is the mean ± SD of the amount of feed (g) consumed at each gestational window; $n = 4$; $^{\beta}n = 5$.

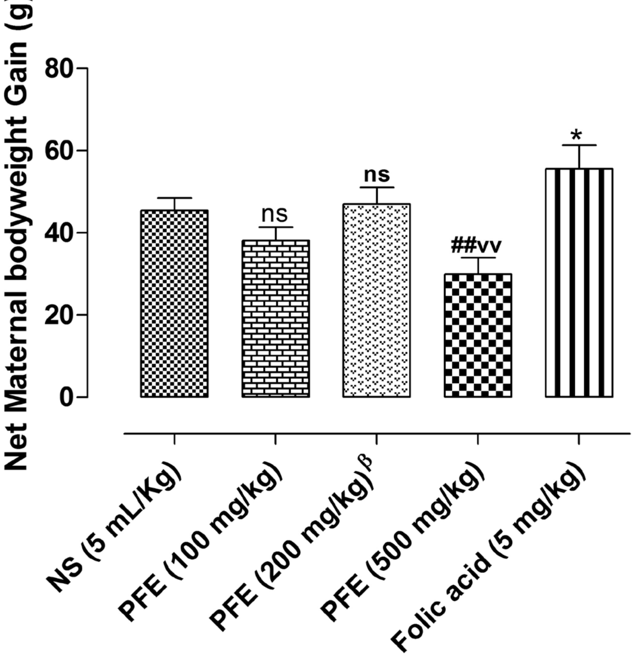

Figure 2. Effect of treatments on net bodyweight gain at GD 15; each bar is the mean ± SD weight gain by dams in each treatment group. $N = 4$; $^{\beta}n = 5$. $P \leq 0.05$ was considered statistically significant in all analysis. $^{vv}P \leq 0.01$ (Folic acid *vs*. PFE); $^{\#\#}P \leq 0.01$ (NS *vs*. PFE); * $P \leq 0.05$ (NS *vs*. Folic acid); ns = not statistically significant ($P > 0.05$).

groups (PFE 100 and 200, and folic acid) except in PFE (500 mg/kg), where two dams were found to be weak, non-responsive to physical touch. No pilo-erection was observed in all groups except the two moribund dams in PFE (500 mg/kg) group. At GD 10, the two moribund dams in the PFE (500 mg/kg) group died leading to a 50% mortality compared to 100% survival rate for control, PFE (100 and 200 mg/kg), and folic acid groups over the same treatment period (Table 4). Autopsy report on the two dead dams from PFE (500 mg/kg) treatment group by two independent pathologists did not implicate PFE treatment but attributed their deaths to mishandling during drug administration.

Effect of PFE treatment on liver function

Alanine transaminase (ALT) was comparable across all treatments. Aspartate transaminase (AST) significantly ($P \leq 0.05$) decreased in low dose PFE (100 mg/kg) group relative to control, but marginally increased in median and high dose PFE (200 and 500 mg/kg) groups relative to control and folic acid groups (Table 5). Total protein (TP) and globin (GLO) marginally increased in low-and median-dose PFE (100 and 200 mg/kg) groups relative to control and folic acid groups but decreased in high-dose PFE (500 mg/kg) relative to control. Albumin (ALB) marginally increased in PFE (100–500 mg/kg) groups relative to control. Both total

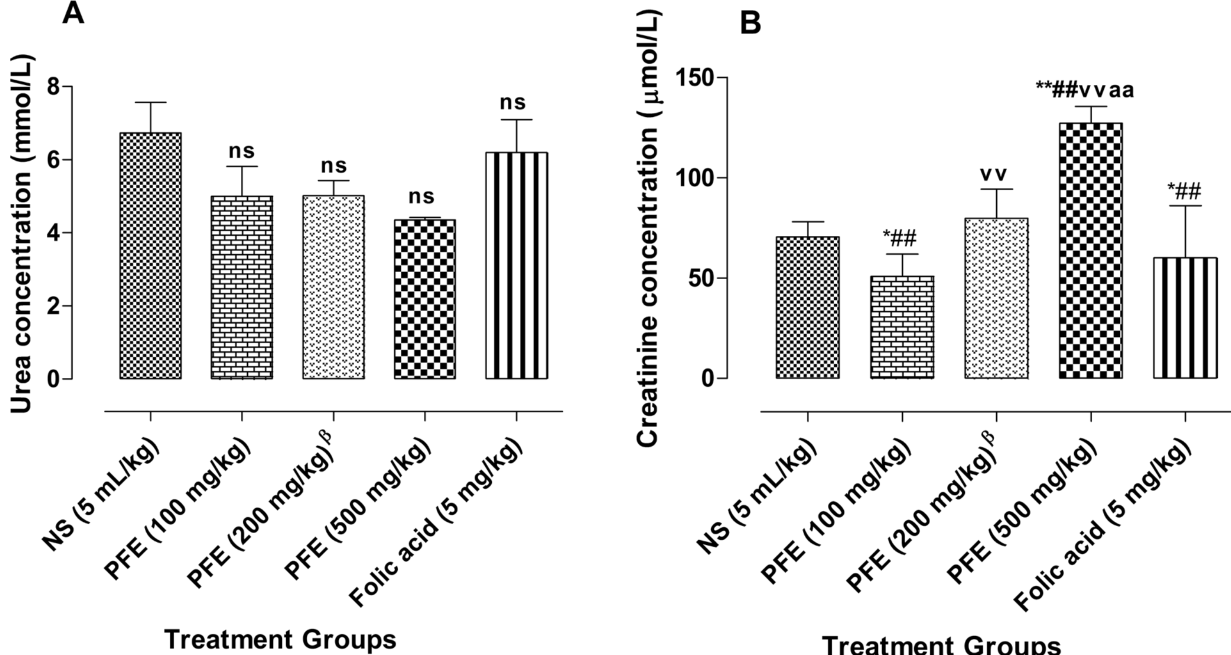

Figure 3. The effect of treatments on serum urea (A) and creatinine (B) levels determined at GD 15. Each bar is the mean ± SD. $n = 4$; $^{\beta}n = 5$. Values are Mean ± SD. $n = 4$; $^{\beta}n = 5$. $P \leq 0.05$ was considered statistically significant for all statistical analyses. ns = not significant; $^{*}P \leq 0.05$ (NS *vs*. PFE); $^{**}P \leq 0.01$ (NS *vs*. PFE); $^{\#\#}P \leq 0.01$ (PFE *vs*. Folic acid); $^{vv}P \leq 0.01$ PFE (100 *vs*. 200 mg); $^{aa}P \leq 0.01$ PFE (200 *vs*. 500 mg).

Table 3. Effect of treatments on organ/bodyweight ratio at GD 15.

Treatment groups	Maternal bodyweight at GD 15 (g)	*Relative organ weight × 10^{-2} (%)			
		Liver	Heart	Right kidney	Left kidney
NS (5 ml/kg)	213.77 ± 18.114	3.71 ± 0.335	0.32 ± 0.017	0.33 ± 0.031	0.30 ± 0.026
PFE (mg/kg)					
100	194.99 ± 5.817$^{\#}$	3.89 ± 0.686^{ns}	0.36 ± 0.121^{ns}	0.33 ± 0.071^{ns}	0.30 ± 0.074^{ns}
200$^{\beta}$	190.29 ± 20.998$^{\#\#}$	4.17 ± 0.830^{ns}	0.36 ± 0.055^{ns}	0.37 ± 0.058^{ns}	0.35 ± 0.055^{ns}
500	207.44 ± 6.463^{ns}	3.80 ± 0.049^{ns}	0.31 ± 0.007^{ns}	0.32 ± 0.042^{ns}	0.30 ± 0.028^{ns}
Folic acid (5 mg/kg)	194.20 ± 30.345*	3.89 ± 0.347^{ns}	0.31 ± 0.081^{ns}	0.32 ± 0.044^{ns}	0.28 ± 0.033^{ns}

Each value is the mean ± SD. *N* = 4; $^{\beta}n$ = 5. one-way ANOVA followed by Bonferroni's multiple comparison test was used to analyze mean differences between groups. $P \leq 0.05$ was considered statistically significant for all comparisons. ns = not statistically significant ($P > 0.05$). *maternal bodyweight at GD 15/respective mean organ weight (liver, heart, right, and left kidneys)/group × 100.

and direct bilirubin (TB and DB) marginally increased in low-and median-dose PFE (100 and 200 mg/kg) and folic acid groups relative to control, but significantly ($P \leq 0.05$) increased in PFE (500 mg/kg) group compared to control. Indirect bilirubin (IB) marginally increased in PFE (100–500 mg/kg) and folic acid groups compared to control. Alkaline phosphatase (ALP) significantly ($P \leq 0.05$) decreased in PFE (100–500 mg/kg) groups relative to control and folic acid groups. Gamma-glutamyltransferase (GGT) marginally decreased in low-dose PFE (100 mg/kg) but increased marginally in PFE (200 and 500 mg/kg) and folic acid groups compared to control (Table 5).

Effect of PFE treatment on kidney function

Urea concentration decreased in PFE (100, 200, and 500 mg/kg) especially in PFE (500 mg/kg) compared to control and folic acid groups; however, the mean difference was statistically insignificant ($P \geq 0.05$) (Fig. 3A). Folic acid and PFE (100 mg/kg) decreased mean creatinine concentration compared to control, though the mean difference was not significant statistically ($P \geq 0.05$). PFE (200 and 500 mg/kg) treatments increased significantly ($P \leq 0.05$) mean creatinine concentration compared to control-and folic acid-treated dams (Fig. 3B).

Effect of PFE treatment on post-implantation development

Mean gravid uterine weight was not significantly ($P \geq 0.05$) different across treatment groups (PFE and folic acid) and control. There was post-implantation loss in high-dose PFE (500 mg/kg) relative to control, folic acid, and low-and median-dose PFE (100 and 200 mg/kg). Except high-dose PFE (500 mg/kg) group, all the remaining treatment groups (low- and median-PFE, 100 and 200 mg/kg and folic acid) together with control group had 0% post-implantation loss (Table 6). A gross physical examination of embryos across all groups showed no observable anatomical malformations or deformations that could be related to treatment.

Histological assessment of brain and spinal cords of embryos after "in utero" exposure to treatments

Histological examination of brain and spinal cord sections of representative embryos showed no histo-morphological alterations of serious toxicological relevance except extensive eosinophilic cytoplasm and basophilic nuclei (Fig. 4).

Discussion

This study assessed the safety of PFE in pregnancy; specifically maternal toxicity and embryo-fetal toxicity post—"in utero exposure" was investigated. Hand-in-hand use of herbal medicine and herb-related adverse events have increased worldwide [4]. Crude preparations of *P. fruticosa* is used as a herbal medicine by all class of people including special populations (pregnant mothers, children, and the elderly) in Afro-Asian countries to treat many diseases including upper respiratory disorders [25,30] mostly by ingestion of decoc-

Table 4. Effect of treatments on liver indices of dams at GD 15

Treatment groups	Number of pregnant rats	Deaths during treatment	Number of survived pregnant rats	Live index (%)
NS (5 ml/kg)	4	0	4	100.00
PFE (mg/kg)				
100	4	0	4	100.00
200*	5	0	5	100.00
500	4	2	2	50.00
Folic acid (5 mg/kg)	4	0	4	100.00

**n* = 5.

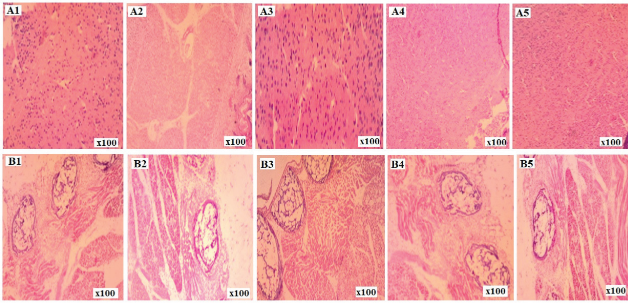

Figure 4. Each histomicrograph is a representative section of embryo-fetal brain (A1–A5) and spinal cord (B1–B5) after dams were gestationally exposed to treatments for 15 days and fetuses removed and examined histologically. Generally sections showed normal eosinophilic cytoplasm with basophilic nuclei and normal vascularization with normal nuclei within the soma of the neurons surrounded by glial cells and developing neuroglia. However, there was marked cellular disorganization and distortion of cortical architecture with interspersion of neuronal and glial cells layers in A2–A4 (PFE treatments) compared to A1 (normal control) and A5 (folic acid). Also, focal areas in A3 and A4 showed remarkable vacuolation compared to A1 and A5. (B) Histomicrographs of representative sections of fetal vertebral column showing unremarkable histomorphology with moderate mineralization (X) in B2, B3, and B4 compared to B1 and B5. A1 and B1 = normal saline (5 ml/kg *po*), A2 and B2 = PFE (100 mg/kg *po*), A3 and B3 = PFE (200 mg/kg *po*), A4 and B4 = PFE (500 mg/kg *po*), and A5 and B5 = folic acid (5 mg/kg *po*).

tions. Although scientific evidence abound to support the folk use of *P. fruticosa* for the treatment of many common diseases [24,37,38], no study assessed its safety in pregnancy. Previously acute and sub-acute toxicity studies on crude preparations of *P. fruticosa* conducted on non-pregnant rodents showed that it was safe at 2 g/kg [24], however, the present study showed that gestational exposure of dams to PFE at doses >100 mg/kg may increase the risk of post-implantation loss and renal injury in pregnant rats.Maternal toxicity encompasses changes in motor activity, bodyweight loss, decline in feed and water consumption, changes in external body structures, e.g., hair, mucous membrane, moribundity, and finally death upon gestational exposure to a known drug or chemical entity [39–42]. In this study, although feed consumption decreased across all groups (treatment and control dams) and then stabilized between GD 10 and 15 as pregnancy advanced, this could not be related to treatment in view of the physiological demands of pregnancy [43] and the fact that decrease in food consumption failed to reflect in bodyweight loss as is often the case [44]. Dams in all groups gained weight and the differences in weight gain among the groups were not statistically significant compared to that of control dams except in the case of high-dose PFE (500 mg/kg) group, but as far as there was a gain in weight this could not be related to maternal toxicity as reported elsewhere [44]. Also, there were comparable relative organ weights across all treatment groups relative to control indicating that treatments did not produce organ hypertrophy and edema. This observation complements an earlier safety study on *P. fruticosa* in non-pregnant animals, where body and organ weights and other safety indices did not change across treatment groups compared to controls [25,30], suggesting that *P. fruticosa* is safe at least with respect to body and organ weight changes but what may happen at the cellular and tissue levels remained veiled.

To investigate any possible tissue/organ injury in relation to treatment, liver and kidney enzyme activities were monitored. Sudden increase in some tissue-specific enzymes is indicative of direct cell/tissue injury [45]. In this study, although PFE treat-

Table 5. Effect of treatments on liver enzyme activity at GD 15.

Parameter	NS (5 ml/kg)	PFE (mg/kg)			Folic acid (5 mg/kg)
		100	200$^{\beta}$	500	
ALT (μ/l)	123.75 ± 22.29	112.75 ± 8.66^{ns}	123.40 ± 7.02^{ns}	123.00 ± 2.83^{ns}	112.75 ± 5.56^{ns}
AST (μ/l)	208.00 ± 53.93	165.25 ± 22.77$^{\#\#}$	215.60 ± 20.62^{vvaa}	236.50 ± 4.95^{vvaa}	145.75 ± 20.66***
TP (g/l)	67.00 ± 8.87	72.25 ± 3.95^{ns}	71.40 ± 4.83^{ns}	62.00 ± 1.41^{ns}	59.25 ± 5.56^{ns}
ALB (g/l)	33.50 ± 5.26	38.00 ± 2.58^{ns}	36.20 ± 1.48^{ns}	35.50 ± 3.54^{ns}	30.50 ± 2.65^{ns}
GLO (g/l)	33.50 ± 5.26	34.25 ± 2.06^{ns}	35.20 ± 3.56^{ns}	26.50 ± 2.12^{ns}	28.75 ± 3.59^{ns}
TB (μmol/l)	5.10 ± 0.63	6.05 ± 0.79^{ns}	6.76 ± 2.75^{ns}	13.65 ± 2.05**	7.78 ± 1.55^{ns}
DB (μmol/l)	2.83 ± 0.21	3.63 ± 0.44^{ns}	4.56 ± 2.57^{ns}	9.85 ± 0.354**	3.33 ± 1.59^{ns}
IB (μmol/l)	2.28 ± 0.75	2.43 ± 0.43^{ns}	2.20 ± 0.264^{ns}	3.80 ± 1.70^{ns}	4.45 ± 1.10^{ns}
ALP (μ/l)	180.50 ± 14.48	92.75 ± 12.92$^{**\#\#vv}$	157.80 ± 17.56^{vvaa}	151.50 ± 13.44^{vvaa}	224.00 ± 45.48***
GGT (μ/l)	0.47 ± 0.050	0.34 ± 0.077^{ns}	0.50 ± 0.032^{ns}	0.50 ± 0.099^{ns}	0.52 ± 0.083^{ns}

Values are Mean ± SD. $N = 4$; $^{\beta}n = 5$. The level of significance was established using one-way ANOVA followed by Bonferroni's multiple comparison test. $P \leq 0.05$ was considered significant for comparison of mean differences between treatments. ns = not statistically significant ($P > 0.05$). $^{\#\#}P \leq 0.01$ (NS *vs*. PFE); $^{**}P \leq 0.05$ (NS *vs*. Folic acid); $^{vv}P \leq 0.01$ (Folic acid *vs*. PFE); $^{aa}P \leq 0.01$ [PFE (100 mg) *vs*. PFE (200 and 500 mg)].

Table 6. Developmental toxicity assessments after daily gestational drug exposure to dams for 15 days.

Parameter	NS (5 ml/kg)	PFE (mg/kg)			Folic acid (5 mg/kg)
		100	200[β]	500	
Gravid uterine weight (g)	19.68 ± 0.622	15.90 ± 0.421	17.58 ± 0.723	15.63 ± 0.944	21.70 ± 0.859
Total implantation sites	8.25 ± 0.304	7.25 ± 0.957	6.20 ± 0.387	8.00 ± 0.000	7.75 ± 0.5
No. of embryos	8.25 ± 0.304	7.25 ± 0.957	6.20 ± 0.387	5.00 ± 0.243*	7.75 ± 1.5
[a]Post-implantation loss	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	3.00 ± 0.243	0.00 ± 0.000
[b]% post-implantation loss	0.00	0.00	0.00	37.5*#	0.00
Embryo weight (g)	9.87 ± 0.407	6.58 ± 0.201	8.76 ± 0.017	6.12 ± 0.243	10.86 ± 0.590
Average weight per embryo (g)	1.26 ± 0.224	0.90 ± 0.065	1.38 ± 0.230	1.35 ± 0.297	1.44 ± 0.174
Embryo weight to mother weight (%)	0.60 ± 0.008	0.47 ± 0.006	0.73 ± 0.021	0.65 ± 0.027	0.76 ± 0.165

Each value is the mean ± SD. $n = 4$; [β]means $n = 5$. The level of significance was established using one-way ANOVA followed by Bonferroni's multiple comparison test. [a]Total implantation sites minus number of embryos; [b]Number of resorptions/total implantation sites × 100; $P \leq 0.05$ was considered statistically significant for comparison of mean difference between treatments.*$P \leq 0.05$ (NS *vs*. PFE); #$P \leq 0.05$ (Folic acid *vs*. PFE).

ment marginally increased serum levels of some liver enzymes compared to control, the differences were not significant statistically; therefore, could not be inferred to indicate liver injury particularly in view of the fact that these liver enzymes are not liver-specific. However, PFE significantly decreased the serum levels of key liver enzymes conventionally used to assess liver function, including ALP and AST. Of note, increase in serum ALP is reported to be characteristic of bile duct obstruction, intrahepatic cholestasis, and hepatic damage [46]. Also, serum ALP levels at any point reflect combined activity of many ALP isoenzymes from many organs including the liver, bone, kidneys, placenta, and intestinal lining; therefore, PFE treatment-related decrease in ALP suggest that PFE have no adverse effects on the liver, placenta, and other organs during pregnancy in view of the multi-organ source of ALP and the fact that during pregnancy, the placenta may account for about half the total serum ALP [47]. AST partakes in energy production via its role in the Kreb's cycle. In cells, AST is located in the cytoplasmic and mitochondrial compartments. It is widely distributed in many cells including liver, heart, kidney, pancreas, skeletal muscles, as well as in red blood cells [48]. Cellular damage causes release of AST in serum. Serum AST level at any point is proportional to the degree of cellular damage and may fluctuate in sharp response to cellular damage and disease severity [49]. In the present study it was observed that, low-dose PFE (100 mg/kg) decreased serum AST compared to control suggesting that PFE at doses ≤100 mg/kg may be tolerated in pregnant animals without liver toxicity concerns. However, increasing PFE doses (200 and 500 mg/kg) marginally increased serum AST levels relative to control, perhaps indicating that PFE may be hepatotoxic at doses ≥200 mg/kg in pregnant animals contrary to previous report in non-pregnant animals [30].Urea undergoes renal excretion as the main means of eliminating nitrogen. Renal impairment causes blood urea concentration to rise exponentially, while creatinine accumulates in blood in sharp response to renal damage. Results from this study showed decreased urea concentration in PFE (100, 200, and 500 mg/kg) groups relative to control-and folic-acid groups showing that with specific reference to urea PFE may not pose risk of renal toxicity in pregnancy. However, with respect to creatinine concentration, PFE exhibited a biphasic effect in that at low-dose PFE (100 mg/kg) it decreased creatinine concentration relative to control but at higher doses (200–500 mg/kg) increased creatinine concentration relative to control suggesting that safety of PFE with respect to kidney function in pregnancy is dose-related; and therefore, gestational exposure to PFE above 100 mg/kg should be avoided.

During pregnancy, maternal conditions affect the developing neonate since the neonate depends directly on the mother for nutrients and excretion of waste materials via the placenta. Folic acid is conventionally used during pregnancy in humans for many reasons including prevention of neural tube disorders [4,50], and normal maintenance of key physiological processes for proper development of the neonate [51]. In the present study, folic acid group was used as a positive control for assessment of PFE use during pregnancy. In this study, all the embryo-fetal indices did not show significant differences across treatments and control groups except in % post-implantation loss and brain/spinal cord histological assessments which showed that high PFE dose (≥500 mg/kg) may produce risk of implantation loss and embryotoxicity.Effects of

herbal drugs just as their conventional counterparts relate not only to their chemical nature but also their chemical composition. The present results showed that PFE contains cyanogenic glycosides, saponins, alkaloids, and sterols and these observations support earlier reports [25,30]. GC–MS analysis of PFE showed 12 peaks, out of which 8 matched library compounds. PFE contains cyanogenic glycosides, and it has been shown that cyanogenic glycosides may hydrolytically release endogenous hydrocyanic acid (HCN) at levels that may cause toxicity [52]. HCN is readily absorbed and distributed in systemic circulation. HCN competitively inhibits cytochrome C oxidase, a crucial enzyme in the electron transport chain, leading to blockade of oxygen-reducing cofactor of proteins [52]. HCN-dependent cessation decreases oxygen utilization and triggers compensatory mechanisms such as activation of inefficient alternative energy generation such as anaerobic respiration. As a result, there is energy depletion, accumulation of lactic acid, metabolic acidosis, and cell death. The potential toxicity of cyanogenic glycosides especially in economically important crops such as *Manihot esculenta* necessitated issuance of acute reference doses for cyanide in cyanogenic glycoside-containing products by the Joint Expert Committee on Food Additives. Another phytocomponent of PFE was saponins, which is generally shown to interact chemically with cholesterol to form complexes believed to be responsible for hemolysis [53]. Also, saponins interact with living cell membranes to produce immunologic effects [53]. From our results, we suggest that *P. fruticosa* should not be used during pregnancy at any dose because of the following reasons. Put together, we suggest that *P. fruticosa* should not be used in pregnancy despite the fact that it did not produce maternal and post-implantation loss at 100 mg/kg.

Conclusion

Gestational exposure of PFE to pregnant dams at doses above 100 mg/kg may produce renal injury and post-implantation loss in pregnant animals; therefore, *P. fruticosa* should be avoided in pregnancy.

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Conflict of Interest

The authors declare no conflict of interest.

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