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Phytochemical, elemental, antioxidant, antimicrobial and hypoglycemic studies of a mixed herbal product used for the management of diabetics

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Abstract
Medicinal plants are important sources of disease-preventing compounds, which are important for the treatment of various health challenges such as diabetes. On an aqueous extract of a herbal product (HP) used for the management of diabetes, total phenolic and flavonoid contents were determined by Folin-Ciocalteu reagent and $AlCl_3$ method respectively. Microbiological evaluation was done by determining the total viable, yeast, mould and coliform bacteria count. The elemental analysis was carried out using atomic absorption spectrometer. The acute toxicity was done using Organization for Economic Cooperation and Development guideline while the hypoglycemic activity was evaluated using alloxan-induced diabetic rats. Flavonoids, saponins, alkaloid, cardiac glycoside, steroids and terpenoids were detected in the HP. Total flavonoid and phenolic contents obtained was 1.58±0.001mg/g quercetin equivalent and 10.84±0.003 mg/g gallic acid equivalent respectively. Heavy metals Fe and Zn were present while Cu, Cd, Cr and Pb were absent. Na and K were also present at concentrations of 3.90 and 2.20mg·kg^{-1} respectively. The total viable and coliform counts were found to be 1.34 x $10^5$and 9.0 x 10^4 cfu/g respectively while there was absence of mould and yeast in the HP. The LD_{50} of the HP was found to be above 5000 mg/kg. At dose of 125 mg/kg, the HP significantly (P<005) reduced glucose level to 143 mg/dL after 4 hours and to 123 mg/dL after 8 hours. The phytochemicals present, safety and the anti-diabetic activity justify the use of this HP in the management of diabetes.

Keywords: Phytochemical screening; Elemental analysis; Herbal product; Diabetes

INTRODUCTION

Diabetes mellitus is the most common endocrine-metabolic disorder characterized by chronic hyperglycaemia. It is associated with risk of microvascular and macrovascular damage, with associated reduced life expectancy and diminished quality of life. Diabetes occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. This leads to an increased concentration of glucose in the blood (hyperglycaemia) [1]. Symptoms of high blood sugar include frequent urination, increased thirst, and

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increased hunger and it can cause many complications if left untreated [2].

Nigeria, the 7th most populous nation on earth and the most populous nation in Africa with approximate population is 170 million and counting, with 76 million adults and 3.9 million people with diabetes [3,4] is the leading country in Africa in terms of the number of people with diabetes. Nigeria also accounts with 105,091 diabetes-related deaths in 2013 [5]. Diabetes related admissions of all hospital in-patients over a 10 year period account for 15% of all medical admissions in South South of Nigeria [6] with the same pattern in South East Nigeria [7,8]. Today it is estimated that 415 million people live with a form of diabetes [9]. In 2015, an estimated 1.6 million deaths were directly caused by diabetes [1].

The increase in prevalence and mortality due to diabetes imposes huge financial costs to households and governments [10] while placing immense pressure on the already overstretched healthcare systems in Africa [11]. As more and more people are diagnosed with diabetes, costs associated with treating both the disease and its complications become more and more burdensome. The objectives of this research therefore are: to establish, if any, its toxicity, provide baseline data on the phytochemicals, elemental, antioxidant and nutrition facts of a Herbal Product (HP); support or refute the acclaimed anti-diabetic properties. The HP is a combination of three plant; mango, bitter melon and ginger with trade name "Naturebetics".

EXPERIMENTAL

Materials. Normal saline, Ibuprofen (Sigma), Quercetin (sigma), Garlic acid (Sigma), $AlCl_3$ (Sigma), Rats, Stopwatch, Albumin, Electronic weighing balance. Thin layer chromatographic plates (MERCK, silica gel 60 F_{254} 0.2 mm), nutrient agar, Microbact GMB 24E kits, Muller Hilton Agar, Folin Ciocalteu reagent (Sigma).

Herbal product collection. The HP was collected from the production unit at Ilorin, Kwara State, Nigeria in 2015 and stored at room temperature.

Preliminary phytochemical screening of HP. Basic phytochemical screening to detect the presence or absence of plant chemical constituents such as alkaloids, tannins, saponins, anthraquinones, flavonoids, carbohydrates, cardiac glycoside, steroids and triterpene were carried out using standard procedures [12-14] on aqueous preparation of the HP.

Determination of total phenolic and flavonoid contents

Total phenolic content: Total phenolic contents was evaluated with Folin-Ciocalteu's phenol reagent [15]. A 1 ml quantity of the extract solution in methanol was mixed with 1 ml Folin-Ciocalteu reagent previously diluted with water (1:9 v/v). After 5 minutes, 0.8 ml of 7% Na_2CO_3 solution was added with mixing. The tubes were vortexed for 5 seconds and allowed to stand for 30 min at 40°C for color development. Absorbance was then measured at 765 nm using the Hewlett Packard UV-vis spectrophotometer. Samples of extract were evaluated at a final concentration of 0.1 mg/ml. Each test was done in triplicate. Gallic acid was chosen as a standard. Total phenolic content was expressed as mg/g gallic acid equivalent based on the calibration curve using the following equation: $y = 0.1216x$, $R^2 = 0.9365$, where y was the absorbance x was the concentration.

Total flavonoid content: Colorimetric aluminum chloride method was used for flavonoid determination [15]. A solution of the HP in methanol was separately mixed with 1.5 ml of 2% aluminum chloride. After one hour at room temperature, the absorbance

was measured at 420 nm. A yellow color indicated the presence of flavonoids. HP samples were evaluated at a final concentration of 0.1 mg/ml. Quercetin was chosen as a standard. Total flavonoid content were calculated as quercetin equivalents (mg/g) using the following equation based on the calibration curve: $y = 0.0255x$, $R^2 = 0.9812$, where y was the absorbance and x was the concentration. Each test was done in triplicate.

Total alkaloid determination [16]: A 0.1 g quantity of the each extract was weighed into a sample bottle and 4 ml of 20 % acetic acid in ethanol was added. The sample bottle was covered and allowed to stand for 4 hours. It was then filtered and the extract was concentrated on a water bath at 55°C to one quarter of its original volume. Concentrated ammonium hydroxide was added dropwise to the extract until precipitation was complete. The precipitate was collected from the solution, washed with dilute ammonium hydroxide, and filtered. The residue, which contained the alkaloid, was weighed after dryness to completion and the percentage was calculated using the formula:

$$\text{amount of alkaloid (\%)} = \left(\frac{\text{Weight of alkaloid precipitate}}{\text{Weight of powdered sample}}\right) x \frac{100}{1}$$

Total saponin determination [17]: A 0.1 g quantity of the extract was dissolved in 2 ml of 20 % ethanol and transferred into separator funnel. Diethyl ether (2 ml) was added and shaken vigorously. The ether layer was discarded, while the purification process was repeated. 6 ml of n-butanol was added and the extracts were washed twice with 1 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the sample was dried in the oven to a constant weight. The saponin content was calculated according to the equation:

$$\text{amount of saponin (\%)} = \left(\frac{\text{weight of residue}}{\text{weight of sample}}\right) x \frac{100}{1}$$

Elemental analysis. Eleven elements namely Ca, Cu, Fe, Zn, Cd, Cr, Na, K, and Pb were assessed quantitatively using the method of Association of Official Analytical Chemist [18] with the aid of Atomic Absorption Spectrometer (AAS) GBC Avanta Model. Standards and digested sample were aspirated and the mean signal responses were recorded at each of the element respective wavelengths.

Microbiological analysis. A 10 mL quantity of HP was homogenized with 90 ml of distilled water by shaking for several minutes. A 1 mL quantity was taken from the suspension and transferred to another tube to make serial dilution up to 10^6. Total viable count per ml was determined by pour plating of suitable dilutions on melted nutrient agar, and incubated aerobically after solidification at 37°C for 72 hours [19]. Yeast and Moulds were enumerated according to Harrigan and MacCance [20] using potato dextrose agar (PDA). The plates were incubated at 25°C for 3-5 days, plates having between 30 and 300 colonies were count as colony forming units (cfu/ml). Coliform bacterial count was determined according to literature [20,21] using MacConkey broth. The tubes were incubated at 37°C for 48 hours. Positive tubes gave gas in Durham tubes. The positive tubes were sub cultured into EC broth medium and then incubated at 44°C for 24 hours, to determine coliform bacteria, the tubes showing gas production were considered positive.

Acute toxicity study. The fixed dose method of the Organization for Economic Cooperation and Development (OECD) Guideline was employed using eight male and female albino rats weighing 80-100 g. Two treatment groups: Group A and Group B randomly containing three animals per group

were used. After fasting the animals overnight, the treatment groups (Groups A and B) received the HP extracts by gavage at single doses of 2000 and 300 mg/kg respectively. The animals were observed continuously for the first four hours and after a period of 24, 72 and 168 hrs, they were observed for signs of lethality or death. This was repeated with one rat per group and all observations were recorded. The dose that resulted in 50% mortality was determined [22].

Diabetic Study

Experimental animals. Thirty healthy (25) adult Wistar rats of both sexes (160 ± 20g) obtained from animal house, University of Ilorin, were used in this study. The animals were allowed to acclimatize for about 1 weeks, kept under a 12-hour light and dark cycle, fed on animal cubes (Feeds Nigeria Ltd) and provided with water *ad libitum*. The study was performed in accordance with the National Institute of Health (NIH) "Guide for the Care and Use of Laboratory Animals"1985.

Study design. Diabetes mellitus was experimentally induced after a 12-hour fast overnight by intraperitoneal administration (i.p.) of 60 mg/kg. After 72 hours, the blood sugar level was monitored with a glucometer (*Accu-Chek*, Roche Diagnostics) and rats with plasma glucose level >200mg/dl were classified as diabetic [23] and included in the study. A total of six groups containing rats of both sexes per group were used. Five groups of the animals were diabetic and one group was used as normal positive control (non-diabetic group). The rats were treated as follows:

Group I: Non-diabetic rats treated with 0.5 mL distilled water

Group II: Diabetic rats treated once with Glibenclamide 5 mg/kg body weight (b.wt)

Group III, IV and V: Diabetic rats treated once with different doses of the HP (125, 250 and 500 mg/kg b.wt/p.o. respectively).

The blood glucose level of the animals was then monitored after 4 and 8 hours of various treatment using a glucometer [24].

Statistical Analysis. Results obtained were expressed as mean ± standard error of mean. ANOVA was carried out using Graph Pad prism 7(Graph Pad prism 6 software, Inc, USA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Preliminary phytochemistry. HP was found to contain steroids, terpenoids, saponins, alkaloids, flavonoids and cardiac glycosides as found in Table 1. The HP was found to contain the following elements Ca, Fe, Na, and K in quantities below permissible limit. Cu, Cd, Cr and Pb were found to be absent as shown in Table 2. The total alkaloid, saponins, phenolic and flavonoid contents were found to be 67.85 ± 0.12 %, 31.25 ± 0.09 %, 1.58 ± 0.001 mg/g gallic acid equivalent and 10.84 ± 0.003 mg/g quercetin equivalent respectively (Table 3).

Microbial analysis. The total viable count, yeast count and mould count were all below permissible limits by WHO, except total coliform, which was highly above the permissible limits (table 4). The total viable count and total coliform was higher than the permissible limits set by the American herbal product association (AHPA) while total yeast and mould count was below the permissible limit [25].

Acute oral toxicity. In the acute oral toxicity study, the animals in the test manifested no change in behavior or physiology. There was no sign of lethality or death at dose level of 2000mg/kg. The LD_{50} was found to be >5000 mg/kg.

Antidiabetic property of HP. Evaluation of oral hypoglycemic activity of HP in alloxan-induced diabetic rats showed the HP reduced blood glucose levels significantly at 125 and 250 mg/kg b.wt and also suppressed postprandial hyperglycemia in rats. Conversely, a significant increase was observed at 500 mg/kg. HP reduced the blood glucose level at 125 mg/kg b.wt to 143 and 123 mg/dL after 4 and 8 hours post treatment respectively (table 5). However, the blood glucose levels of 409.92 and 260.20mg/dL were observed at 500mg/kg after 4 and 8 hours' post-treatment respectively.

Table 1: Phytochemicals present in the aqueous solution of HP

Phytochemicals	Method	HP
Steroids	Liebermann-Burchard	+
Terpenoids	Liebermann-Burchard	+
Saponins	Frothing	+
Alkaloids	Meyer	+
	Wagner	+
	Dragendorff	+
Tannins	Pb subacetate	-
	$FeCl_3$	-
Flavonoids	Shinoda	+
	NaOH	+
Cardiac glycosides	Keller-Killiani	+

Key: + = present, - = absent

Table 2: Elemental analysis

Parameters	HP (mg/kg)	WHO Limits (mg/kg)
Calcium	3.1	NE
Copper	0.00	NE
Iron	0.30	NE
Zinc	0.04	NE
Cadmium	0.00	0.30
Chromium	0.00	NE
Sodium	3.90	NE
Potassium	2.20	NE
Lead	0.00	10.00

NE = not established yet by WHO [27]

Table 3. Total phenolic and flavonoid content in aqueous HP

Constituent in HP	Mean ± Standard error of mean
Total Phenolic content	1.58 ± 0.001 mg/g gallic acid equivalent
Total Flavonoid content	10.84 ± 0.003 mg/g quercetin equivalent
Alkaloid	67.85 ± 0.12 %
Saponins	31.25 ± 0.09 %

Table 4: total coliform, viable count, yeast and mould count found in HP.

S/No.	Parameters	Naturebetic	Limits according to AHPA[25]	Limits according to WHO [27
1	Total viable count (*cfu*/g)	1.34×10^5	10^4	10^7
2	Total *coliform* (*cfu*/g)	9.0×10^4	10^2	10^4
3	Total yeast count (*cfu*/g)	Nil	10^3	10^4
4	Total *mould* count (*cfu*/g)	Nil	10^3	10^4

Table 5: Antidiabetic activity of HP

Group/dose	Glucose level (mg/dL)	
	4 hours	8 hours
Negative control	226.00 ± 9.27	224.20 ± 7.48
125 mg/kg	143.80 ± 27.22*	123.60 ± 36.34*
250 mg/kg	109.60 ± 3.08*	383.60 ± 38.64
500 mg/kg	409.92 ± 27.78*	260.20 ± 44.42
Glibenclamide 5mg	207.00 ± 10.7	145.60 ± 14.91

Data are expressed as mean ± SEM., (n= 5), ($p<0.05$), * = statistically significant when compared with control

DISCUSSION

Extraction is the main step for isolating and recovering phytochemicals from plant materials whose efficiency is affected by the chemical nature of secondary metabolites and the extraction method used [26]. The extraction yield depends on the polarity of the solvent, extraction time, temperature, and composition of the sample. The presence of flavonoid and saponins in the HP is due to the use of water in extraction and implies they are highly polar compounds.

Contaminants in herbal medicines can be classified into physicochemical and biological contaminants. Contamination can be removed or controlled through good collection, agricultural and manufacturing practices for herbal medicines. Chemical and microbiological contaminants can result from the use of animal manures, sewage as fertilizers, and human excreta. Majority of potentially hazardous contaminants are found in herbal materials thus resulting in their presence in herbal products. Post-harvest processing (e.g. drying) may change the level of some contaminants present in extracts, and in finished herbal products during the manufacturing process [27]. The total viable, Total coliform, Total yeast and Total mould count of the HP was less than that specified by WHO showing that the HP was clear of such contaminant. In contrast, according to AHPH, the HP did not confirm to standard as the values for total coliform and total viable count was above the limits. To ensure improve quality of the HP required the services of trained personnel for plant materials collection, processing and storage under the supervision of specialists (Pharmacognosist, Botanist etc.) in herbal production.

Some literature [28] have shown herbal medicines having total viable count within the range 0 - $1.5x10^4$ cfu g^{-1} and total fungi count ranged from 20 - 10^5 cfu g^{-1}. The total viable count in this study is higher than that of herbal product reported by Alwakeel [28] while the fungi ranged is lower. The differences between the results of this study and those of other studies could be attributed to the different solvents used in extraction, which resulted in differences in compositions and the difference in plant matrix [29].

The results in this study corroborate those of previous investigations, where it was observed that almost all herbal samples collected for herbal preparations were contaminated with molds and aerobic bacteria [30] though that of this study did not exceed the WHO limits.

The content of essential and trace elements in medicinal plants vary according to geoclimatic conditions, geochemical characteristics of the soil, anthropogenic activities, plant species, and the part of the plant used for preparing the herbal medicine [31]. Physicochemical properties (presence or absence of other elements, characteristics of soil or sediments, exposure period, pH level, and dispersion range) also influence the level to which metals accumulate in plants [32]. Plants assimilate trace elements through the roots as well absorbing through the leaves. Additional sources of trace elements for plants include atmospheric dusts, rainfall, fertilizers, and plant protection agents [33].

Low concentration level of trace elements have health benefits while higher levels may pose health risks [34]. Heavy metal concentrations could reach levels with potentially hazardous effects on human health due to their cumulative properties and toxicity. The presence of Zn and Fe in this HP is of clinical significance in addition to the product claim as these elements play essential roles in body functions and development. In addition, the level of Na in the HP suggests caution in its use in the management of co-morbid patients suffering from high blood pressure.

Alloxan induces diabetes by destroying the pancreatic beta cells that secrets insulin resulting in hyperglycemia and hypoinsulinemia [35]. The production of free radicals is one of the intracellular phenomena for its cytotoxicity [36]. This study demonstrates that the effectiveness of the HP in controlling blood glucose levels in alloxan-induced diabetic rats at 125 and 250mg/kg b.wt may be by inhibition of production of free radicals and prevention of destruction of pancreatic beta cells. The observed anti-diabetic effects of HP could be due to the presence of flavonoids and alkaloids such as purine, pyrrolizidine, berberine [37-39] that had been reported to possess antidiabetic activities. However, the significant increase in blood glucose level at 500 mg/kg b.wt could be due to the presence of some secondary metabolites whose antagonistic activity have full expression at high doses, thus, effective doses between 125 and 250mg/kg b.wt are recommended for better therapeutic outcome.

Some herbal products have also been reported to possess anti-diabetic activities, for instance, American ginseng acts as a glucose-reducing agent in patients with type 2 diabetes mellitus due to increase in the expression of adiponectin and a reduction in lipid accumulation in the 3T3-L1 cells [40,41]. Flavonoids have been proven to be effective compounds for glucose absorption in the small intestine. A kinetic study on the effects of flavonoid compounds, such as quercetin-4'-glucoside and quercetin-3-glucoside, on the inhibition of glucose uptake showed that these compounds prevented the absorption of methyl α-D-glucopyranoside [42].

Conclusion. The HP contains secondary metabolites with antidiabetic activity mediated via regulation of blood glucose metabolism. The herbal product was safe and devoid of contamination. This study justifies its use in the management of diabetes.

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