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ARTICLE

Antioxidant and Antidiabetic Potentials of a Standardized Polyherbal Mixture Used in Traditional Medicine

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Abstract: Polyherbal preparations have gained much attention as a potential source for discovering new drug therapeutics for the treatment of diabetes mellitus. The present investigation aims to determine *in vitro* and *in vivo* antidiabetic activity and the antioxidant potential of hexane, ethyl acetate and methanol extracts of a polyherbal mixture prepared from equal amounts of garlic cloves (*Allium sativum* L.), curry leaves (*Murraya koenigii* L. Sprengel), black pepper seeds (*Piper nigrum* L.) and rath goraka fruits (*Garcinia quaesita* Pierre). The standardization and *in vitro* antioxidant activity and antidiabetic activities were determined using standard methods. The *in vivo* acute antihyperglycemic activity of the hexane, ethyl acetate and methanol extracts was determined using an oral glucose tolerance test in streptozotocin-induced diabetic Wistar rats. Highest significant *in vitro* antioxidant capacity in terms of DPPH free radical scavenging (24.71 ± 0.01 ppm, p = 0.02) and reducing antioxidant power of ferric ion (23.29 ± 0.78 mol/dm^3, p = 0.01) and *in vitro* antidiabetic properties in terms of α-amylase (25.74 ± 0.60 ppm, p = 0.01) and α-glucosidase (22.37 ± 0.06 ppm, p = 0.02) inhibition activities were observed in the hexane extract when compared with the respective standard compounds, ascorbic for antioxidant (DPPH 6.78 ± 0.03 ppm; FRAP 25.02 ± 0.21 mol/dm^3) and acarbose for antidiabetic activity (α-amylase 5.68 ± 0.35 ppm; α-glucosidase 17.11 ± 0.62 ppm). In the glucose tolerance test, a significant improvement of glucose tolerance was found in the hexane (17.38%) and ethyl acetate (15.81%) extracts-treated groups at the therapeutic dose against the diabetic control group (p < 0.05). The results obtained from the present evaluation showed that the hexane and ethyl acetate extracts of the polyherbal mixture could be considered as a potential source for developing antidiabetic agents targeting the management of diabetes mellitus.

Keywords: Diabetes mellitus, Antioxidant, Garlic, Curry leaves, Black pepper, Rath goraka, Glucose tolerance test.

Introduction

The use of medicinal plants for the treatment of diabetes mellitus is very popular, especially in Asian countries. Ayurveda is the most practiced and well-developed therapeutic system in Sri Lanka[1]. In Ayurveda, medicinal plants have been used as a combination of plants/drugs, a therapeutic model referred to as polyherbalism or polypharmacy[2]. The associated synergism found in polyherbal mixtures can be described as positive herb-herb interactions which lead to the maximum therapeutic effects on diseases, compared to the sum of their individual effects[3]. Additionally, this combined drug therapy can focus on multiple targets at the same time to obtain maximum effectiveness[4]. This concept is

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of particular interest in designing novel therapies targeting the management of diabetes mellitus[5].

In the clinical arsenal of medicines, diabetes has been managed mainly with oral antidiabetic agents, like metformin, sulfonylureas, thiazolidinedione, biguanides and glibenclamide[6]. However, most of the synthetic drugs which have been used to treat diabetic patients have caused adverse side effects and various complications[7]. Recent findings indicated that presently used hypoglycemic agents are focused only on a single therapeutic target; however, it may not be sufficient considering the multifactorial nature of diabetes and its complications[8]. Therefore, many researchers are interested in novel combined drug therapeutics that can address the multiple disease pathogenesis of diabetes while minimizing the side effects associated with the therapy[9]. As a primary solution, Ayurvedic polyherbal preparations for the management of diabetes mellitus have gained increasing attention worldwide[10]. A home-made Ayurvedic remedy made of the cloves of *Allium sativum* L. (AS) (common name; garlic) which belongs to the Liliaceae family, the leaves of *Murraya koenigii* L Sprengel (MK) (common name; curry leaves) which belongs to the Rutaceae family, the seeds of *Piper nigrum* L (PN) (common name; black pepper) which belongs to the Piperaceae family and the fruits of *Garcinia queasita* Pierre (GQ) (common name; rath goraka) which belongs to the Clusiaceae family has been practiced for the treatment of hyperglycemia and dyslipidemia by Ayurveda medical practitioners in Sri Lanka[11]. Several previous studies has investigated the antidiabetic potential of the above individual plants, plant parts and their isolated compounds[12–15]. However, there are limited details on the blood glucose lowering property of GQ except in the one reported study of hypoglycemic activity of three extracts of the plant and its isolated compound of garcinol[16]. In addition, our group recently identified that cold water, hot water and water acetone extracts of the above mentioned polyherbal mixture have no acute or subchronic toxicity[17]. Further, we have observed that the water-acetone extract of the polyherbal mixture has strong antihyperglycemic and antihyperlipidemic effects than the water extracts on diabetes-induced Wistar rats[18]. We anticipated that it could be due to the compounds present in this mixture, which have been extracted by the organic solvents (acetone) compared to water and hence the higher activity. However, the crude extract which is rich in antidiabetic and antioxidant compounds has not been studied. Therefore, in this research, we aimed to study the *in vitro* and *in vivo* glucose lowering effect of the sequentially extracted standardized extracts of hexane, ethyl acetate and methanol extracts of the above-mentioned polyherbal mixture. In addition, *in vitro* antioxidant activity, as well as the HPLC fingerprints, were also studied for all three extracts.

Materials and Methods

Chemicals and reagents

(L)-Ascorbic acid, *α*-glucosidase from *Saccharomyces cerevisiae*, Folin-Ciocalteu reagent, 2,2-diphenyl-2-picryl-hydrazyl (DPPH), glibenclamide, porcine pancreatic *α*-amylase (PPA) and streptozotocin (STZ) were acquired from Sigma-Aldrich, USA. Acarbose was purchased from MP Biomedicals, France and a glucose assay kit from Biorex, UK. 3,5-Dinitrosalicylic acid (DNSA) was obtained from Sigma-Aldrich, India. Sodium potassium tartarate and starch (potato) were from DAEJUNG, Korea. All other general solvents, reagents and chemicals used in the present study were of analytical grade.

Instruments

A microplate reader (UVM 340, Cambridge) and a UV-visible spectrophotometer (Shimadzu, Japan) were used for the measurement of biochemical parameters. A rotatory evaporator (Heidolph, Germany) was used in the plant extracts preparation and the HPLC fingerprint profiles were obtained using an Agilent 1100 instrument (USA).

Collection and Preparation of Plants

The fresh leaves of MK and fruit rinds of GQ were collected from the home garden in Peradeniya, Sri Lanka. The cloves of AS and dried seeds of PN were bought from the Kandy market, Sri Lanka. Each plant material was collected in April 2018. The MK plant was like a shrub that reached about 5–6 m in height. The plant had a dense shady crown and had a short trunk with nearly 20 cm diameter and greenish, middle-age matured fresh leaves. The GQ was a large tree (height about 20–25 m) with a round head and a rough bark. Fruits were large, ≈ 5–6

cm heigh, more or less of globular shape, ripened fruits red orange in colour and depressed. The GQ fruits were variable in form with 8–10 of deep vertical grooves forming as blunt lobes. The PN plant was like a woody climber and they reached heights about 10 m. The black pepper seeds are the dried fruit of PN plant. A single stem contained about 20–25 spikes of fruits. The spikes were sun-dried to separate the peppercorns from the spikes and to form air-dried black pepper seeds. The AS was a bulbous plant grown up to 1 m in height. One garlic bulb contained a segmented part called cloves and about 10–15 cloves were in a single bulb. Plant parts were submitted for the authentication to the herbarium of the Royal Botanic Gardens, Peradeniya, Sri Lanka and only one Voucher was obtained for G. *quaesita* (Voucher No: 6/01/H/03) as it was the only plant that was having similar varieties. The collected and authenticated plant materials were thoroughly washed with water and air-dried (curry leaves air dried for one day and used with the greenish colour) before extraction.

The plant mixture was prepared by grinding an equal quantity (500 g each) of air-dried plant samples together and extracted into hexane (3 × 24 h), ethyl acetate (3 × 24 h) and methanol (3 × 24 h) sequentially in a bottle shaker at room temperature (27 °C). Solvents were evaporated using a rotary evaporator and further dried by a freeze dryer to gain dry powder forms from hexane, ethyl acetate and methanol extracts. These samples were stored in a refrigerator in the Department of Chemistry, Peradeniya University at 4 °C until further use (about two months).

Standardization of the Polyherbal Mixture

Physico-chemical characterization of the crude extracts of the polyherbal mixture was carried out in terms of moisture content and ash values, such as the content of total ash, the content of acid-insoluble ash and the content of water-soluble ash[19].

High-performance liquid chromatography (HPLC) fingerprint profiles of the crude extracts were determined on an HPLC-UV/DAD instrument connected to a diode-array detector (DAD 1100 series) and controlled by Agilent software. The solvents were filtered through a PTFE filter (pore size 0.45 μm) before HPLC analysis. One milliliter of each sample (1000 ppm) was filtrated and injected (10 μL) into a C18 column (Agilent, 100 Å, 4.6 × 150 mm, 5 μm). The samples were eluted with a mobile phase, using gradient and isocratic solvent systems comprised of water (A) acetonitrile (B) and methanol (C). Method profile: 0–4 min: isocratic, 25% of A and 75% of C; 4–6 min: linear, 25–0% of A and 75–100% of C; 6–15 min: isocratic, 100% of C; 15–20 min: linear, 0–100% of B and 100–0% of C. The flow rate was maintained at 0.5 mL/min during the time range 0–8 min, gradient from 0.5–1.0 mL/min in the range 8–10 min and at 1.0 mL/min in the range 10–20 min. The HPLC fingerprint spectra were monitored at 260 nm for the three extracts of the polyherbal mixture.

Qualitative phytochemical screening of the polyherbal mixture was conducted using the standard procedures of Zohra[20] and Bansode[21].

Antioxidant Activity of the Polyherbal Mixture

2.2-Diphenyl-2-picryl-hydrazyl (DPPH) Free Radical Scavenging Activity

The reported method of DPPH assay was applied to estimate the free radical scavenging antioxidant potential of the crude extracts of the polyherbal mixture[22]. DPPH free radical (0.3 mM) dissolved in methanol (1.0 mL) was mixed with 2.5 mL of a concentration series of the crude extract mixture. The solution mixture was shaken well and incubated for 30 minutes at 25 °C in a dark area. L-ascorbic acid was used as the positive control in the assay. The absorbance of the solution mixture was measured using a UV-visible spectrophotometer at a wavelength of 517 nm. The antioxidant activity of crude extracts of the polyherbal mixture was calculated as a percentage using the following formula:

$$Radical\ Scavenging\ activity\ \% = \left(1 - \frac{A_S - A_B}{A_C}\right) \times 100\%$$

A_S, A_B and A_C correspond thereby to the absorbance by sample, blank and control, respectively. Fifty percent inhibition concentration (IC_{50}) of the DPPH free radical by the crude extract of the polyherbal mixture was determined from the graph plotted with the concentration of crude extract along the x-axis and the percentage inhibition along the y-axis ($y = 0.813\ x + 18.02$, $R^2 = 0.9595$).

Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant activity of the crude extracts of the polyherbal mixture was estimated using the method described by Berker *et al.*[23] with a few changes. The FRAP solution was prepared freshly by adding 300 mM of sodium acetate buffer at pH 3.6, 10 mM of 2,4,6-Tris(2-pyridyl)-s-triazine diluted in 40 mM of HCl and 20 mM of $FeCl_3$ dissolved in distilled water in the ratio of 10:1:1, respectively. Three milliliters of the FRAP reagent were added to 100 µL of the crude extract of the combined plant mixture and the solution mixture was mixed well. The samples thereafter were kept at 27 °C for 30 min and the absorbance of the resultant mixture was recorded at 593 nm using a UV-visible spectrophotometer. $FeSO_4$ was used as the standard compound. The FRAP value was determined using the regression equation of the $FeSO_4$ standard curve and is expressed in terms of ferrous equivalents.

Estimation of Total Polyphenolic Content (TPC)

The polyphenolic content of the crude extracts of the polyherbal mixture was estimated using the Folin-Ciocalteu method[24]. The reaction mixture was prepared by mixing a methanolic solution of the crude extracts (0.5 mL), 10% Folin-Ciocalteu's reagent (2.5 mL) dissolved in distilled water and 7.5% Na_2CO_3 solution (2.5 mL). The test samples were kept for incubation in an oven at 27 ^oC for two hours. The absorbance value of the test sample was recorded using a spectrophotometer at a wavelength of 765 nm. The same method was followed for the gallic acid standard and a calibration line was plotted for the gallic acid standard series. The polyphenol concentration of the crude extracts was estimated from the regression equation ($y = 0.0961\ x + 0.0394$, R^2=0.9973). The total polyphenol concentration of crude extracts is presented as gallic acid equivalents in mg GAE/g.

***In vitro* Antidiabetic Assays**

α-Amylase Inhibition Assay

The α-amylase inhibition assessment was carried out for the crude extracts of the polyherbal mixture by using the procedure reported by Apostolidis *et al.* with slight changes[25]. Briefly, 100 µL of 20 mM phosphate buffer (pH 6.9 with 6.7 mM sodium chloride) containing α-amylase solution (1 mg/mL) and different concentrations of the stock solution of extracts (1.563–1000 µg/mL) were kept for incubation for 30 min at 25 ^oC. After the incubation, 100 µL of 1% solution of starch was added to each sample and 100 µL of DNSA colour reagent was mixed with a blank sample at a time frame. The solution mixture was then incubated for 3 min at 25 ^oC. The reaction process was stopped by adding 100 µL of DNSA reagent to each sample and 100 µL of starch to its blank after three minutes. Thereafter, the samples were incubated in a water bath at 85 ^oC for 15 min and after 15 min, they were allowed to cool to 25 ^oC. The solution mixture was diluted by adding 900 µL ultra-pure water and the absorbance was measured at a wavelength of 540 nm using a microplate reader. Acarbose was used as a positive control in this assay procedure. The results were recorded in terms of IC_{50} values.

Inhibition of α-glucosidase Enzyme

The α-glucosidase enzyme inhibition assay was carried out as previously described by Sagbo *et al.*[26] with minor modifications. In a 96-well microplate, the solution mixture containing 100 µL of phosphate buffer (30 mM, pH = 6.8), 10 μL of the crude extract of the polyherbal mixture (1.563–1000 µg/mL) was kept in an oven at 37 °C for 5 min with 10 μL of 82.8 μg/mL α-glucosidase solution dissolved in the phosphate buffer. After 5 min of incubation, 20 µL of 1 mM *p*-nitrophenyl α-D-glucopyranoside reagent was mixed with the reaction mixture and further incubated for 30 min at 37 °C. Thereafter, 40 μL of 200 mM Na_2CO_3 solution was added to the mixture and the absorbance of each sample was measured at 405 nm using a microplate reader. The sample and control blank were also prepared by adding 10 μL of phosphate buffer instead of glucosidase enzyme solution. The assay protocol was repeated for acarbose, the positive control. A graph was plotted with concentration (ppm) along the x-axis and percentage inhibition (%) along the y-axis to obtain the IC_{50} value.

***In vivo* Acute Antidiabetic Experiments**

All animal protocols in experiments, their maintenance and handling were carried out according to the 3R concept of replacement, reduction and refinement involving accepted animal ethics. Free accesses were provided to Wistar rats for water and standard pellet food *ad libitum* during the maintenance. They were maintained under standard laboratory conditions

and rats were allowed one week of acclimatization before the commencement of experiments. The Ethical Review Committee (ERC) (Ref. No. 09.03.2016.3.8) granted ethical approval for the use of animals in the experiments.

Adult healthy male Wistar rats (220 ± 20 g, 12–14 weeks of age) were obtained from the Animal Breeding Unit and the animals were housed in the Animal Vivarium of the Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Sri Lanka. The animal experiments and relevant bioassays were conducted at the Research Laboratory of the said institution.

Development of Hyperglycemic Condition in Wistar Rats

The male rats fasted overnight and diabetes was induced in the rats by intraperitoneal administration of a dose of 65 mg/kg of STZ drug, dissolved in freshly prepared buffer solution of 0.1 M citric acid/0.1 M sodium citrate (pH 4.5)[27]. After three days of the injection of STZ, the fasting blood/serum glucose concentration of experimental animals was evaluated using the glucose-oxidase method following an enzyme assay protocol (Biorex diagnostics). The rats with a fasting blood/serum glucose level of > 11.1 mmol/L were considered in the state of hyperglycemic condition and they were used in the present study[28].

Hypoglycemic Activity of the Polyherbal Mixture

Overnight fasted healthy male rats were divided randomly into four groups (n = 6/group). Group one was considered as the untreated healthy group and received water. A single therapeutic dose at 25 mg/kg of hexane extract, 30 mg/kg of ethyl acetate extract and 95 mg/kg of methanol extracts of the polyherbal mixture dissolved in corn oil was introduced orally to healthy rats in groups two, three and four, consecutively. An oral glucose tolerance test (OGTT) was conducted for each group. Serum glucose concentration was estimated at fasting, 1 hour, 2 hours, 3 hours and 4 hours. The calculated total area under curve (TAUC) values were used to assess the hypoglycemic effect over the experimental period. The OGTT and collection of blood for estimation of blood glucose concentration were carried out following the protocol described by Attanayake *et al.*[29].

Acute Antihyperglycemic Activity/Dose-response Study of the Polyherbal Mixture

Overnight fasted male rats were grouped randomly into twelve cages. The rats in the first group (n = 6/group) were healthy untreated and the rats in the second group (n = 6/group) were diabetic untreated/control. Both groups of rats received distilled water. Hexane, ethyl acetate and methanol extracts of the polyherbal mixture were given orally to diabetic rats in groups three to eleven (n = 6/group) and they were treated with different doses as low, therapeutic and high, based on the percentage yield in each crude extract in order to identify the maximum effective dose. The therapeutic dose of each crude extract was determined based on the percentage yield of each crude extract[30]. Three doses of each crude such as low (≈ 1/3 of therapeutic dose), therapeutic and high (≈ 3× of therapeutic dose) were designed according to Ayurvedic guidelines for the way of administration of herbal extract to human[31].

Accordingly, groups 3 to 5 were hexane extract-treated diabetic rats at the doses of 8, 25 and 75 mg/kg, groups 6 to 8 were ethyl acetate extract-treated diabetic rats at the doses of 10, 30 and 90 mg/kg and groups 9 to 11 were methanol-extract treated diabetic rats at the doses of 32, 95 and 285 mg/kg, respectively. Glibenclamide at the dose of 0.5 mg/kg was administered orally to STZ-induced diabetic rats in the twelfth group (n = 6/group), which served as the positive control. The OGTT was carried out for each group and assessed through TAUC values.

Statistical Analysis of Data

The quantitative data of all described experiments was performed in triplicates. The data/results obtained are presented as mean ± SEM (standard error value of the mean). Statistical analysis of the data was carried out by one-way ANOVA followed by Dunnett's *post hoc* test using the SPSS software (version 22) and values of $p < 0.05$ were accepted as statistically different (p).

Results

Standardization of the Polyherbal Mixture

Physicochemical parameters, like moisture level, total ash content, acid insoluble and water-soluble ash content for the crude extracts of the polyherbal mixture, are shown in Table 1. The

moisture contents results showed that both hexane and ethyl acetate extracts of the combined plant mixture had lower moisture content than the standard value. The total ash content of the hexane and ethyl acetate extracts used were at acceptable levels when compared with the standard values. The water-soluble and acid-insoluble ash contents of the crude extracts of the polyherbal mixture used in the present study were in the recommended range.

Examination of the HPLC chromatograms of the hexane extract showed that it contains eight characteristic peaks with different retention times (RT) at 3.68, 4.13, 4.29, 4.83, 9.56, 12.42, 13.18 and 13.30 min. Moreover, the HPLC fingerprint profile of the ethyl acetate extract assigned four characteristic peaks with the RT values at 2.94, 3.64, 4.79, 11.45 min and the characteristic peaks of methanol extract were at 1.39, 3.06, 3.76 and 4.97 min. Work is going on to identify all these components.

Phytochemical screening experiments show that alkaloids, tannins, sterols/steroids, triterpenoids, phenolic compounds and flavonoids are present in the crude polyherbal extracts, while saponins, reducing sugars and starch are present only in very low amounts or totally absent.

Table 1. Physico-chemical parameters of the crude extracts of the polyherbal mixture.

Parameter	Hexane extract	Ethyl acetate extract	Methanol extract	Standard value % w/w
Moisture content/%	7.71 ± 0.83	7.61 ± 0.92	17.82 ± 1.21	NMT 10%
Total ash content/%	8.07 ± 0.28	8.58 ± 0.71	11.78 ± 0.40	NMT 10 %
Acid insoluble ash content/%	0.02 ± 0.01	0.05 ± 0.02	0.13 ± 0.01	NMT 3%
Water soluble ash content/%	6.21 ± 0.28	6.73 ± 0.62	9.21 ± 0.74	NMT 10%

Data is represented as Mean ± SEM. NMT; not more than. (Standard values from reference [32]).

In vitro Antioxidant Activity of the Polyherbal Mixture

In the current research work, we investigated the *in vitro* antioxidant potentials of the polyherbal mixture, in terms of DPPH (free radical) scavenging assay, FRAP assay, Folin-Ciocalteu assay for the total polyphenolic content and the data is summarized in Table 2. In this method, the fifty-percent inhibition concentration of DPPH free radical (IC_{50}) by the sample was determined; a low IC_{50} value would indicate high antioxidant potential and *vice versa*. The highest antioxidant activity of the polyherbal mixture was obtained for the hexane extract (IC_{50} = 24.71 ± 0.01 ppm), while the standard compound L-ascorbic acid attained 6.78 ± 0.03 ppm (Table 2). Moreover, the statistical analysis of the results of the DPPH assay showed that a significant difference existed only between the methanol extract of the polyherbal mixture and L-ascorbic acid ($p < 0.05$). Similarly, the statistical results revealed that the hexane and ethyl acetate extracts of the polyherbal mixture had similar free radical scavenging potential when compared with the standard compound.

The FRAP value was estimated using the regression equation of $FeSO_4$ standard curve ($y = 0.0009\ x + 0.2961$, R^2 = 0.9858) and was expressed in terms of the ferrous equivalent (Table 2). The highest reducing power was observed in the hexane extract (23.29 ± 0.78 mol/dm^3), while the lowest was observed in the methanol extract (5.57 ± 0.36 mol/dm^3) of the polyherbal mixture. Further, hexane and ethyl acetate extracts of the polyherbal mixture have a significant difference in reducing power ($p < 0.05$) compared with the methanol extract.

The TPC of crude extracts of the polyherbal mixture was estimated by the regression equation of gallic acid standard ($y = 0.0961\ x + 0.0394$, R^2 = 0.9973). The results of the Folin-Ciocalteu assay denoted that the highest TPC was in the hexane extract, while the lowest value was recorded in the methanol extract (Table 2).

In vitro Antidiabetic Potential of The Polyherbal Mixture

In the present evaluation, *in vitro* antidiabetic potential of the polyherbal mixture was screened using the assay protocols of α-amylase and α-glucosidase enzyme inhibition. The results of α-amylase and α-glucosidase enzyme inhibition by

various crude extracts are summarized in Table 3. The highest IC_{50} value indicates the lowest α-amylase and α-glucosidase enzyme inhibition property and *vice versa*. Acarbose (positive control) inhibited the α-amylase activity with an IC_{50} value of 5.68 ± 0.35 ppm, while the IC_{50} values of hexane, ethyl acetate and methanol extracts were found to be 25.74 ± 0.60, 27.11 ± 1.16 and 360.23 ± 0.58 ppm, respectively. The hexane and ethyl acetate extracts showed significant inhibitory action of the α-amylase enzyme with respect to the positive control, acarbose. The α-glucosidase inhibitory potential of acarbose was found to be 17.11 ± 0.62 ppm, while the IC_{50} values of hexane, ethyl acetate and methanol extracts were 22.37 ± 0.06, 33.70 ± 0.22 and 179.62 ± 0.85 ppm, respectively. The results obtained for α-glucosidase inhibition assay of the crude extracts varied in a similar manner to the α-amylase inhibition activity. In this investigation, hexane and ethyl acetate extracts of the polyhedral mixture were discovered to possess significant inhibitory activity on the carbohydrate digestive enzymes ($p < 0.05$).

Table 2. Antioxidant activities of the different crude extracts of the polyherbal mixture.

Plant extract/ standard drug	DPPH (IC_{50} ppm)	FRAP ($moldm^{3-}$)	TPC (mg $GAEg^{-1}$)
Hexane extract	24.71 ± 0.01^{a}	23.29 ± 0.78^{a}	10.26 ± 0.28
Ethyl acetate extract	39.34 ± 0.09^{a}	8.50 ± 0.36^{a}	8.47 ± 0.09
Methanol extract	440.81 ± 0.04^{ab}	5.57 ± 0.36^{b}	4.28 ± 0.02
Ascorbic acid	6.78 ± 0.03^{ac}	25.02 ± 0.21^{ac}	

Data is represented as mean ± SEM. The same letter in the same column shows no significant difference and different letters show a significant difference ($p < 0.05$).

Table 3. Inhibitory activities of crude extracts of the polyherbal mixture on α-amylase and α-glucosidase enzymes.

Sample	α-amylase IC_{50} /ppm	α-glucosidase IC_{50} /ppm
Hexane extract	25.74 ± 0.60^{a}	22.37 ± 0.06^{a}
Ethyl acetate extract	27.11 ± 1.16^{a}	33.70 ± 0.22^{a}
Methanol extract	360.23 ± 0.58^{ab}	179.62 ± 0.85^{ab}
Acarbose	5.68 ± 0.35^{ac}	17.11 ± 0.62^{ac}

Data is represented as mean ± SEM. The same letter in the same column represents no significant difference and different letters show a significant difference ($p < 0.05$).

In vivo Acute Antidiabetic Activity of the Polyherbal Mixture

The *in vivo* acute antidiabetic activity was evaluated under hypoglycemic and antihyperglycemic activity. The blood glucose concentration reduction in oral glucose tolerance of the crude extracts of the polyherbal mixture over four hours was evaluated using the TAUC values. Low TAUC values represent high improvement or efficacy in the oral glucose tolerance of the extract. The mean TAUC values for healthy rats treated, with the crude extracts of the polyherbal mixture are summarized in Table 4. The hexane extract showed the optimum effectiveness at the therapeutic dose while the methanol extract exhibited minimum potency to develop hypoglycemia. The improvement percentage of oral glucose tolerance at the therapeutic dose was in the descending order of hexane extract (7.08%), ethyl acetate extract (3.82%) and methanol extract (2.10%). However, the TAUC values achieved for the combined plant mixture-treated groups were not statistically significant ($p > 0.05$) compared to the healthy control group.

The effect of crude extracts of the polyherbal mixture on the oral glucose tolerance in streptozotocin-induced diabetic rats is shown in Table 5. The oral administration of the crude extracts at the lowest dose of each reduced the serum glucose concentration by 14.22, 12.30 and 6.20%, respectively, in diabetic rats. At the therapeutic dose of each extract, the serum glucose concentration was reduced in diabetic rats by 19.23, 17.44 and 9.32%, respectively, in the OGTT. At the highest dose, a significant lowering of the serum glucose concentration was observed in all extracts-treated groups showing 22.78, 20.11 and 13.46% reduction, respectively, when compared to the diabetic control group. Glibenclamide was the standard drug used in the current study. The glibenclamide-treated diabetic rats showed a TAUC value of 77.46 mmol/L.h and an improvement of 24.46% in glucose tolerance. The capacity of the oral glucose tolerance of the glibenclamide-treated group was significantly different in the methanol extract-treated group at the dose of 32 mg/kg ($p < 0.05$). The oral administration of the standard drug of glibenclamide also reduced the serum glucose concentration by 33.36% in diabetic rats. The mean TAUC value and fasting blood/serum glucose concentrations were significantly improved by 318% and 270.73%, respectively, in diabetic untreated rats when compared to healthy untreated rats ($p < 0.05$). A significant improvement in oral glucose tolerance was observed for the hexane extract at the doses of 8, 25 and 75 mg/kg, the ethyl acetate extract at the doses of 10, 30 and 90 mg/kg and the methanol extract at the dose of 285 mg/kg treating diabetic rats ($p < 0.05$). The highest antihyperglycemic effect at the therapeutic dose, however, was recorded in the hexane extract-treated diabetic rats and the observed glucose tolerance was 17.38%.

Table 4. The area under the oral glucose tolerance curve values (OGTT) and the total area under the curve values (TAUC) of crude extracts of the polyherbal mixture in healthy rats.

Group	The area under the OGTT curve values (mmol/L.h)				TAUC (mmol/L.h)
	1 hour	2 hours	3 hours	4 hours	
Healthy control rats	6.04 ± 0.14	6.94 ± 0.25	5.74 ± 0.24	5.11 ± 0.23	23.84 ± 0.81
Hexane extract	5.67 ± 0.13	6.12 ± 0.11	5.40 ± 0.10	4.96 ± 0.11	22.15 ± 0.37
Ethyl acetate extract	5.60 ± 0.08	6.35 ± 0.12	5.70 ± 0.12	5.28 ± 0.10	22.93 ± 0.31
Methanol extract	5.82 ± 0.17	6.68 ± 0.31	5.84 ± 0.22	5.01 ± 0.14	23.34 ± 0.76

Data is represented as mean ± SEM (n = 6/group).

Discussion

Several studies have shown a certain upward propensity of the prevalence of diabetes mellitus throughout the world[33]. For the successful management of diabetes and its complications, it requires mediation on diet plans, lifestyle changes, oral hypoglycemic agents, lipid-lowering agents, …*etc*. Further, early diagnosis and treatment of diabetes mellitus are also significant parts of the management and lead to preventing complications associated with diabetes mellitus[34]. The present investigation was mainly focused on the antidiabetic potential of the selected polyherbal mixture extracts obtained *via* a sequential extraction process.

Standardization is important for maintaining and estimating the quality, purity, efficacy and safety of a polyherbal drug formulation, as these are combinations of more than one herb to obtain the maximum therapeutic effect[35]. According to the WHO guidelines for herbal drugs, chemical evaluation and standardization, the quality and standards of a herbal drug can be evaluated under the following categories: the identity of the drug, the physicochemical character of the drug and the pharmacological parameters[36]. Hence, the standard parameters, such as physicochemical properties, HPLC fingerprint profiles and phytochemical constituents, were determined to evaluate the quality, purity, safety, compound composition and therapeutic effect of the selected polyherbal mixture used in the present study. Physicochemical evaluation of herbal drugs is important for detecting inorganic contaminants present in the drugs. According to WHO, the excess moisture content in herbal plant materials leads to microbial and insect contamination, deterioration and microbial

growth[37]. The hexane and ethyl acetate extracts, therefore, are in a safe margin in terms of minimizing microbial contamination and deterioration. The total ash content is an essential part of the evaluation of the purity, authenticity and quality of drugs[38]. Usually, it indicates the presence of phosphates, carbonates and silicates as inorganic contaminants in a drug[39]. Thus, a high total ash value represents contaminations and impurities in the samples. Therefore, the low total ash content in the hexane and ethyl acetate extracts indicates fewer inorganic impurities and the high quality of the extracts. As well, very low values for acid insoluble ash and water-soluble ash indicate the presence of the least amounts of impurities in the sample and confirm further the low possibility of adulteration and contamination of the samples[40,41]. These low values recorded in the physicochemical evaluation, therefore, confirm the high quality and purity of the polyherbal mixture used in the study. The HPLC analysis of the three extracts of the polyherbal mixture aimed to identify the major peaks and screen the phytochemical composition. The HPLC fingerprint technique is a more precise standardization method and it has been applied to study the change of phytocomponents of the sample with time[42]. According to literature reports, mahanimbine, isomahanimbine, koenimbidine, murrayacine and koenimbine have been isolated as the major compounds from the hexane extract while myricetin and quercetin have been isolated as the major compounds from the ethyl acetate

Table 5. Values of the area under the oral glucose tolerance curve (OGTT) and values of the total area under the curve (TAUC) of crude extracts of the polyherbal mixture in streptozotocin-induced diabetic rats.

Group	Area under the OGTT curve values (mmol/L.h)				TAUC
	1 hour	2 hours	3 hours	4 hours	(mmol/L.h)
Healthy control rats	6.33 ± 0.19	7.08 ± 0.25	5.86 ± 0.24	5.26 ± 0.16	24.53 ± 0.66[a]
Diabetic control rats	23.76 ± 1.36	28.73 ± 0.82	26.42 ± 0.99	23.64 ± 1.26	102.54 ± 2.26[c]
Diabetic rats+ Hexane extract (8 mg/kg)	22.00 ± 0.72	24.15 ± 0.81	21.07 ± 1.11	18.83 ± 1.13	86.04 ± 1.64[b]
Diabetic rats + Hexane extract (25 mg/kg)	20.87 ± 0.50	23.26 ± 0.43	21.14 ± 0.34	19.45 ± 0.33	84.72 ± 1.55[b]
Diabetic rats + Hexane extract (75 mg/kg)	20.82 ± 0.20	22.67 ± 0.25	21.23 ± 0.17	19.51 ± 1.00	84.23 ± 1.42[b]
Diabetic rats + Ethyl acetate extract (10 mg/kg)	22.99 ± 0.59	24.24 ± 0.10	20.80 ± 0.10	19.14 ± 0.87	87.15 ± 1.42[b]
Diabetic rats + Ethyl acetate extract (30 mg/kg)	21.30 ± 0.25	23.67 ± 0.97	21.75 ± 0.09	19.60 ± 0.98	86.33 ± 2.05[b]
Diabetic rats + Ethyl acetate extract (90 mg/kg)	21.07 ± 0.29	23.63 ± 0.54	21.16 ± 0.30	19.27 ± 0.23	85.12 ± 1.07[b]
Diabetic rats + Methanol extract (32 mg/kg)	23.21 ± 1.18	26.38 ± 0.94	23.41 ± 0.70	21.25 ± 0.75	94.25 ± 1.98[c]
Diabetic rats + Methanol extract (95 mg/kg)	21.70 ± 0.87	25.89 ± 0.19	22.92 ± 0.80	20.07 ± 0.62	90.57 ± 1.62
Diabetic rats + Methanol extract (285 mg/kg)	21.62 ± 0.77	24.67 ± 0.94	21.98 ± 0.93	19.35 ± 0.83	87.62 ± 1.40[b]
Diabetic rats + Glibenclamide (0.5 mg/kg)	18.83 ± 0.03	20.05 ± 0.15	19.60 ± 0.15	18.98 ± 0.17	77.46 ± 0.46[b]

The values are expressed as mean ± SEM (n = 6/group). [a]- significance at $p < 0.05$ for TAUC value of healthy group *vs.* TAUC value of diabetic control group; [b]- significance for TAUC value of crude extracts polyherbal mixture-treated group *vs.* TAUC value of diabetic control group at $p < 0.05$; [c]- significance at $p < 0.05$ for TAUC value of diabetic control and crude extracts-treated group *vs*. TAUC value of glibenclamide-treated group. Each data point is expressed as the mean ± SEM.

extract of MK leaves[43,44]. Further, diallyl sulfides, diallyl disulfide, diallyl trisulfide and dimethyl disulfide have been isolated as major compounds from both hexane and ethyl acetate extracts of AS cloves[45]. From the hexane extract, piperine and β-pinene have been recorded as the major isolated compounds, while β-caryophyllene has been isolated as the major compound from the ethyl acetate extract of PN seeds[46–48]. Garcinol has been recorded as the major isolated compound from the hexane extract of GQ fruits, as per literature reports[16]. Those characteristic peaks in the HPLC profile of hexane extract and ethyl acetate extract, therefore, may be due to the presence of either of the aforementioned compounds. In addition, these characteristic peaks would be beneficial to identify the existing contaminants or adulterants in the sample during the used time[49]. Further, if the sample is contaminated during the time of the experiment, these characteristic peaks with specific retention time may be changed or disappear in the next fingerprint profile of the sample. Moreover, the time of year at which the samples were collected and the geographical variations could also affect the phytochemicals present in herbal mixtures[50]. Hence, it is of utmost importance to record the HPLC fingerprint profile when promising activities are reported to maintain the reproducibility of the recorded features of the polyherbal composition. Further, this qualitative data may be beneficial for the proper characterization of a polyherbal formulation to obtain the maximum therapeutic effect of a drug designed for a specific disease and is important to ensure the drug quality and purity before introducing a new formulation to the pharmaceutical industry.

Phytochemical components present in the herbal plant are considered bioactive secondary metabolites and are important in different biological activities, such as antidiabetic, antioxidant, antifungal, anticancer antimicrobial, …*etc.*[51]. Previous studies on individual plants, that were used in the study except for GQ, have shown that different types of phytochemical groups, such as phenolics, alkaloids, flavonoids, steroids, glycosides, triterpenoids, saponins, …*etc.* are present[52–54]. These phytochemical constituents perform a significant function in the pharmaceutical merit of plant varieties. Especially, phenolic compounds are good sources of antioxidants and show a vast range of pharmacological characters, like antidiabetic, anti-inflammatory and anticancer effects[55]. Moreover, phytochemicals, like flavonoids and phenolic compounds, have been found to act as very active antioxidant groups and have therapeutic potential for diseases resulting from oxidative stress. Many of the above-mentioned phytoconstituents are involved in minimizing potent glucose suppressive activities caused by oxidative stress due to stimulation of insulin hormone secretion from pancreatic beta cells accelerating glucose fixation by the cells and/or suppressing glucose absorption through the intestine wall[56,57]. The antidiabetic effects of the selected polyherbal mixture used in the current study may be due to the presence of antidiabetic phytoconstituents. Therefore, this polyherbal mixture can be justified to be a valuable collection of compounds of considerable medicinal merit.

Oxidative stress can be one of the causes for diabetes mellitus and related side effects[58]. Oxidative stress is caused by an imbalance between antioxidants and the formation of free radicals[59]. Several scientific studies have justified the correlation between antioxidants activity *vs.* diabetes mellitus and antioxidant consumption is shown to be an effective treatment for diabetes mellitus[60]. In ethnopharmacological research, *in vitro* antioxidant activity studies are used often to screen for the therapeutic potential of plant extracts[61]. The scavenging activity of medicinal plants is determined widely by the method of DPPH (free radical) scavenging assay. The principle behind this phenomenon explains that DPPH free radicals react with antioxidant components available in the sample and thereafter, the antioxidant donates hydrogen to DPPH free radical and converts it into the reduced form[62]. The color change observed from violet to light yellow with the addition of crude extract was observed at 517 nm. The results in this radical scavenging assay, therefore, suggest that hexane and ethyl acetate extracts of the polyherbal mixture could be useful in developing a potential drug for diabetes mellitus and the associated oxidative stress.

Ferric reducing antioxidant power (FRAP) is also a very popular method in investigating the antioxidant capacity of plants having phenolic compounds[62]. The mechanism of this method is different from the DPPH assay and describes the reducing power of antioxidants. In the FRAP assay, reductant antioxidants present in the crude

extract reduce the ferric ion-TPTZ complex to ferrous ion-TPTZ complex and form a Prussian blue color complex of Fe^{2+}-TPTZ. According to the results, we prefigure that the antioxidant potential based on the reducing potential of the hexane and ethyl acetate extracts was probably due to the presence of high polyphenolic components. The presence of polyphenolic compounds in the crude extracts was detected by the color change of the sample from the yellow phosphomolybdate-phosphotungstate complex to a blue-color complex. The obtained results from the present study corroborated the polyphenol content of the polyherbal mixture, which might contribute to an increment in the *in vitro* antioxidant activity. Previous studies on antioxidant activity reported that the antioxidant activity of the sample is highly correlated with its phenolic content[63,64]. Thus, the polyphenolic components of the extracts used in the present study could be the pivotal phytoconstituents contributing to the antioxidant potential in radical scavenging and reducing power. In summary of antioxidant assays, hexane and ethyl acetate extracts exhibited comparatively higher antioxidant capacity, in terms of the capability of DPPH free radical scavenging, reducing antioxidant power of ferric ion and total polyphenolic content. The presence of significant antioxidant activities in these two extracts may be due to the presence of highly active and concentrated antioxidant compounds. This was further correlated with the finding of Liyanagamage *et al.* in their study on the fruit rinds of GQ which have shown higher antioxidant activity in the hexane extract as well and the high antioxidant active compound gacinol isolated in high yield from the same extract[65].

The antidiabetic effects of herbal extracts and polyherbal mixtures are mainly *via* induction of insulin hormone secretion, minimization of the demand for insulin, multiplication of the activity of insulin at specific tissues or cells and the inhibition of carbohydrate digestion enzyme activity[66]. Enzyme inhibition is a major factor involved in the management of diabetes and this approach has been used by many scientists for the detection of *in vitro* and *in vivo* antidiabetic activity of selected synthetic drugs as well as natural products. Eddouks *et al*. described that antihyperglycemic potential is linked to the capability to halt digestion of polysaccharides to small sugar molecules and the absorption of these digested molecules, consequently avoiding a sudden rise in serum glycemic level after the diet[67]. The *in vitro* α-amylase inhibition activity signifies the abundance of reducing sugar remaining after the treatment of polysaccharides (starch) and plant material with the α-amylase enzyme. The presence of α-amylase enzyme inhibitors in the plant material inhibits the action of α-amylase which is important for the digestion of starch into di- and/or oligosaccharides. α-amylase inhibitors contribute to a delay in the glucose absorption rate further allowing to maintain the serum blood glucose in the human body[68]. Therefore, the hexane and ethyl acetate extracts could be used as polysaccharides, mainly starch blockers by impeding the digestion of polysaccharides into their reducing forms like maltose and other simple sugars. These results, therefore, justified the presence of α-amylase inhibitors in hexane and ethyl acetate extracts and further described their antidiabetic activity. α-Glucosidase activity is measured *in vitro* by the determination of the reducing sugars arising from hydrolysis of sucrose *via* the enzyme. α-Glucosidase is responsible for the digestion of disaccharides and oligosaccharides into the smallest sugar molecules[69]. The inhibitors of the α-glucosidase enzyme deputize one group of medicines known in the therapies of diabetes mellitus. In this process, when the enzyme activity is inhibited by the inhibitors, this leads to a decrease in the blood glucose level, because the glucose absorption is minimized due to the low production of the simple sugar molecules[70]. The hexane extract, however, showed better α-amylase and α-glucosidase enzymes inhibitory activity, possibly due to the presence of high concentrations of phytochemical constituents which could have behaved as effective enzyme inhibitors in the management of diabetes mellitus.

In summary, the outcomes of this work implied that the polyherbal mixture has no potency to develop hypoglycemic conditions in healthy rats after the administration of the polyherbal mixture and would be a satisfactory therapeutic agent at the particular dose. During the diabetic induction process, STZ drug was used to develop type-1 diabetes mellitus in Wistar rats. The STZ is the most used chemical for type-I diabetic induction, which leads to selective pancreatic β-cell destruction by increasing the release of nitric oxide in the medium. This results in gradual depletion in pancreatic β-cell pyridine nucleotide amount and

subsequent β-cell death. Thus, it results in an increase in blood glucose concentration in STZ-induced diabetic rats. According to the anti-hyperglycaemic study, all the extracts showed dose-dependent reduction of the serum glucose concentration in OGTT at doses of hexane extract of 8, 25 and 75 mg/kg, ethyl acetate extract of 10, 30 and 90 mg/kg and methanol extract of 32, 95 and 285 mg/kg in diabetes-induced rats. This might be due to the presence of antidiabetic and antioxidant compounds and inhibitors of carbohydrate digestion enzymes. These results corroborate with those of other *in vitro* studies and the antihyperglycemic effect of hexane and ethyl acetate extracts would be due to the availability of a higher number of antidiabetic compounds, such as mahanimbine, isomahanimbine, koenimbidine, murrayacine koenimbine, myricetin, diallyl sulfide, diallyl disulfide, diallyl trisulfide, piperine and garcinol, present in the selected plants and the synergism of the active phytoconstituents[16,71–76].

Conclusions

In conclusion, standardization of a polyherbal mixture made from a mixture of equal amounts by weight of *Allium sativum* L. (garlic) cloves, *Murraya koenigii* L. Sprengel leaves (curry leaves), *Piper nigrum* L. (black pepper) seeds and *Garcinia queasita* Pierre (rath goraka) fruits was carried out based on the physicochemical properties, HPLC profiles and phytochemicals. Physico-chemical data indicates that the crude extracts obtained during the extraction process have no contaminations. The HPLC data concluded that hexane and ethyl acetate extracts of the polyherbal mixture possessed a high profile of bioactive compounds. The results revealed that the hexane and ethyl acetate extracts of the polyherbal mixture exerted both *in vitro* antioxidant potential and antidiabetic properties which may be due to the high profile of phytoconstituents. This data, therefore, suggested that hexane and ethyl acetate extracts of the above polyherbal mixture might be useful in developing novel therapeutic agents targeting the free radical pathologies in diabetes mellitus and could be used as a potential source to isolate natural antidiabetic agents. Further, the *in vivo* study carried out with streptozotocin-induced diabetic rats revealed that hexane and ethyl acetate extracts, at the equivalent therapeutic dose, had a significant dose-dependent anti-hyperglycemic effect. Future studies on long-term antidiabetic effect of the most active extract and also the potential mechanism of action will be useful to confirm the antidiabetic effect of the polyherbal mixture. Additional efforts on isolation and identification of active components responsible for the reported biological activities are required.

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