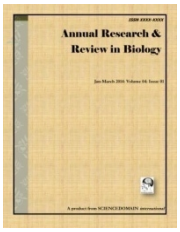

Annual Research & Review in Biology
4(11): 1813-1826, 2014

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Hypoglycemic Potential of Polysaccharides of the Leaf Extract of *Telfairia occidentalis*

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Authors' contributions

Author OAE undertook this work as a USM-TWAS postdoctoral fellow under the supervision of authors MAS and HAR participated in the design of the work and also in providing the materials used. Author AA was involved in the animal work. Author SF performed in the animal study and HPLC analysis. Authors ML, FA, SA were involved in the chromatographic analyses. All authors read and approved the final manuscript.

Original Research Article

Received 17th December 2013
Accepted 23rd January 2014
Published 15th February 2014

ABSTRACT

Context: *Telfairia occidentalis* Hook (Cucurbitaceae), a popular vegetable in West Africa, has been reported to have antidiabetic property. But the active components are not yet known.
Aims: This study was carried out to determine the hypoglycemic potentials and the antidiabetic property of polysaccharides of the leaf of *Telfairia occidentalis*.
Place and Duration of Study: Hypertension and Cardiovascular Physiology Research Laboratory; School of Pharmaceutical Sciences, University Sains Malaysia, Penang, Malaysia, between June 2013 and October 2013.
Methodology: The dried plant material was macerated with water and extracted with ethyl acetate to obtain ethyl acetate and aqueous residue fractions. To obtain the crude polysaccharides, the plant material was boiled with water. Ethanol (96%) was added to the extract. The precipitate obtained was successively washed with ethanol (99%), ether and acetone. Crude proteins were obtained by ammonium sulphate precipitation. The various fractions (250 and 500mg/kg) were orally administered to normoglycemic and STZ-induced diabetic rats. Blood glucose was evaluated with a glucometer at 0, 1, 2 and 4 hours, after administration. Glibenclamide (4mg/kg) was used as the positive control. Column

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chromatography and HPLC analyses of the aqueous residue and crude polysaccharides were carried out.
Results: None of the fractions at the two dose levels affected blood glucose concentration of the normoglycemic rats. However, the aqueous residue and crude polysaccharides significantly (p=0.05) reduced glucose level at 4 hours in the diabetic rats from 100% at 0 hour to 74.28 and 60.87% at 4 hours, respectively. Based on the column chromatography and HPLC profiles, the hypoglycemic activity of the aqueous residue and crude polysaccharides fractions could be attributed to their carbohydrates content.
Conclusion: Polysaccharides from the leaf of *Telfairia occidentalis* possess hypoglycemic activity and have the potential of being useful in the management of diabetes. Further work needs to be done to characterize the active polysaccharides.

Keywords: Telfairia occidentalis; Polysaccharides; Diabetes; HPLC; Streptozotocin; Blood glucose.

1. INTRODUCTION

Diabetes continues to be a ravaging disease and remains one of the leading causes of heart attack, blindness, kidney failure and lower limb amputation. It is the fourth major cause of death in developed countries. 5-10% of the world's health care budget is spent on the management of diabetes. Some countries may spend as much as 40% of their budget to manage the scourge of diabetes in 2025. Because, according to International Diabetes Federation, the global prevalence of diabetes will reach about 439 million by the year 2035, if care is not taken [1,2].

There is an increasing demand for the use of antidiabetic herbs in the treatment of diabetes, especially in developing countries, for many reasons. It is now known that neither insulin nor the oral hypoglycemic agents are capable of restoring a normal pattern of glycemic control, particularly in type 2 diabetes which accounts for over 90% of all cases of diabetes. The exorbitant cost, lack of availability, side effects and toxicity of these synthetic hypoglycemic drugs also limit their use. In developing countries, religion, folklore, tradition and poverty compel patients to patronize traditional herbal remedies. In recognition of this growing trend, the World Health Organization has made a call for greater recognition of herbal medicine [3,4].

Telfairia occidentalis Hook (Cucurbitaceae), popularly known as fluted pumpkin, is widely cultivated and consumed in West Africa mainly because of its nutritious leaves [5]. The medicinal potential of the plant has also been receiving increasing attention in recent times. The antioxidant, hepatoprotective, hematological, antiplasmodial, antimicrobial, testiculoprotective, anticancer, anti-inflammatory, anxiolytic and sedative properties of the plant have been documented [6]. The hypoglycemic and antidiabetic properties of the plant have also been reported [7-12].

There is paucity of information on the phytochemical constituents of the leaf of *Telfairia occidentalis*. However, it has been reported that the leaf contains tannins, flavonoids, alkaloids, saponins, steroids, anthraquinones, and glycosides [13,14]. The presence of long chain n-3-unsaturated fatty acid from the leaf has also been reported. Palmitoleic acid (16.62%) and elaidic acid (0.85%) are the predominant omega 9 fatty acid present in the leaf [15]. The carbohydrate content of the leaf is 25% [16,17].

Most of the reports of the pharmacological activities of the plant are on the aqueous, methanolic and ethanolic extracts. The aim of this study was therefore to investigate the hypoglycemic and antidiabetic properties of the whole aqueous extract, ethyl acetate fraction, aqueous residue (obtained after ethyl acetate extraction of the aqueous extract), crude polysaccharides and crude proteins of the leaf of *Telfairia occidentalis* in both normoglycemic and STZ-induced diabetic SD rats. The choice of water, and not organic solvents, as the extraction medium in this work was based on the fact that water is the common solvent used to prepare the plant for consumption.

2. MATERIALS AND METHODS

2.1 Plant Materials

Fresh leaves of *Telfairia occidentalis* were collected at the Medicinal plant garden of the Faculty of Pharmacy, University of Uyo, Nigeria. The plant was identified in the same Faculty and was assigned Voucher number UUH28(d). The fresh leaves were washed with distilled water, air dried and pulverized with a grinding machine.

2.2 Plant Extraction

The powdered plant material (400g) was macerated in distilled water (4 liters) at 50ºC for 12 hours. The extract was filtered with Whatman filter paper, concentrated in an oven (Binder, USA) at 30ºC and freeze-dried in a freeze dryer (LACONCO Freeze dry/Shell, LACONCO Corporation, USA). The freeze dried extract was kept at -29ºC in a deep freezer (SANYO Biomedical Freezer) until it was used.

2.3 Fractionation of Water Extract

The freeze dried whole water extract (50 g) was partitioned repeatedly with ethyl acetate (200ml) at room temperature with a separatory funnel until the solvent was no longer coloured. The ethyl acetate fraction and the residue of the water extract were concentrated *in vacuo* to obtain the ethyl acetate fraction and aqueous residue, respectively.

2.4 Preparation of Crude Polysaccharides

The powdered plant material (50g) was boiled in distilled water (500ml) for 4 hours. The extract obtained was filtered with Whatman filter paper and concentrated at 30ºC to 100ml. Ethanol (96%, 400ml) was added to the concentrated extract. The mixture was kept at room temperature for 12 hours. The ethanol supernatant was decanted and the precipitate was successively washed with ethanol (99%), ether and acetone to obtain the crude polysaccharides (18). Each washing was done three times with 200ml of the solvent.

2.5 Preparation of Crude Proteins

The dried plant material (50g) was macerated with distilled water (500ml) at room temperature for 24 hours. The extract was filtered and concentrated in vacuo. Ammonium sulphate (75.61g) was added to the water extract (100ml) at 20ºC in small quantities while stirring carefully after every addition. Each batch was allowed to dissolve before adding the next batch. The precipitated proteins in the fully saturated solution were removed by centrifugation at 12000xg for 10minutes. The precipitated protein was re-suspended in

distilled water and dialysed with Fisherbrand^R regenerated cellulose dialysis tubing (Nominal MWCO 12000-14000, 20µm wall thickness, vol/cm: 6.42ml, Fisher Scientific, USA) against water at room temperature for 24 hours. The water was changed four times during the period. The crude protein obtained after dialysis was freeze- dried.

2.6 Column Chromatography of Aqueous Residue and Crude Polysaccharides

Sephadex LH-20 (Fluka Biochemika) (30g) was added to excess water in a beaker and allowed to swell for 4 hours. Excess water was decanted to obtain slurry in a ratio of 75% settled sephadex to 25% water. The slurry was carefully stirred with a glass rod to re-suspend it. It was then poured into the glass tube (column) in one continuous motion with a glass rod held against the wall of the column. The column reservoir was filled with water and the tap opened to allow water to run through until the Sephadex had reached a constant height (52cm). The aqueous residue was dissolved in water, filtered with filter syringe (Minisart R, 0.2m pore size) and loaded into the column. The column was eluted with distilled water. Forty five eluents (6ml/tube) were collected. Protein and carbohydrate content of each tube was monitored. The same process was followed for crude polysaccharides.

2.7 Evaluation of Carbohydrates and Proteins Contents

Carbohydrates content of the eluents were determined using the modified Phenol-sulphuric acid method [19]. Each sample (50µl) was pipetted into a microplate well. Concentrated sulphuric acid (150µl) was rapidly added to each well to cause mixing. Phenol (5%, 30µl) was immediately added. The microplate was incubated for 5 mins at 90ºC in a static water bath by floating the microplate carefully. The microplate was removed, cooled in another water bath for 5 minutes and dried. Absorbance was measured at 490nm by a microplate reader (TECAN infinite M200 PRO, USA). Absorbance of each eluent was plotted against tube number. This same procedure was followed to evaluate the carbohydrate content of the aqueous residue and crude polysaccharides from a glucose standard.

Lowry method [20] was used to monitor the protein content of each tube. Freshly prepared alkaline Copper sulphate reagent (2ml) was mixed with 0.2ml of sample and incubated for 10minutes at room temperature. Folin ciocalteau reagent (0.2ml) was added to the mixture. The mixture was incubated for 30 minutes at room temperature. The reaction mixture (200µl) of each of the eluent was added into a microplate well and their absorbance measured at 660nm by a microplate reader. This same procedure was followed to evaluate the protein content of the aqueous residue and crude polysaccharides from a standard curve obtained using different concentrations of Bovine serum albumin (standard).

2.8 HPLC Profile of the Aqueous Residue, Crude Polysaccharides and Crude Proteins

HPLC profile of the plant materials was obtained using Shimadzu (Japan) HPLC which was equipped with Prominence Degasser (DGU-20A_5), Prominence pump (LC-20AD), Prominence CBM(CBM-20A), Prominence autosampler (SIL-20A_{HT}), column oven (CTO-10ASvp), fluorescence detector (RF-10AXL) and Hypersil Gold column (4.6x250cm, 5µm, Thermo scientific, USA). The plant materials (1mg/ml of water) were filtered with syringe filter (0.45µm pore size). Injection volume was 20µl and oven temperature was 30^{0}C. Isocratic elution was done using filtered distilled water as mobile phase. Flow rate was

1ml/minute and run time was 50minutes. Detection was done at excitation/emission wavelengths of 280/350nm and 330/400nm.

2.9 Animals

Male Sprague-Dawley (SD) rats weighing 200-300g were obtained from the animal house of Universiti sains Malaysia (USM, Malaysia). They were given free access to food (standard rat pellet diet) and water and were kept under a 12:12 hour light/dark cycle at ambient temperature of about 25ºC. All authors hereby declare that the "principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed. All experiments were examined and approved by the animal ethics committee of Universiti Sains Malaysia (Animal Ethics Approval No. USM/animal ethics approval/2013/(86)(439).

2.10 Induction of Diabetes

Overnight fasted rats were injected intraperitoneally with 55mg/kg of Streptozotocin (Sigma, USA). The Streptozotocin (STZ) was freshly dissolved in freshly prepared ice-cold citrate buffer (0.1M, pH 4.5) and was injected within 15minutes after dissolution [21]. The injected rats were given glucose (5%) for the first 48 hours after injection to ameliorate the initial hypoglycemic shock. After 7 days, the blood glucose level of the rats was evaluated. A pilot study earlier undertaken showed that almost all the rats became highly diabetic (with glucose level > 20mmol/L) but many of them soon afterwards showed signs of recovery from diabetes. This necessitated a follow up injection of 40mg/kg of STZ two weeks after the first injection. Rats with blood glucose concentration above 17mmol/L were used in this experiment.

2.11 Experimental design

2.11.1 Effect of plant materials on normoglycemic rats

Normal (normoglycemic) rats were fasted overnight and divided into twelve groups (five rats per group). The first five groups were treated with 250mg/kg each of the whole extract, aqueous residue, crude polysaccharides, crude proteins and ethyl acetate fraction, respectively. Another five groups received 500mg/kg each of the whole extract, aqueous residue, crude polysaccharides, crude proteins and ethyl acetate fraction, respectively. The last two groups which served as the positive and negative control received Glibenclamide (4mg/kg) and Vehicle (Tween 80 (1%)), respectively. All materials administered were done through the oral route using a gavager.

2.11.2 Effect of plant materials on diabetic rats

Twelve groups of overnight fasted diabetic rats were also used and were treated the same way as in the normoglycemic rats.

2.11.3 Evaluation of blood glucose

In both the normoglycemic and diabetic rats blood was collected from the tail vein at 0, 1, 2 and 4 hrs after treatment and blood glucose concentration was evaluated by the glucose oxidase method with a commercial glucometer (Accu-chek^{TM}, Roche, Germany).

2.12 Statistical Analysis

Data obtained are presented as Mean±SEM and were analysed using One way ANOVA, followed by Turkey's post test with the aid of GraphPad prism 5.0 (GraphPad Software Inc., 2007.). Values with p=0.05 were considered significant.

3. RESULTS AND DISCUSSION

Percent extraction yield for the whole water extract, aqueous residue, crude polysaccharides, crude protein and ethyl acetate fraction were 3.65, 99.1, 4.8, 1.2, 0.9%, respectively. Results of the effect of the plant materials on blood glucose of normoglycemic and diabetic rats are shown in Figs. 1 and 2, respectively. None of the plant materials at the two dose levels (250 and 500mg/kg) significantly affected blood glucose concentration in the normoglycemic rats. However, Glibenclamide (4mg/kg) significantly reduced blood glucose level to 68.27 and 60.17% at 2 and 4hrs after administration, respectively (Figs. 1a and 1b).

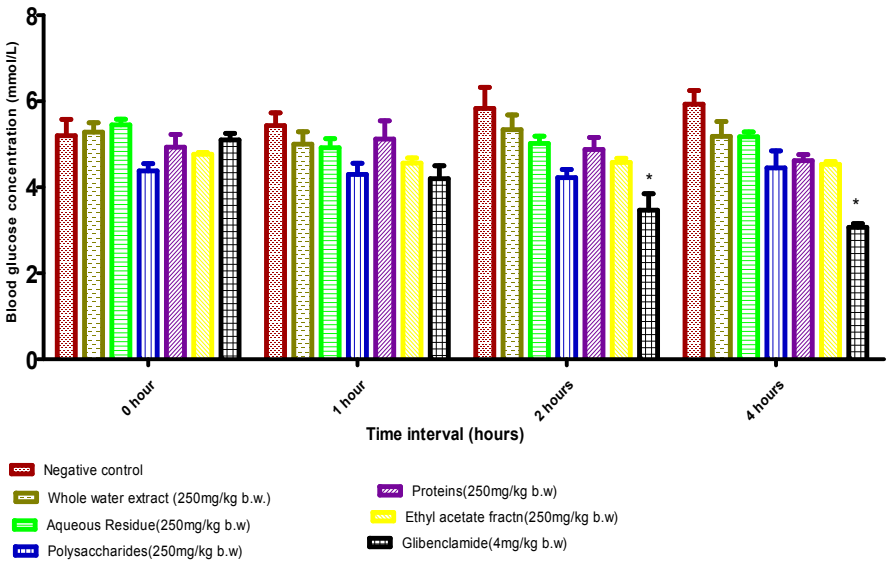

Fig. 1a. Effect of various fractions (250mg/kg) of *Telfairia occidentalis* on blood glucose concentration in normoglycemic rats.

*Test drugs: significant from normal control, * P < 0.05;*
Mean ± S.E.M = Mean values ± Standard error of means of five animals

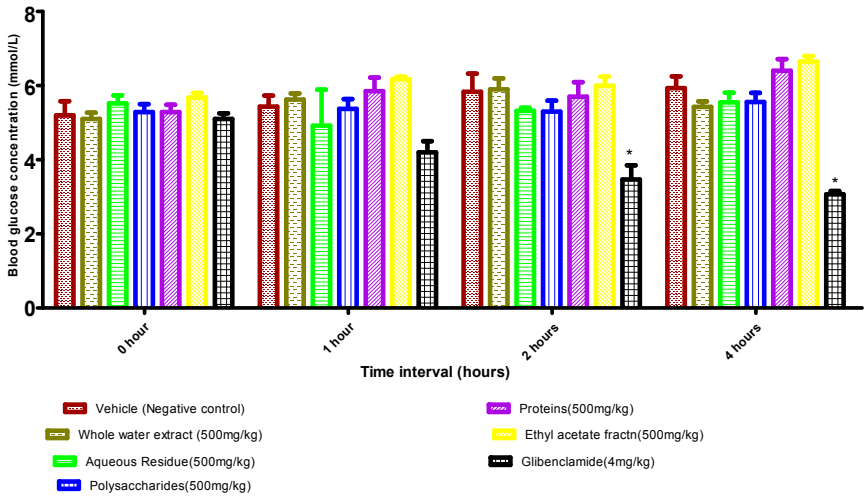

Fig. 1b. Effect of various fractions (500mg/kg) of *Telfairia occidentalis* on blood glucose concentration in normoglycemic rats.

*Test drugs: significant from normal control, * P < 0.05;*

Mean ± S.E.M = Mean values ± Standard error of means of five animals

In the diabetic animals, the aqueous residue and crude polysaccharides significantly reduced blood glucose level at the dose of 500mg/kg, four hours after administration from 100% at 0 hour to 74.28 and 60.87%, respectively. But Glibenclamide did not reduce glucose level in the diabetic rats (Figs. 2a and 2b).

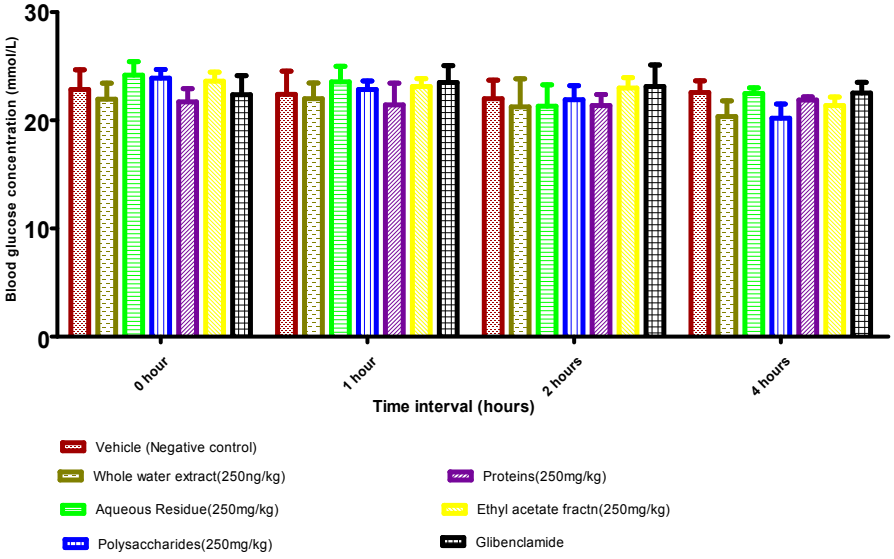

Fig. 2a. Effect of various fractions (250mg/kg) of *Telfairia occidentalis* on blood glucose concentration in diabetic rats.

*Test drugs: significant from normal control, * P < 0.05;*

Mean ± S.E.M = Mean values ± Standard error of means of five animals

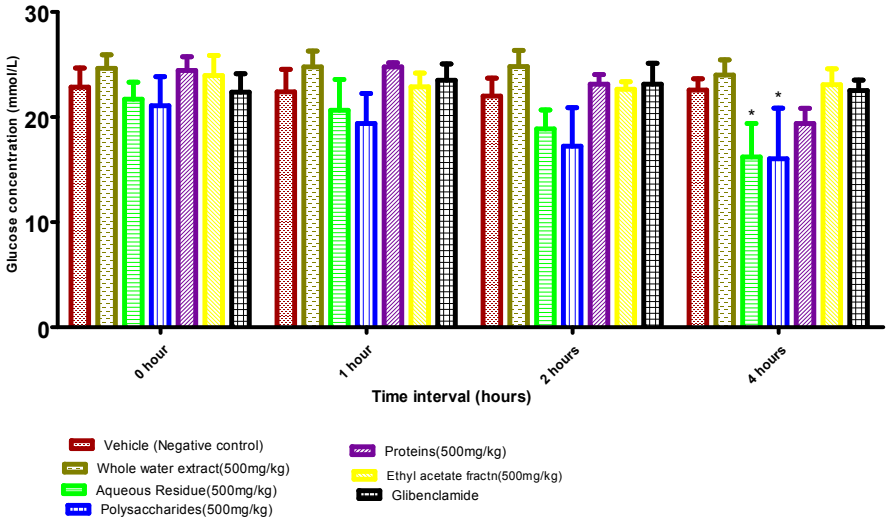

Fig. 2b. Effect of various fractions (500mg/kg) of *Telfairia occidentalis* on blood glucose concentration in diabetic rats.

*Test drugs: significant from normal control, * P < 0.05;*
Mean ± S.E.M = Mean values ± Standard error of means of five animals

The carbohydrate contents of the aqueous residue and crude polysaccharides obtained by extrapolation from the glucose standard curve (r^2=0.9948) using the phenol-sulphuric acid method are 424.76 and 346µg/ml, respectively. While the protein content evaluated from Bovine serum albumin standard curve (r^2=0.9786) using the Lowry method for aqueous residue, crude polysaccharides and crude proteins are 465, 580 and 750µg/ml, respectively. The results of column chromatography of aqueous residue and crude polysaccharides are presented in Figs. 3 and 4, respectively.

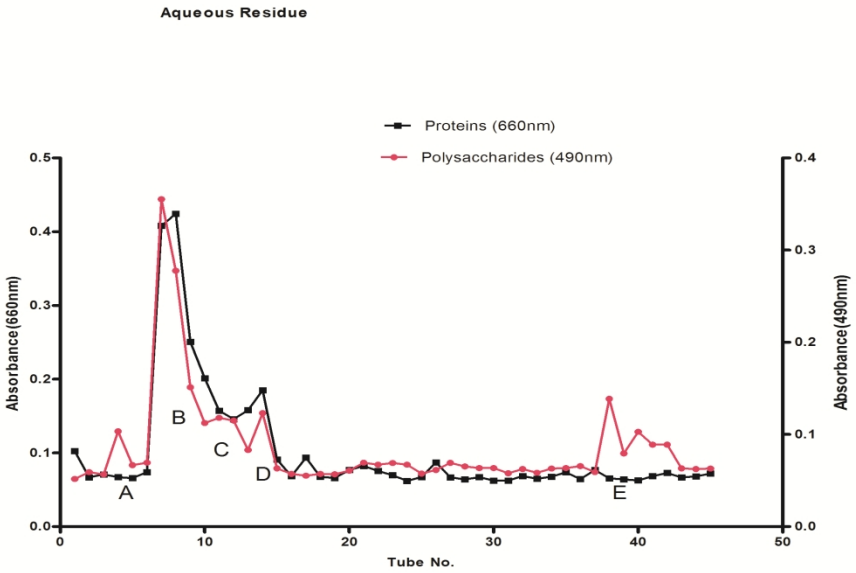

Fig. 3. Column chromatography profile of Aqueous Residue

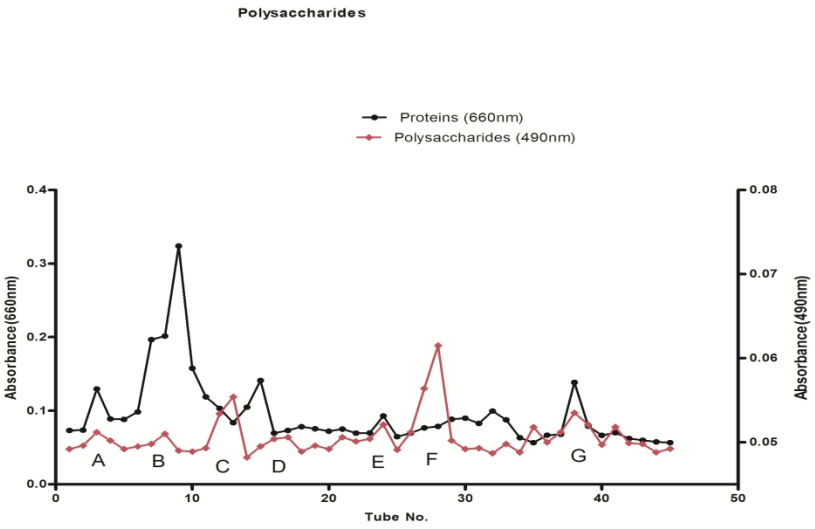

Fig. 4. Column chromatography profile of crude polysaccharides

The HPLC profiles of the aqueous residue, crude polysaccharides and crude proteins at the two different excitation and emission wavelengths are show in Figs. 5(a, b, c) and 6(a, b, c).

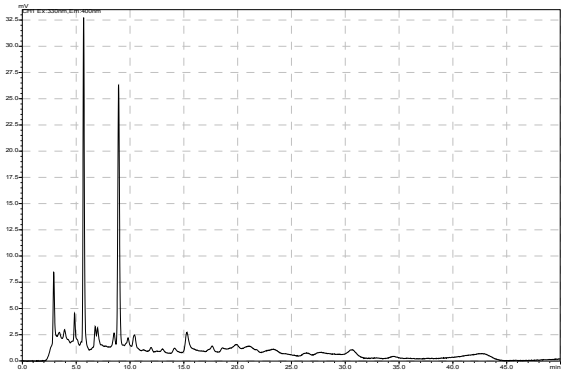

Fig. 5a. HPLC profile of aqueous residue at 330/400nm (excitation/emission wavelengths)

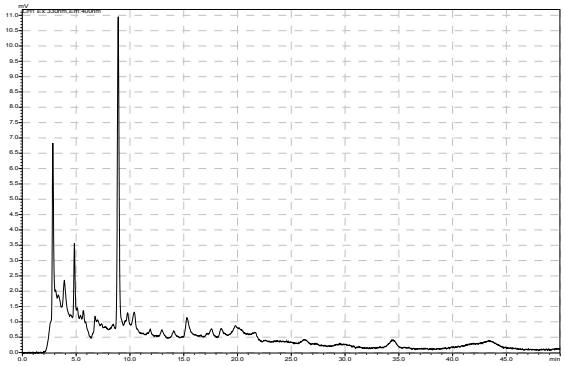

Fig. 5b. HPLC profile of crude polysaccharides at 330/400nm (excitation/emission wavelengths)

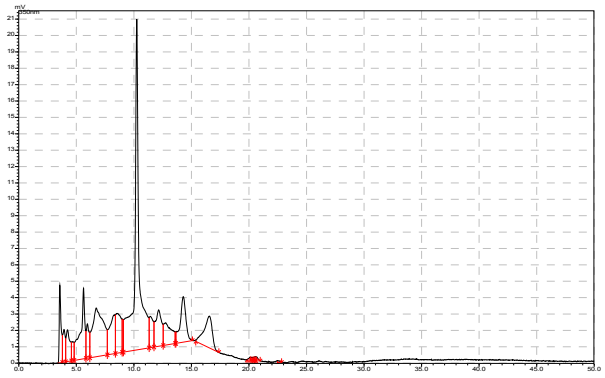

Fig. 5c. HPLC profile of crude proteins at 330/400nm (excitation/emission wavelengths)

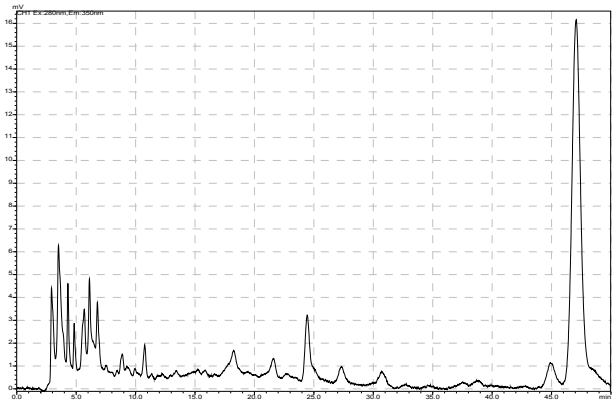

Fig. 6a. HPLC profile of aqueous residue at 280/350nm (excitation/emission wavelengths)

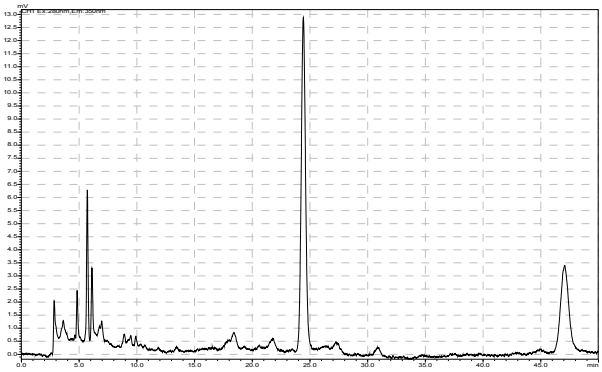

Fig. 6b. HPLC profile of crude polysaccharides at 280/350nm (excitation/emission wavelengths)

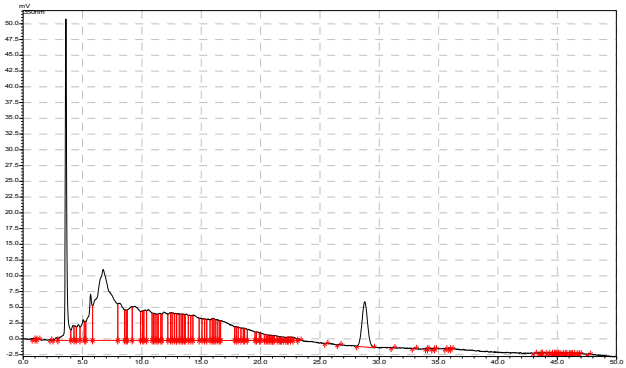

Fig. 6c. HPLC profile of crude proteins at 280/350nm (excitation/emission wavelengths)

The blood glucose reducing effect of glibenclamide in the normoglycemic rats is in line with the mechanism of action of the drug. Glibenclamide, a sulphonylurea, acts by stimulating the pancreatic β cell to secrete insulin, thereby reducing blood glucose level. Failure of the plant materials to reduce glucose level is an indication that, unlike glibenclamide, they do not possess the ability to enhance the stimulation of pancreatic β cell to secrete insulin.

Streptozotocin, a nitro derivative of glucosamine, is a pancreatic beta cell toxin that causes a rapid necrosis of the beta cells. It is a toxic analogue of glucose which accumulates selectively in the pancreatic beta cells through the glucose transporter. Inside the beta cell, STZ molecule dissociates into glucose and methylnitrosourea. Methylnitrosourea alkylates and modifies biomolecules, breaks down DNA and destroys beta cell, thereby causing diabetes [22]. In this study, the rats obviously did not respond to the beta cell stimulating activity of glibenclamide. This could be a consequence of the destruction of the beta cells in the diabetic rats by the administration of STZ, unlike in the normoglycemic rats whose beta cells were intact. By implication, the reduction of blood glucose level by the aqueous residue and crude polysaccharides could not be through pancreatic beta cell stimulation but rather through some extra pancreatic mechanisms.

The carbohydrate and protein contents of the aqueous residue as shown in the column chromatography profile (Fig. 3) have similar features, especially from tubes 6-12 and 13-16 (peaks B and D). In the crude polysaccharides profile (Fig. 4) the relationship between carbohydrates and proteins is not as close as in the aqueous residue. However, the major protein peaks in both the aqueous residue and crude polysaccharides are similar. The ethyl acetate fraction and crude proteins did not show any antidiabetic activity. This suggests that the antidiabetic effect of the aqueous residue and crude polysaccharides could be attributed to their polysaccharides (or carbohydrates) content. The aqueous residue contains more polysaccharides than the crude polysaccharides fraction. Apart from the likely presence of proteoglycans in the crude polysaccharides fraction, protein impurities may be responsible for its high protein content.

HPLC profiles of the aqueous residue and crude polysaccharides showed two identical major peaks at 2.908 (2.826) and 8.934 (8.900) minutes when detected at 330/440 nm (excitation/emission wavelengths). But the aqueous residue has an additional peak at 5.685minutes. When detected at 280/350nm, both the aqueous residue and crude polysaccharides again showed a similar pattern, with distinct peaks at 24.422(24.433) and

47.054(47.077) minutes. But, while the peak at 47.077mins is the larger one for the aqueous residue, the peak at 24.422 mins is the larger one for the crude polysaccharides (Figs. 6a and 6b). The crude proteins showed only one major peak at 10.425minutes at 330/440 nm (Fig. 5c) and also only one major peak at a retention time of 3.591min at 280/350nm (Fig. 6c). This major peak is different from those shared in common by the aqueous residue and crude polysaccharides.

Column chromatography and HPLC profiles of the fractions show that the aqueous residue and crude polysaccharides are similar in content. The HPLC profiles of these two fractions are clearly different from that of the crude proteins. None of the HPLC peaks of the aqueous residue and crude polysaccharides correspond to those of crude proteins. This shows that the major contents of the aqueous residue and crude proteins are not proteins. This leads to the conclusion that the protein content of the aqueous residue and crude polysaccharides is as a result of proteins bound to the polysaccharides, rather than free proteins. It is therefore highly probable that the major carbohydrates in the aqueous residue and crude polysaccharides are proteoglycans. This is supported by the column chromatography profiles of the two fractions which show similarity in the carbohydrate and protein profiles, especially in the aqueous residue.

These results lead to the inference that the blood glucose reducing effect of the aqueous residue and the crude polysaccharides fraction could be attributed to their carbohydrates (polysaccharides) content.

Polysaccharides are known to possess antidiabetic activity. Ivorra et al. [23] in their review gave a list of plant polysaccharides with antidiabetic activity. The list includes Aconitans A, B, C and D; Arborans A and B; Oryzabrans A, B, C and D; Ganoderans A and B; Discorans A, B, C, D, E and F; Saccharans A, B, C, D, E and F; Moran A; Ephedrans A, B, C, D and E and Panaxans A, B, C, D and E [23]. The antidiabetic property of polysaccharides from the fruit of pumpkin (*Cucurbita moschata*), a plant belonging to the Cucurbitaceae like *Telfairia occidentalis* has also been reported [24-26]. Further work needs to be done to isolate and identify the antidiabetic polysaccharides (or carbohydrates) in the leaf of *Telfairia occidentalis*.

4. CONCLUSION

The results of this study show that polysaccharides from the leaf of *Telfairia occidentalis* possess antidiabetic potential, acting through extra hepatic mechanism, and could be useful in the management of diabetes. Further work should therefore be carried out to isolate, characterize the active polysaccharide, and also elucidate its mechanism of action.

ETHICAL APPROVAL

All authors hereby declare that the "principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed. All experiments were examined and approved by the animal ethics committee of Universiti Sains Malaysia (Animal ethics approval No. USM/Animal ethics approval/2013/(86)(439).

ACKNOWLEDGEMENT

The authors are grateful to Universiti sains Malaysia and Third World Academy of Science for the award of postdoctoral fellowship to Dr O. A. Eseyin.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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