ORIGINAL ARTICLE ASIAN JOURNAL OF MEDICAL SCIENCES

Detection of protein glycation inhibitory potential of nine antidiabetic plants using a novel method

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Submitted: 13-06-2014 Revised: 14-08-2014 Published: 31-10-2014

ABSTRACT

Background: Protein glycation is a major cause of chronic diabetic complications. Medicinal plants with protein glycation inhibitory potential will be beneficial to prevent or delay diabetic complications. **Objective:** The objective of the study was to analyze protein glycation inhibitory potential of ten plants among which nine are well known for their antidiabetic effects. **Methods:** Methanol extracts were prepared using parts from nine plants namely, *Coccinia grandis, Ficus racemosa, Gymnema lactiferum, Gymnema sylvestre, Musa paradisiaca, Phyllanthus debilis, Phyllanthus emblica, Strychnos potatorum* and *Tinospora cordifolia*. Dried latex of *Pterocarpus marsupium* was used without further extraction. Glycation inhibitory potential of the extracts was analyzed using bovine serum albumin (BSA) and fructose, incubated in the presence or absence of plant extracts for 4 week. Appropriate controls and blanks and the standard inhibitor aminoguanidine were included. A novel native polyacrylamide gel electrophoresis (PAGE) method established recently was used to detect the effect of plant extracts on the BSA glycation. **Results:** Previously we have demonstrated that the BSA migration towards the anode is increased proportionate to the degree of glycation. This increase was comparatively less when the glycation was inhibited. Accordingly, most promising antiglycation effects were observed with *P. debilis* (whole plant), *P. emblica* (fruit) and *P. marsupium* (latex). *F. racemosa* (stem bark) showed the highest inhibition among the remaining extracts. *G. sylvestre* (leaf), *M. paradisiaca* (yam) and *T. cordifolia* (leaf) also showed promising inhibition. *C. grandis* (leaf) and *S. potatorum* (seed) showed lower inhibition. Lowest inhibition was observed with *G. lactiferum* (leaf). **Conclusion:** We have demonstrated the antiglycation potential of some antidiabetic plants, using a novel method developed by us recently. Extracts of *P. debilis, P. emblica and P. marsupium* showed a strong protein glycation inhibitory potential.

Access this article online

Website: http://nepjol.info/index.php/AJMS

DOI: 10.3126/ajms.v6i2.10584

Key words: AGE, Glycation inhibition, PAGE, Antidiabetic plants

INTRODUCTION

Protein glycation is a non enzymatic process which leads to the formation of stable irreversible group of compounds known as advanced glycation end products (AGEs). Amount of AGEs formed on a protein depends on the reactivity of specific amino groups, reactivity of the sugar, sugar concentration and the half-life of the protein.[1] Chronic hyperglycaemia associated with diabetes mellitus and the oxidative stress accelerates AGE production. AGEs are fluorescent and non fluorescent complex adducts which are accumulated predominantly on long-lived proteins such as collagen, compromising their physiological functions.[1] Acceleration of AGE levels in the body is a major cause for micro and macro vascular complications such as retinopathy, nephropathy and atherosclerosis associated with diabetes mellitus.[1-4] Several lines of evidence suggest the deleterious effects of AGEs leading to endothelial dysfunction and pathogenesis of vascular complications.[4]

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Scientific literature identifies inhibition of AGE formation as one of the therapeutic approaches to prevent the progression of diabetic complications. Several synthetic compounds have been evaluated for their inhibitory effects against the formation of AGEs. However, many synthetic inhibitors were not successful at clinical trials due to safety issues.[1] Numerous medicinal plants are being used to treat diabetes from ancient times with little or no side effects.[5] However, majority of traditional antidiabetic plants await proper scientific evaluation for their ability to prevent the chronic diabetic complications. Plants with protein glycation inhibitory potential will be beneficial in this regard.[1] Glycation inhibitors present in antidiabetic plants appear important candidates for the development of new therapeutics against diabetic complications which will also provide protection against hyperglycaemia and possibly the oxidative damage, as part of the overall diabetes management. However, adequate work has not yet been done in on this area especially in Sri Lanka. Analytical techniques available to identify protein glycation inhibitors require expensive specialized equipment[6-8] which might be one of the limiting factor regarding glycation studies. The objective of the study was to analyze protein glycation inhibitory potential of ten plants among which nine are well known for their antidiabetic effects using a relatively simple novel method established by us recently.

MATERIALS AND METHODS

Parts from ten plants were collected namely, *Coccinia grandis* (L.) J. Voigt *(Kowakka)* leaves, *Ficus racemosa* L. *(Attikka)* stem bark, *Gymnema lactiferum* (L.) R. Br. ex Schult *(Kurinnan)* leaves, *Gymnema sylvestre* (Retz.) R. Br. ex Schult *(Masbedda)* leaves, *Musa* X *paradisiaca* L. *(Alu kesel)* yam, *Phyllanthus debilis* Klein ex Willd *(Pitawakka)* whole plant, *Phyllanthus emblica* L. *(Nelli)* fruit, *Pterocarpus marsupium* Roxb. *(Gammalu)* latex, *Strychnos potatorum* L. f. *(Ingini)* seeds and *Tinospora cordifolia* (Willd.) Hook.f. & Thoms. *(Rasakinda)* leaves. Plants were authenticated and specimens were deposited at the National Herbarium, Royal Botanical Garden, Peradeniya. *P. marsupium* latex was dried under shade without further processing. Rest of the plant materials were cleaned, dried under shade and ground to a fine powder using a grinder. Dry powder (10 g) was extracted with methanol (100 ml) using the ultrasonicator. Methanol extract was filtered and the solvent was evaporated by rotary evaporator (Buchi RII) at 45-50°C. These crude extracts were stored at room temperature until further analysis.

Protein glycation was carried out using bovine serum albumin (BSA) and fructose (0.5 M).[7] Protein and sugar incubations were carried out in the presence of plant extracts [5 mg, 0.5 mg/ml in phosphate buffer (pH 7.4) and other suitable concentrations depending on the inhibitory activity of the extract] at 37°C for 4 week. BSA and fructose mixture in absence of plant extract was used as the positive control. Corresponding test blanks were prepared in the absence of fructose. Standard inhibitor aminoguanidine (AG) (10 mM) was included. Aliquots were collected at intervals for further analysis with polyacrylamide gel electrophoresis.

A novel native PAGE method established by us was used to detect the effect of plant extracts on the BSA glycation.[9] Polyacrylamide gels (10%) were prepared according to the standard technique. Samples were loaded under native conditions. Electrophoresis was carried out using Enduro Vertical Gel Electrophoresis system- E2010-P according to the standard Laemmli method.[10] After separation at pH 8.6, protein bands were visualized by staining with Coomassie brilliant blue. Changes in the migration position of BSA bands were compared. Approximate percentage inhibition of glycation was assessed based on the decrease in migration of BSA in the presence of plant extract, in comparison to the positive control.

RESULTS

Previously we have demonstrated that the increase in BSA migration towards the anode in presence of fructose (compared to the migration of test blank), is proportionate to the degree of glycation.[9] According to the findings of the present study with different concentrations of plant extracts used [5 mg/ml (Figure 1), 0.5 mg/ml (Figure 2A), 50 μg/ml (Figure 2B left) and 2 mg/ml (Figure 2B right)], most promising antiglycation effects with almost 100% inhibition were observed with *P. debilis* whole plant, *P. emblica* fruit (Figure 2B left panel) and *P. marsupium* latex (Figure 3). *F. racemosa* (stem bark) showed the highest inhibition among the remaining seven plant extracts (Figure 2 A right panel). Glycation inhibition with *P. debilis*, *P. emblica*, *P. marsupium and F. racemosa* extracts at 0.5 mg/ml were greater than that of AG (Figure 2A). *G. sylvestre* (leaf), *M. paradisiaca* (yam) and *T. cordifolia* (leaf) also showed nearly 100% inhibition at 2 mg/ml (Figure 2B Right panel) However, *G. sylvestre* and *M. paradisiaca* did not show any inhibition at 0.5 mg/ml extract while *T. cordifolia* showed only a mild inhibition (Figure 2A). Lower inhibitions were observed with *C. grandis* (leaf) and *S. potatorum* (seed) (Figure 2B Right panel and Table 1). Lowest inhibition found was with *G. lactiferum* (leaf) (Figure 1, 2B Right panel and Table 1). Summary of the results is shown in the table 1.

Table 1: Glycation inhibitory activity (only as an approximate percentage) at different concentrations of the plant extract

Plant part	5 mg/ml	2 mg/ml	0.5 mg/ml	50 µg/ml
Coccinia grandis leaves	>50%	~20%	0	
Ficus racemosa bark	Some interference		~ 50%	~20%
Gymnema lactiferum leaves	<50%	<20%	0	
Gymnema sylvestre leaves	>50%	~80%	0	
Musa paradisiaca yam	>50%	~80%	0	
Phyllanthus debilis plant	Interference		~100%	~100%
Phyllanthus emblica fruit	Interference		~100%	~100%
Pterocarpus marsupium latex	Interference		~100%	~100%
Strychnos potatorum seeds	~50%	~20%	0	
Tinospara cordifolia leaf	Some interference	~80%	~20%	

Approximate percentage inhibitions are stated based on the differences in the degree of the BSA movement towards the anode, compared to that of uninhibited control. Concentrations of plant extracts used were at 5, 2, 0.5 mg/ml and 50 µg/ ml. Interference: BSA migration was affected at 5 mg/ml plant. Empty spaces indicate that the experiments were not conducted at the respective concentrations of the extracts

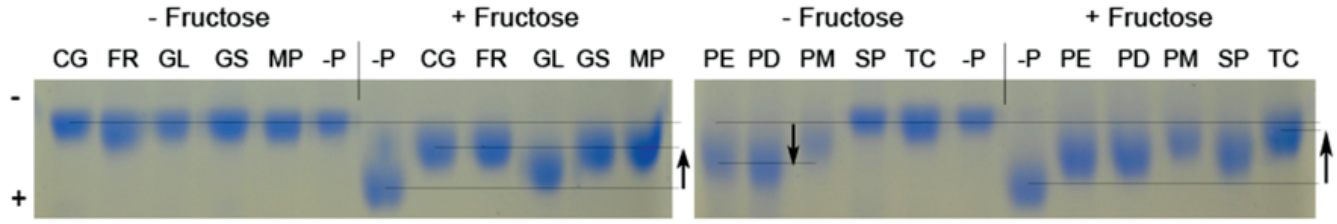

Figure 1: Effect of presence of plant extracts (5 mg/ml) on BSA migration. PAGE was conducted. -P: in absence of plant. Rest of the incubations was conducted in presence of following. AG: Aminoguanidine (10 mM), *CG: C. grandis, FR: F. racemosa, GL: G. lactiferum, GS: G. sylvestre, MP: M. paradisiaca, PE: P. emblica, PD: P. debilis, PM: P. marsupium, SP: S. potatorum and TC: T. cordifolia*. Plant concentration used was 5 mg/ml. Incubations were carried out in presence of fructose (+ Fructose) or in absence of fructose (- Fructose). Incubation period was 27 days. Arrow to the top shows the decrease in BSA migration in presence of plant extracts. Downward arrow shows the increase in BSA migration in presence of higher concentrations of PD, PE and PM extracts

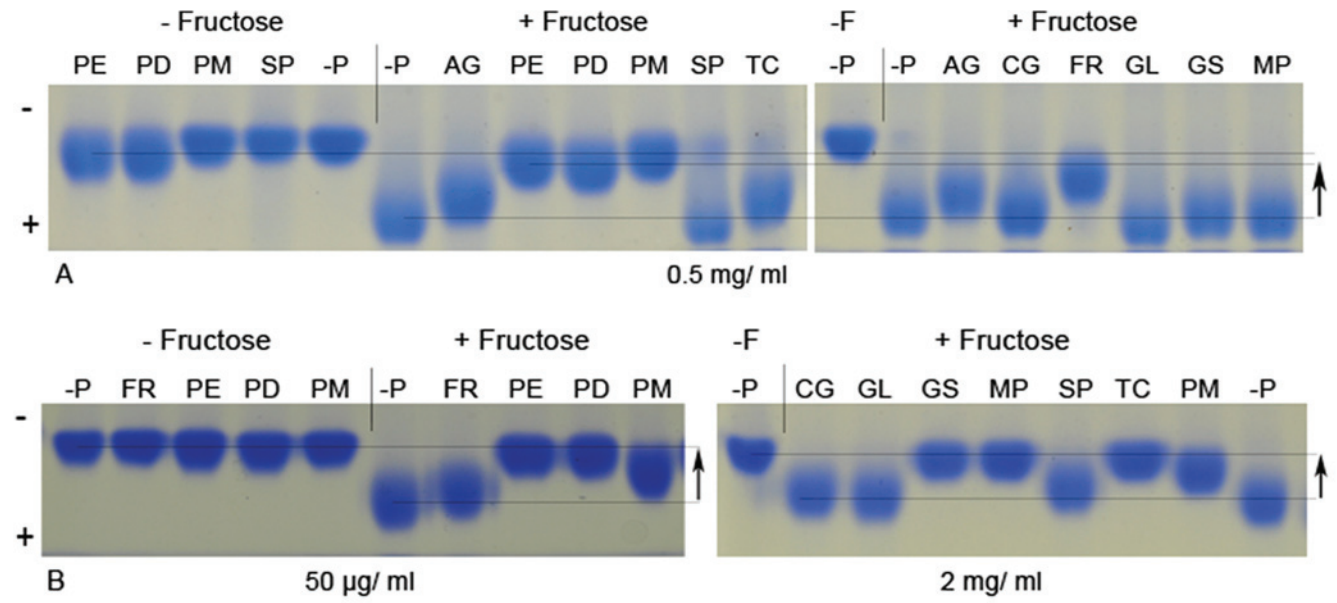

Figure 2: Effect of presence of plant extracts (0.5 mg, 50 µg and 2 mg/ml) on BSA migration. PAGE was conducted. -P: in absence of plant. Rest of the incubations was conducted in presence of following. AG: Aminoguanidine (10 mM), *CG: C. grandis, FR: F. racemosa, GL: G. lactiferum, GS: G. sylvestre, MP: M. paradisiaca, PE: P. emblica, PD: P. debilis, PM: P. marsupium, SP: S. potatorum and TC: T. cordifolia*. Incubations were carried out in presence of fructose (+ Fructose) or in absence of fructose (-F or - Fructose). Arrows show the decrease in BSA migration in presence of plant extracts. 2A: Plant concentration used was 0.5 mg/ml with all ten extracts. Incubation period was 15 days. 2B: Plant concentration used was 50 µg/ml with FR, PD and PE (2B left panel), < 50 µg/ml with PM (2B left and right panels) and others with 2 mg/ml (2B Right panel). Incubation period was 7 days

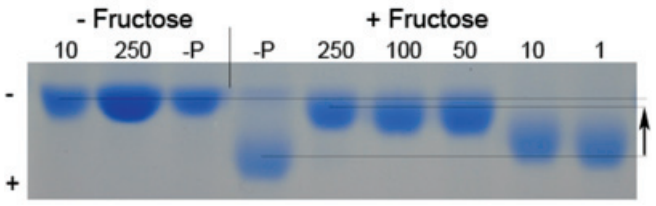

Figure 3: Effect of presence of *P. marsupium* on BSA migration. PAGE was conducted. -P: in absence of plant. Incubations were carried out in presence of fructose (+ Fructose) or in absence of fructose (- Fructose). Concentrations of the extract used were 1, 10, 50, 100 and 250 µg/ml. Incubation period was 14 days. Arrow shows the decrease in BSA migration in presence of PM latex. Inhibition observed was nearly 100% with the extract at 50 µg/ml and above

At higher concentrations (5 mg/ml) *P. debilis*, *P. emblica* and *P. marsupium* showed an increase in the BSA movement even in absence of fructose (Figure 1-downward arrow). This interference was gradually lost, when the concentrations of these extracts were reduced to 0.5 mg (Figure 2A) and 50 µg/ml (Figure 2B left panel).

DISCUSSION

All the plants tested in our study, other than *G. lactiferum* are being used to treat diabetes since ancient times[5] and almost all are proven for their hypoglycaemic potential experimentally too.[5,11,13] The PAGE method used to detect the inhibition of glycation was a novel method developed by us recently which can be conducted without expensive equipment. Previously we have demonstrated that the increase in BSA migration towards the anode in comparison to that of test blank was proportionate to the degree of glycation.[9] This increase was comparatively less in a dose-dependent manner, when the glycation was inhibited.[9] With the present study, we were able to demonstrate the antiglycation potential of the plants tested, identifying an additional beneficial effect which will prevent or delay chronic diabetic complications.

Among the tested, *P. debilis* whole plant, *P. emblica* fruit *and P. marsupium* latex showed a very strong antiglycation potential. They showed almost 100% inhibition even at 50 µg/ml plant. At higher concentrations (5 mg/ml) those three extract showed an increase in the BSA movement independent of fructose, masking their inhibitory effects on glycation. This interference was not there when lower concentrations of extracts (50 µg/ml) were used. Highest inhibitory effects among the remaining seven extracts were seen in *F. racemosa*. These extracts seem to contain phytochemicals which add negative charges to BSA when used at higher concentrations.

There are some studies reporting the antiglycating activity of three plants parts, *C. grandis* leaf, *G. lactiferum* leaf and *P. emblica* fruit, included in our study. A few studies have shown antiglycating effects of different parts obtained from *M. paradisiaca* (inflorescence), *T. cordifolia* (stem) and *P. marsupium* (bark) mostly using animal models. We could not find any previous reports on the antiglycating effects of *F. racemosa, G. sylvestre, P. debilis* and *S. potatorum* plant parts. We found evidence for particularly high antiglycating effects in *P. debilis* and *F. racemosa* in this study.

C. grandis leaves and *P. emblica* fruit were tested previously for their antiglycating activity *in vitro* using the BSA model and quantified the glycation products based on the fluorescent intensity revealing a strong antiglycation potential in *P. emblica* fruit.[7,14] Perera *et al*., revealed that the water extracts of *P. emblica* fruit show significant antiglycation activity with an IC_{50} of around 75 µg/ml.[14] They used BSA and 500 mM glucose incubated under different conditions (at 60°C for 24 h) and quantified relative amounts of glycated BSA, based on the fluorescent intensity using a fluorescent microplate reader. In addition, *P. emblica* was also effective in delaying the progression of diabetic cataract in streptozotocin induced diabetic rats.[15] Our results on *P. emblica* with the novel method support the findings of previous studies carried out *in vitro and in vivo,* showing a strong antiglycating effect. Ethanol extracts of *C. grandis* leaves (0.5 mg) on the inhibition of the middle stage of protein glycation was tested by incubating BSA and methylglyoxal or glucose for 3 days with/without the extract followed by the measurement of fluorescence intensity.[16] Their results indicated a comparatively higher glycation inhibition (96%) in presence of ethanol extracts of *C. grandis* leaf. We observed a good inhibition with methanol extracts of *C. grandis* at 5 mg/ml, but not at 0.5 mg/ml. Our findings with another very sensitive assay conducted on glycation induced protein cross-linking tallied with the results of the present study (unpublished data). *G. lactiferum* leaf was tested for antiglycating effects using type 2 diabetic patients. A significant reduction in the HbA_{1C} levels was observed when a suspension of *G. lactiferum* leaf powder was given twice daily for four weeks.[17] However, in our study, the lowest glycation inhibitory effects were shown by *G. lactiferum*. A comparison cannot be made between the two studies as even a hypoglycaemic effect itself can show an effect on reducing glycation *in vivo*.

M. paradisiaca (inflorescence) has showed effective antiglycation potential as demonstrated by a spectrofluorimeter.[18] In our study methanolic extract of *M. paradisiaca* yam showed a good antiglycation potential. Antiglycating activity of *T. cordifolia* stem-derived alkaloids was evaluated against lens aldose reductase isolated from male Wistar rats.[19] Another study demonstrated the inhibitory activity of *T. cordifolia* stem bark against lens

aldose reductase isolated from normal Wistar rats with an IC_{50} of 103 μg/ml. They also showed preventive effects of *T. cordifolia* stem bark against diabetic neuropathy in female Wistar rats.[20] Our results with the *T. cordifolia* leaf support the glycation inhibitory potential of *T. cordifolia* stem. Aqueous extract of *P. marsupium* bark has shown anti-cataract activity in alloxan diabetic rats.[21]

The antiglycation effects observed under *in vivo* conditions may have resulted partly due to the hypoglycaemic effects of those plant extracts. In our study, sugar concentration was similar in the incubation mixtures in presence and absence of the extract. Hence the antiglycation activities observed are independent of the possible hypoglycaemic effects of these extracts.

A good correlation has been shown to exist between free radical scavenging capacity and antioxidant activity of the plant extract and glycation inhibitory activity.[22] Tannins and certain flavanoids are known to be potential free-radical scavengers. It is evident that a positive correlation exists between the total phenolic content of the plant extract and their glycation inhibitory potential.[22] Some studies have shown free radical scavenging and antioxidant capacity in several plants used in our study. *In vitro* free radical scavenging and antioxidant activities of *C. grandis* leaf extract were observed which was suggested to be attributed to the presence of phenolic and flavanoid compounds in the leaf.[23] Tannin fraction of *Ficus racemosa* possessed a significant antioxidant potential *in vitro*.[24] Ethanol extract of *F. racemosa* bark exhibited concentration dependent hydroxyl radical and superoxide radical scavenging and inhibition of lipid peroxidation with an IC_{50} comparable with tested standard compounds.[25] A significant antioxidant activity was found in alcoholic extract of *G. sylvestre* which was suggested to be partly due to the presence of phenolic compounds.[26] Methanolic extract of *M. paradisiaca* inflorescence has showed effective antioxidant potential.[18] *In vitro* antioxidant activities of of hydromethanolic extracts of *C. Grandis* leaves was shown. Ethanolic extracts of *P. emblica* had a strong antioxidant activity and high total polyphenol and tannin content.[27] Oral administration of *P. emblica* extracts to streptozotocin-induced diabetic rats reduced the level of 5-hydroxymethylfurfural which is an indicator of oxidative stress.[28] Strong antioxidant potential and a correlation between the antioxidant activity and total phenolic content were observed in *P. debilis*.[29] Aqueous extract of *P. marsupium* stem bark showed high *in vitro* antioxidant activity in all assays used and also protected rat liver mitochondria against oxidative damage.[30] *P. marsupium* latex resin is known to contain about 75% of tannic acid which may have contributed to the strong antiglycation activity we have observed. Ethanolic extract of *S. potatorum* seeds showed normalization of *in vivo* antioxidant activity with a significant reduction of blood glucose level in streptozotocin-induced diabetic rats.[31] The alterations of blood glucose level and antioxidant status in alloxan induced diabetes rats have been restored to normal levels by administration of *T. cordifolia* stem methanol extract, indicating the protective role of *T. cordifolia*.[32] Even though previous studies demonstrated free radical scavenging and antioxidant capacities of some plant parts used, assessment of those activities in the methanol extracts of all ten plant parts used in our study is necessary to find out the correlation of those activities with the antiglycation activity observed.

CONCLUSIONS

We were able to demonstrate the antiglycation potential of some medicinal plants with known hypoglycemic effects, using a novel method developed by us recently. *P. debilis* whole plant, *P. emblica* fruit *and P. marsupium* latex showed a strong glycation inhibitory potential using a novel method established by us. *F. racemosa* stem bark showed the highest inhibition among the remaining seven plant extracts.

ACKNOWLEDGEMENTS

Financial assistance from National Science Foundation grant RG/2012/BS/01, Prof. R. Sivakanesan for revising the manuscript, Mr. A.M.P.S.T.M. Bandara and Ms. W. K. V. K. Premadasa for assistance with electrophoresis, Mr. G. Gunasekera for photography and Deputy Director, National Herbarium, Peradeniya for authenticating the plants.

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Authors Contribution:
HKIP – Concept and design of the study, obtaining grants, data collection, literature search, analysis and interpresrctation, manuscript preparation and critical revision of the manuscript. **CSH –** Plant collection, conducting experiments and data collection.

Source of Support: National Science Foundation grant RG/2012/BS/01, **Conflict of Interest:** None declared.