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In Vitro Glucose Entrapment and Alpha-Glucosidase Inhibition of Mucilaginous Substances from Selected Thai Medicinal Plants

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Abstract

As a continuous searching for anti-diabetic(type II) substances, seven mucilage polysaccharides from selected plants were studied as follow: aerial parts of *Basella alba* Linn., fruits of *Hibiscus esculentus* Linn., leaves of *Litsea glutinosa* (Lour.) C.B. Robinson, seeds of *Ocimum canum* Sims., seeds of *Plantago ovata* Forssk., fruits of *Scaphium scaphigerum* G. Don. and seeds of *Trigonella foenum-graecum* Linn. The bioactive properties for entrapping glucose, inhibiting enzyme alpha-glucosidase and free radical scavenger were *in vitro* studied compared to glucomannan. The physical characteristics for water holding capacity and viscosity were determined. The chemical characteristics were assayed for monosaccharide composition using methanolysis, TMS-derivatization and gas chromatography. *O. canum* mucilage superiorly entrapped glucose compared to glucomannan. This activity was relevant to its highly viscous gelation. *S. scaphigerum* showed another property of alpha-glucosidase inhibition. *S. scaphigerum* mucilage (0.5%) inhibited the enzyme activity by 82.6%, compared to 1-Deoxynorjirimycin (by 47.6%). Most mucilages, except *O. canum* and *P. ovata*, showed DPPH scavenging activity higher than glucomannan. Galacturonic acid was found in 3 from 7 mucilages namely *B. alba*, *P. ovata* and *S. scaphigerum*. Whereas rhamnose was common sugar found in all seven mucilages. Monosaccharide components of these mucilages were compared to the results from the previous reports.

Keywords

Mucilage • Polysaccharide • Anti-diabetic potential • Free radical scavenger • Physicochemical property

Introduction

Type 2 diabetes comprises 90% of people with diabetes around the world and is one of the major public health challenges of the 21st century. The number of cases worldwide in 2000 is estimated to be about 171 million and is projected to rise to 366 million in 2030. The World Health Organization (WHO) projects that without urgent action, diabetes-related deaths will increase by more than 50% in the next 10 years. Especially in upper-middle income countries, diabetes deaths are projected to increase by over 80% between 2006 and 2015 [1]. This circumstance results that the demand for medical care in type 2 diabetes will continue to increase. The substantial care and cost are due to the management of complications of the disease at both the starting point and the degree of deterioration over time. Macrovascular complications (ischemic heart disease, peripheral vascular disease, and cerebrovascular disease) has been estimated to be the largest cost component followed by microvascular complications (nephropathy, neuropathy and retinopathy) [2]. Endothelial dysfunction is considered to be an integral component of vascular diseases. Impaired endothelial function induces vasoconstriction, inflammatory and proliferative changes in the arterial wall and promotes atherosclerotic lesion growth. Prevention or normalization of endothelial function, contributes to the prevention of vascular lesion progression or destabilization [3]. Hyperglycemia has been proposed to be a crucial factor inducing endothelial dysfunction. High concentration of blood glucose as well as high glucose fluctuation during postprandial period correlates with the increase in reactive oxygen species or oxidative stress. Reactive oxygen species mediates the activation of the imbalance in vasoregulating factors (vasodilators and vasoconstrictors) then affects endothelial homeostasis and triggers atherogenic changes, including increases in low-density lipoprotein oxidation, sympathetic tone, vasoconstriction, and thrombogenicity [4]. Accordingly, glucose control is an important goal to diminish the risk of long term health complications of type 2 diabetes. In addition to glycated haemoglobin and fasting plasma glucose, postprandial glucose is recently recommended as essential target for diabetes management [5]. Alpha-glucosidase inhibition is one of the powerful interventions. Alpha-glucosidase is intestinal enzyme which catalyzes the degradation of diet polysaccharides to absorbable monosaccharide. Natural or synthetic glucosidase inhibitors are of therapeutic interest to delay postprandial hyperglycemia in type 2 diabetes. Amongst these, saccharide derivatives, for example Acarbose and Miglitol, have been approvable for anti-diabetic drugs [6]. Non-starch polysaccharides designated as soluble dietary fibers are also useful functional foods according to their association with the reduced risks of diabetes and cardiovascular diseases [7]. The viscous characteristics due to excellent water-holding and gel-forming capabilities have been proposed as an important mechanistic factor to delay gastric emptying and delay absorption of glucose in gastrointestinal tract [8]. Plant mucilage has been credited as one of plant chemical constituents showing hypoglycemic activity [9, 10]. This study investigated *in vitro* properties for anti type 2 diabetic potential among selected mucilaginous plants compared to a well known soluble dietary fiber, glucomannan. Some physico-chemical characteristics of these mucilages were also characterized.

Results and Discussion

Mucilage extraction

Seven mucilaginous plants were studied as follow: aerial parts of *Basella alba* Linn. (Basellaceae), fruits of *Hibiscus esculentus* Linn. (Malvaceae), leaves of *Litsea glutinosa* (Lour.) C.B. Robinson (Lauraceae), seeds of *Ocimum canum* Sims. (Labiatae), seeds of *Plantago ovata* Forssk. (Plantaginaceae), fruits of *Scaphium scaphigerum* G. Don. (Sterculiaceae) and seeds of *Trigonella foenum-graecum* Linn. (Papilionaceae). The mucilages from selected plants yielded range from 3.5% to 23.0% as shown in table 1.

Tab. 1. Mucilage polysaccharides from selected plants

Plants	Used parts	% Yield
Basella alba Linn.	aerial parts	3.5
Hibiscus esculentus L.	fruits	5.6
Litsea glutinosa Lour.	leaves	12.0
Ocimum canum Sims.	seeds	17.6
Plantago ovata Forssk.	seeds	19.0
Scaphium scaphigerum G. Don.	fruits	23.0
Trigonella foenum-graecum L.	seeds	15.0

In vitro property of entrapping glucose

Dialysis tubing technique is a simple model to evaluate the potential of soluble dietary fibers to additionally retard the diffusion and movement of glucose in the intestinal tract [11]. Movement in this system is not by the true diffusion but is assisted by the convective activity of intestinal contractions [12]. The entrapment ability of mucilage gel resulted in decreasing of glucose diffusion into the external solution. The retardation of the nutrient flow into the external medium is an indication of the modulating effect of that fiber on glucose absorption in the jejunum [11]. From the studied model, all mucilages showed concentration response (0.5, 1.0 and 2.0%w/v) on glucose entrapment activity. The percentage of glucose releasing from 2% mucilage suspension were 61.6, 70.8, 71.7, 80.6, 83.4, 85.8 and 92.8 % for *O. canum, P. ovata, T. foenum-graecum, L. glutinosa, H. esculentus, B. alba* and *S. scaphigerum* respectively. Glucomannan showed 65.4% of glucose releasing at the same concentration (Figure 1). Glucomannan seems to be the most impressive natural fiber with increasing importance in the biomedical and pharmaceutical fields. It has been found to decrease the serum glucose levels and also the postprandial insulin flow which aiding diabetic control [13]. Psyllium seeds from *P. ovata* and fenugreek seeds from *T. foenum-graecum* are also reported as an adjunct to dietary therapy in patients with type II diabetes, to reduce glucose and glycosylated hemoglobin [14, 15]. Figure 1 showed that the retardation effect on glucose movement of the mucilages from *P. ovata* and *T. foenum-graecum* were lower degree than glucomannan. Whilst *O. canum* mucilage of all concentration superiorly entrapped glucose compared to glucomannan. There have been previous studies of hypoglycemic effect of *O. canum* but from leaves extract [16].

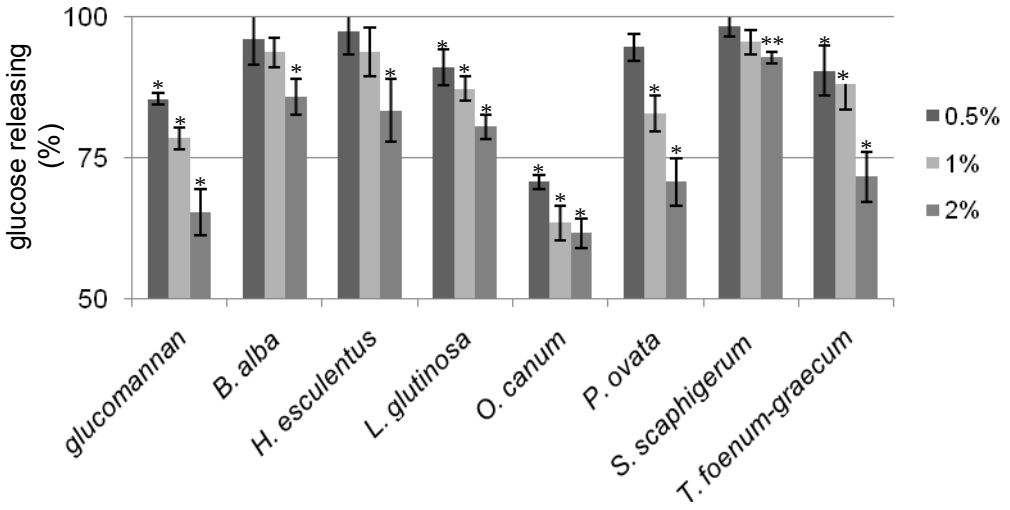

Fig. 1. Percentages of glucose releasing from 0.5, 1, 2% mucilage containing 2% glucose after 2 hr dialysis (n=4). The significance of differences from the control (0% mucilage, 99.8±0.9%) was determined by ANOVA followed by Dunnett's test (*p<0.01, **p<0.05).

In vitro property of alpha-glucosidase inhibition

The effect of selected mucilages against α-glucosidase was evaluated. At the concentration of 0.5% mucilage, *S. scaphigerum*, *L. glutinosa*, *H. esculentus*, *O. canum*, *T. foenum-graecum*, *P. ovata*, *B. alba* and glucomannan showed the inhibitory percentage of 82.6, 41.0, 37.6, 32.8, 30.6, 27.0, 25.0 and 19.7 % respectively. Whereas, 1-Deoxy-norjirimycin at the same concentration showed the inhibition of 47.6% (Figure 2). *S. scaphigerum* mucilage was further investigated and found that the concentration for 50% inhibition of α-glucosidase activity (IC_{50}) was 0.17% (Figure 3). *S. scaphigerum* or Malva nut tree is mostly found in the East of Thailand. The gel made from malva nuts is used as ingredients in dishes and beverages. Malva nut drink is traditionally used to relief coughing and sore throats. The previous study in type 2 diabetic patients reported that after the intake of malva nut drink, fasting plasma glucose and glycosylated hemoglobin decreased significantly [17]. The present study showed a possible mechanism in alpha-glucosidase inhibition**.**

DPPH radical-scavenging activity

Tab. 2. DPPH scavenging activity as IC_{50} among mucilage polysaccharides from selected plants

Mucilages	IC_{50} (mg/ml)	Mucilages	IC_{50} (mg/ml)
B. alba	2.00	*S. scaphigerum*	0.61
H. esculentus	0.70	*T. foenum-graecum*	1.52
L. glutinosa	0.49		
O. canum	>10	Glucomannan	4.15
P. ovata	>10	Ascorbic acid	0.02

[a] mean of 3 runs

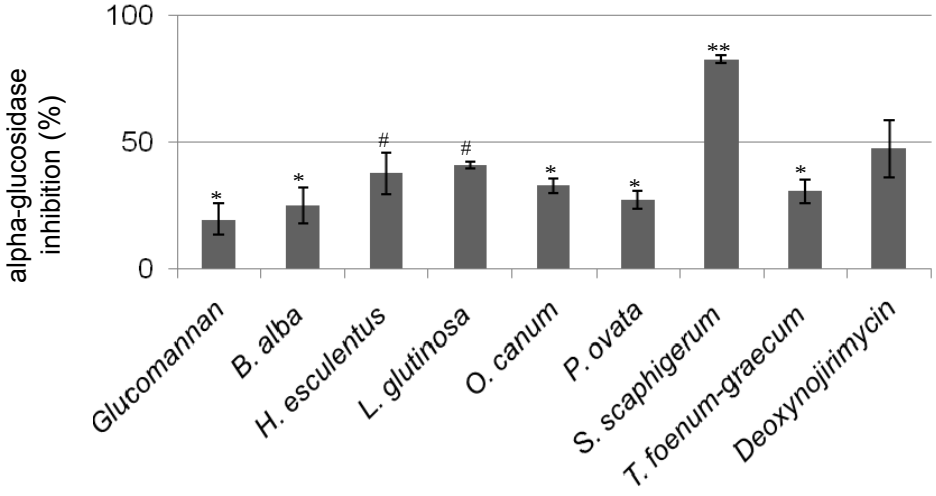

Fig. 2. Percentage of alpha-glucosidase inhibition by 0.5% mucilage (n = 3). # %inhibition was not significantly different from control (Deoxynorjirimycin) (p>0.05); * %inhibition was significantly higher from control (p<0.05); * %inhibition was significantly lower than control (p<0.05) (determined by ANOVA followed by Dunnett's test).

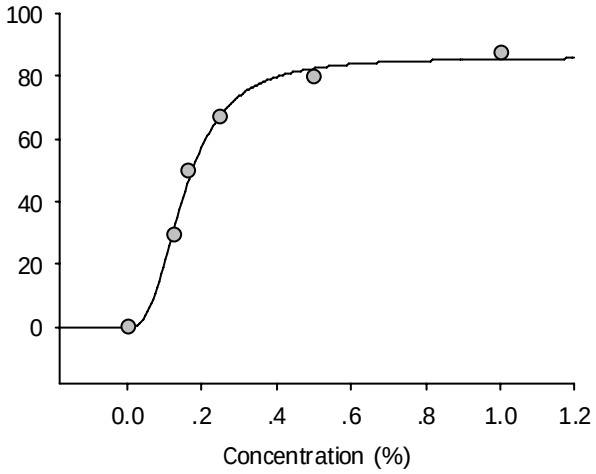

Fig. 3. Percentage of alpha-glucosidase inhibition from various concentration of *S. scaphigerum* mucilage

Endothelial dysfunction is a key factor in all stages of atherosclerosis development. This term refers to an imbalance in the production of vasodilators at the circumstance of high concentrations of reactive oxygen species or oxidative stress [4]. Scavenging of the stable free radical diphenylpicrylhydrazyl (DPPH) is the basis of a common antioxidant assay. There have been widely different protocols which differed in the conditions (i.e. pH,

solvents, wavelength of measurement), yielding different results. In the present study, the polysaccharide samples were incubated with 100 µM DPPH in methanol for 30 min using ascorbic acid as standard antioxidant. The number of DPPH reduced by one molecule of ascorbic acid (antioxidant stoichiometry) was 2.5 which was higher than the theoretic value of 2.0 but close to the previous experiments [18, 19]. Table 2 demonstrated the scavenging activity of the polysaccharide samples on the DPPH radical. Except for *O. canum* and *P. ovat*a, other mucilages showed scavenging activities higher than glucomannan. Especially the mucilages of *L. glutinosa, S. scaphigerum* and *H. esculentus* had IC_{50} less than 1 mg/ml. Although the abilities were lower than that of ascorbic acid, these mucilages counteracted with DPPH as strongly as *Ganoderma* polysaccharides (IC_{50} between 3–13 mg/ml) [20].

Swelling measurements and viscosity

There are a series of physical interactions in the gastrointestinal tract which affect absorption, as follow: diffusion of nutrients from the bulk solution to the intestinal epithelia, the rate of removal of waters of hydration from a complex nutrient solvent system, counter diffusion of nutrients away from the intestinal surface and diffusion of nutrients along the epithelial surface to an appropriate absorptive site [21]. Water can be held within the polysaccharide matrix causing considerable swelling and viscous solution or gelation. Viscous polysaccharide gels may slow absorption by trapping nutrients, digestive enzymes or bile acids in the matrix and by slowing mixing and diffusion in the intestine [22]. These physical properties of selected mucilages were studied (Table 3). *O. canum* and *S. scaphigerum* mucilages showed highest values of swelling volume (SV) and water absorption index (WAI) followed by *P. ovata* and *T. foenum-graecum* respectively. Other mucilages showed the same values of SV and WAI as glucomannan. Viscosity was determined using falling ball viscosity method. *O. canum* mucilage had highest viscosity value even at low concentration (0.5%). Viscous character seemed to be a prominent factor affected the hypoglycemic potential of *O. canum* but not *S. scaphigerum* mucilage.

Tab. 3. Swelling volume (SV), water absorption index (WAI) and viscosity among mucilage polysaccharides from selected plants

Mucilages	SV (ml/g)	WAI (g/g)	Viscosity (mPa s)		
			0.5%	1.0%	2.0%
glucomannan	23.9 ± 1.9	22.1 ± 1.9	21.1 ± 0.3	143.2 ± 8.9	4582.8 ± 60.3
B. alba	25.7 ± 6.3	15.8 ± 3.0	2.2 ± 0.3	3.5 ± 0.4	7.0 ± 0.4
H. esculentus	22.2 ± 4.6	20.7 ± 4.3	7.7 ± 0.7	17.1 ± 0.9	45.1 ± 2.0
L. glutinosa	27.5 ± 8.5	20.6 ± 4.6	1.8 ± 0.2	5.9 ± 0.6	19.1 ± 2.6
O. canum	115.9 ± 17.3*	111.1 ± 17.1*	581.3 ± 59.1	>5000	>5000
P. ovata	60.4 ± 7.7*	48.3 ± 3.2*	6.2 ± 0.3	18.5 ± 1.1	1575.3 ± 57.5
S. scaphigerum	210.5 ± 4.3*	102.8 ± 1.6*	0	0	0
T. foenum-graecum	38.9 ± 1.0	19.1 ± 1.4	6.7 ± 0.4	29.6 ± 1.9	213.0 ± 17.9

[a] mean of 3 runs; * the significance of differences of SV and WAI from the control (glucomannan) was determined by ANOVA followed by Dunnett's test (p<0.01).

Monosaccharide analysis and protein content

The selected mucilages as well as glucomannan were analyzed for the primary structures of their monosaccharide compositions as shown in Table 4 and 5. Konjac glucomannan in this study showed the mannose : glucose molar ratio of around 1.6 : 1 which was in the range reported elsewhere. Galacturonic acid was found in 3 from 7 mucilages namely *B. alba*, *P. ovata* and *S. scaphigerum*. Whereas rhamnose was common sugar found in all studied mucilages. Chemical analyses of the mucilages in this study showed some differing results from the previous reports. This was due to the differences in either plant origin or methodology of extraction and analysis. Literature reviews of monosaccharide composition among these mucilages were summarized in Table 6. *H. esculentus* mucilage from this study had highest glucose (79%) composition compared to 44% and 5% from the literatures. *L. glutinosa* mucilage from the leaves in this study had xylose/ arabinose ratio differed from the reported barks. *P. ovata* and *T. foenum-graecum* mucilages displayed the typical characters of arabinoxylan and galactomannan respectively. The water extracts of *S. scaphigerum* mucilages in this report had similar ratio of monosaccharide with the alkaline extracts reported elsewhere. *B. alba* mucilage was firstly revealed the composition of arabinose, rhamnose, galactose, galacturonic acid and glucose (24:5:41:13:16). Total protein contents in these crude polysaccharides ranged from 2% in *P. ovata* to 38% in *L. glutinosa* (Table 4).

Tab. 4. Monosaccharide composition and total protein content (μg/mg) among mucilage polysaccharides from selected plants

	Ara[b]	Rham	Xy	Man
glucomannan				493.8 ± 11.6
B. alba	43.5 ± 0.8	10.7 ± 0.1		
H. esculentus		27.8 ± 0.6		
L. glutinosa	84.1 ± 1.2	6.0 ± 0.2	56.4 ± 0.5	9.7 ± 0.1
O. canum	47.8 ± 1.1	28.0 ± 0.7	98.43 ± 2.5	37.5 ± 1.5
P. ovata	165.2 ± 13.5	52.0 ± 3.4	697.5 ± 1.3	
S. scaphigerum	121.7 ± 3.4	155.2 ± 4.8	20.8 ± 0.7	
T. foenum-graecum		18.8 ± 0.4	20.4 ± 0.2	310.4 ± 1.2
	Gal	**GalN**	**Glu**	**TP[c]**
glucomannan			309.6 ± 3.6	107.8 ± 0.04
B. alba	88.7 ± 1.0	33.7 ± 1.3	34.6 ± 0.8	235.5 ± 0.04
H. esculentus	46.5 ± 1.4		285.7 ± 1.4	152.7 ± 0.04
L. glutinosa	29.7 ± 0.6		88.9 ± 0.5	375.1 ± 0.01
O. canum	156.2 ± 0.8		66.30 ± 0.2	81.8 ± 0.02
P. ovata		51.7 ± 3.0		20.3 ± 0.02
S. scaphigerum	144.6 ± 0.3	173.4 ± 2.9	21.1 ± 0.2	195.3 ± 0.03
T. foenum-graecum	302.2 ± 1.2		82.2 ± 0.5	209.5 ± 0.01

[a] mean of 3 runs; [b] monosaccharides found in these mucilages included arabinose (ara), rhamnose (rham), xylose (xy), mannose (man), galactose (gal), galacturonic acid (galn) and glucose (glu). Fucose (fu) and glucuronic acid (glun) were absent; [c] Total protein.

Tab. 5. Monosaccharide composition (% mole ratio) among mucilage polysaccharides from selected plants

	Ara	Rham	Xy	Man	Gal	Galn	Glu
glucomannan				61			39
B. alba	24	5			41	13	16
H. esculentus		8			13		79
L. glutinosa	33	2	22	3	10		29
O. canum	12	6	25	8	34		14
P. ovata	18	5	74			4	
S. scaphigerum	23	24	4		23	23	3
T. foenum-graecum		3	3	42	41		11

Tab. 6. Monosaccharide composition among mucilage polysaccharides from literatures

glucomannan	man : gluc 1.6 : 1	[23]
H. esculentus	rham : gal : galn: glu : glun 1 : 2.5 : 1.8 : 0.3 : 0.2	[24]
	ara : rham : xyl : man : gal : galn : glu : glun 5 : 3 : 5 : 3 : 17 : 16 : 44 : 7	[25]
L. glutinosa	ara : xy 3.4 : 1.0 (barks)	[26]
O. canum	ara : rham : xy : man : gal : glu 1 : 2 : 1 : 2 : 5 : 8 (uronic acids 8.15%)	[27]
P. ovata	ara : rham : xy : man : gal : glu 20.7 : 1.1 : 50.3 : 1.1 : 4.8 : 2.0	[28]
S. scaphigerum	ara :rham : gal 1.1 : 1.0 : 1.0 (%w/w)	[29]
T. foenum-graecum	ara: rham : man : gal : glu 0.5 : 0.2 : 31.4 : 26.2 : 0.6	[30]
	gal : man 1.00 : 1.02 to 1.00 : 1.14	[31]

Conclusion

The plants bearing mucilage in this study have been well known in Thailand as both edible and medicinal plants. Mucilagenous typed polysaccharides from these plants were investigated for the biological activities especially antidiabetic potential. Despite the limitations of this *in vitro* study, there seemed to be various mechanisms possibly involved by mucilages due to their physico-chemical characteristics. The *in vitro* models could be beneficial tools for the survey of other potential plant mucilages. Moreover they could refine the possible and capable research designs prior to the expensive further studies of either the animal models or the clinical trials.

Experimental

Seven mucilaginous plants were studied as follow: aerial parts of *Basella alba* Linn. (Basellaceae), fruits of *Hibiscus esculentus* Linn. (Malvaceae), leaves of *Litsea glutinosa* (Lour.) C.B. Robinson (Lauraceae), seeds of *Ocimum canum* Sims. (Labiatae), seeds of *Plantago ovata* Forssk. (Plantaginaceae), fruits of *Scaphium scaphigerum* G. Don. (Sterculiaceae) and seeds of *Trigonella foenum-graecum* Linn. (Papilionaceae). Glucomannan flour (the Siam Konjac Co., Ltd.) was used for comparison. Chemicals and

reagents included *p*-nitrophenyl-α-D-glucopyranoside and α-glucosidase (EC 3.2.1.20) from *Saccharomyces cerevisiae*, 1-Deoxynojirimycin, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), standard monosaccharides (Sigma Chemical Co. Ltd, St. Louis, MO.); methanolic HCl, trimethylchlorosilane, hexamethyldisilazane (Supelco, Bellefont, PA); Glucose Liquicolor kit (Human Gesellschaft für Biochemica und Diagnostica mbH, Germany); Lactated Ringers Buffer pH 7 (General Hospital Product Public Co., LTD., Thailand), All other chemicals were analytical grade. Dialysis tubing cellulose membrane (molecular weight cut off = 12,000 Da) was from Sigma Chemical Co. Ltd, St. Louis, MO. Dialysis tubing cellulose membrane (molecular weight cut off = 3,500 Da) was from Spectrum Medical Industries, Inc., Los Angeles, CA.

Mucilage extraction

The mucilage were extracted from the specified plant parts with warm water and concentrated by lyophilization. The lyophilized samples were re-dissolved in water, precipitated twice with 2 volume of 80% ethanol and dialyzed against distilled water in a dialysis tubing cellulose membrane (molecular weight cut off = 3,500 Da). The samples were lyophilized, ground and kept in refrigerator for further studies.

In vitro property of entrapping glucose

The mucilage and glucomannan were dissolved in Ringers buffer. Glucose was added to make the final concentration of 2% glucose and 0, 0.5, 1 and 2 % w/v polysaccharide gel. Four milliliter of each concentration was dialysed against 60 ml of Ringers buffer in a dialysis tubing cellulose membrane (molecular weight cut off = 12,000 Da) for 2 hours under rotationally shaking at 150 rpm. The released glucose was determined by glucose oxidase - phenyl ampyrone (GOD-PAP) colorimetric method using Glucose Liquicolor kit according to manufacturer's instructions.

In vitro property of alpha-glucosidase inhibition

Alpha-glucosidase activity was assayed using 0.1M sodium phosphate buffer at pH 6.9, and 1 mM p-nitrophenyl-α-D-glucopyranoside was used as a substrate [32]. The concentration of α-glucosidase was 1 U/mL in each experiment. The enzyme (4 µl) was incubated in the absence or presence of various concentrations of tested polysaccharides at 37 °C. The preincubation time was specified at 10 min and the substrate (95 µL) was added to the mixture. The reaction was carried out at 37 °C for 20 min, and then 100 µL of 1M Na_2CO_3 was added to terminate the reaction. Enzymatic activity was quantified by measuring the absorbance of *p*-nitrophenol at 405 nm. One unit of α-glucosidase is defined as the amount of enzyme liberating 1.0 µmol of *p*-nitrophenol per minute under the conditions specified. 1-Deoxynojirimycin was used as the positive control.

DPPH radical-scavenging activity

The potential antioxidant activity of polysaccharide samples was determined on the basis of the scavenging activity of the stable DPPH free radical [33]. Various concentrations of polysaccharides samples (0.5 ml) were added to 1.5 mL of a 0.004% methanolic solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percent scavenging activity was calculated by the following formula:

$$\textbf{Scavenging effect}\ (\%) = (1 - A_{sample}/A_{control}) \times 100$$

Where $A_{control}$ is the absorbance of control (DPPH solution plus water), A_{sample} is the test sample (DPPH solution plus test sample or positive control) and the percent inhibition activity was calculated. IC_{50} values denote the concentration of sample required to scavenge 50% DPPH free radicals.

Swelling measurements and viscosity

Swelling volume (SV) and water absorption index (WAI) were determined from the ratio of the volume and weight of swollen gel to the dry weight of sample respectively [34,35]. A 0.050 g ground sample (<60 mesh) was suspended in 25 ml of water in a 25-ml graduated cylinder for 2 hours. The volume of swollen gel was measured. The supernatant liquid was removed, the swollen gel was weighed and SV and WAI were calculated. Viscosities at various concentrations were measured with a falling ball viscometer (HAAKE Mess-Technik GmbH u. Co, Germany) at 20 °C.

Monosaccharide analysis and protein content

The polysaccharide samples (1 mg) were subjected to methanolysis with 4 M methanolic HCl at 80°C for 24 h. Mannitol was added as an internal standard. The samples were dried with nitrogen, methanol was added and the samples were dried again. This washing was repeated twice [36]. Prior to gas chromatographic analysis, the samples were trimethylsilylated using trimethylchlorosilane : hexamethyldisilazane : Pyridine 1:2:5 (0.4 ml) at room temperature for 30 min. Instrumentation was performed on a Finnigan Trace GC Ultra with DSQ MS detector and a split–splitless injector. The column was a ZB-5 fused silica capillary column (30 m × 0.25 mm i.d.) with film thickness 0.25 μm. Helium was used as carrier gas at a flow rate of 1.0 ml/min. The injector and detector temperature were 260 and 300°C respectively. The column temperature was initially 140°C, then an increase of 1°C/min to 170°C and followed by 6°C/min to 250°C. The protein contents of the samples were determined by Lowry method using bovine serum albumin as protein standard.

Statistical analysis

The significance of differences between the mean values was determined by analysis of variance (ANOVA), followed by Dunnett's test, and a p value of less than 0.05 was considered statistically significant.

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Authors' Statement

Competing Interests

The authors declare no conflict of interest.

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