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Hypoglycemic activity of polysaccharide fractions containing ß-glucans from extracts of *Rhynchelytrum repens* (Willd.) C.E. Hubb., Poaceae

A.C.C.F.F. De Paula[1], R.V. Sousa[2], R.C.L. Figueiredo-Ribeiro[1] and M.S. Buckeridge[1]

[1]Seção Fisiologia e Bioquímica de Plantas, Instituto de Botânica, São Paulo, SP, Brasil
[2]Setor de Fisiologia e Farmacologia, Departamento de Medicina Veterinária, Universidade Federal de Lavras, Lavras, MG, Brasil

Correspondence
M.S. Buckeridge
Seção Fisiologia e Bioquímica de Plantas, Instituto de Botânica
Caixa Postal 4005
01061-970 São Paulo, SP
Brasil
Fax: +55-11-5073-3678
E-mail: msbuck@usp.br

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Abstract

ß-Glucans are soluble fibers with physiological functions, such as interference with absorption of sugars and reduction of serum lipid levels. The objective of the present study was to analyze the distribution of ß-glucans in different tissues of the African grass species *Rhynchelytrum repens* and also to evaluate their hypoglycemic activity. Leaf blades, sheaths, stems, and young leaves of *R. repens* were submitted to extraction with 4 M KOH. Analysis of the fractions revealed the presence of arabinose, glucose, xylose, and traces of rhamnose and galactose. The presence of ß-glucan in these fractions was confirmed by hydrolyzing the polymers with endo-ß-glucanase from *Bacillus subtilis*, followed by HPLC analysis of the characteristic oligosaccharides produced. The 4 M KOH fractions from different tissues were subjected to gel permeation chromatography on Sepharose 4B, with separation of polysaccharides with different degrees of polymerization, the highest molecular mass (above 2000 kDa) being found in young leaves. The molecular mass of the leaf blade polymers was similar (250 kDa) to that of maize coleoptile ß-glucan used for comparison. The 4 M KOH fraction injected into rats with streptozotocin-induced diabetes showed hypoglycemic activity, reducing blood sugar to normal levels for approximately 24 h. This performance was better than that obtained with pure ß-glucan from barley, which decreased blood sugar levels for about 4 h. These results suggest that the activity of ß-glucans from *R. repens* is responsible for the use of this plant extract as a hypoglycemic drug in folk medicine.

Key words
- ß-Glucans
- Cell wall
- Hypoglycemic activity
- Diabetes mellitus
- Grasses
- *Rhynchelytrum repens*

Introduction

Carbohydrates are extensively used as food for humans and animals. They are very important as sources of raw materials for alcoholic beverages, as food additives and also in the pharmaceutical industry. Plant carbohydrates present in food consumed by humans and animals contain soluble or insoluble fiber-like polymers (1).

There are two types of dietary fibers: insoluble and soluble, that can be distinguished by their solubility in aqueous solutions. Whereas the insoluble fibers (cellulose, lignin and some hemicelluloses) are insoluble in water, the soluble ones (hemicelluloses and pectins) form viscous solutions in water. Soluble fibers can form an unstirred water layer in the gut, which decreases absorption of sugars and lipids. Thus, to some extent, soluble fibers can be used to prevent the postprandial increase of glucose, being therefore useful for the treatment of diabetes at certain levels (2).

ß-Glucans are plant hemicellulose polysaccharides (soluble fibers) that are recognized as hypocholesterolemic compounds (3,4). It has been demonstrated in humans that hyperlipidemic individuals may have a decrease of up to 7.5% in serum cholesterol (5).

ß-Glucans, as well as several other viscous plant polysaccharides (e.g., guar, locust bean and pectin), display physiological effects that are typically attributed to a decrease in postprandial glucose levels in serum. This effect has been related to the property of this polymer to form an unstirred water layer which, by resisting the convective effects of intestinal contractions, decreases sugar absorption by the small intestine (6). This effect has also been associated with foods such as oats, barley, beans, and gums which are known to accumulate relatively large amounts of ß-glucan (7-10).

The mixed-linked (1→3),(1→4)-ß-D-glucan (ß-glucan) is a cell wall polysaccharide found only in grasses and cereals (11). ß-Glucan is not synthesized in dividing cells but accumulates specifically during cell enlargement (12). The ß-glucan structure was established by using a sequence-dependent *Bacillus subtilis* endoglucanase (lichenase) which cleaves (1→4)-ß-D-glucosyl units only if preceded by (1→3) units and yields primarily a diagnostic trisaccharide, cellobiosyl-(1→3)-D-glucose, and a tetrasaccharide, cellotriosyl-(1→3)-ß-D-glucose (13). It is an unbranched glucan and over 90% of the polymer consists of these cellotriosyl (G_4 G_3 G) and cellotetraosyl (G_4 G_4 G_3 G) units at ratios ranging from 2 to 3 in grasses, each connected by single (1→3)-ß-D-linkages (14,15).

Many tropical grass species, especially those from Africa, such as *Rhynchelytrum repens*, have been considered invaders of American natural reserves (16) because of their capacity to replace native species (17).

R. repens is a grass widespread in tropical areas and has been consistently observed as an invasive species in pastures, mainly in open areas and at road sides in the State of São Paulo. The plant is used popularly as a phytotherapeutic remedy for the treatment of diabetes in Brazil and in a recent report the precipitate of the aqueous extract of *R. repens* has been shown to significantly reduce plasma glucose levels when administered to diabetic rats (18). Since high molecular weight polymers are expected to predominate in alcohol precipitate fractions, polymers such as ß-glucan might be thought to be probable candidates to display biological activity. Thus, the objective of the present study was to detect and analyze ß-glucan in different plant parts of the grass species *R. repens*. The study revealed a hypoglycemic effect of the hemicellulose fractions which are richer in ß-glucan.

Material and Methods

Plants of *R. repens* (Willd.) C.E. Hubb. (Poaceae) were cultivated in a greenhouse at an average temperature of 26ºC in October 1998. After 20 days, the shoots of the plants were collected and subjected to extraction and fractionation of the cell wall carbohydrates.

Extraction

Shoots of *R. repens* were divided into expanded leaf blades, sheath, stem, and young leaves. The same procedure was simultaneously performed with coleoptiles of maize

to be used as a standard (19). Maize (*Zea mays* L.) caryopsis were soaked in tap water for 15 h, sown on trays of moist vermiculite, and incubated in the dark at 30ºC for 2 days. The upper two thirds of the coleoptiles were collected and used for cell wall extraction and fractionation.

Different parts of *R. repens* plants and maize coleoptiles were sequentially extracted with 20 ml 0.5% ammonium oxalate, pH 7.0, 0.1 M KOH, 1 M KOH, and 4 M KOH at ambient temperature under an N_2 atmosphere with continuous stirring. All extractions were performed for 1 h, except for the 4 M KOH fractionation, which lasted 15 h. After each extraction, the unextracted cell wall components were pelleted by centrifugation, and the supernatant was filtered through nylon meshes. Alkali-soluble fractions were chilled to ice temperature and acidified with glacial acetic acid to pH 5.0. All fractions were dialyzed extensively against deionized water and freeze-dried. Each fraction was submitted to colorimetric assays for uronic acid (20) and total sugars (21).

Digestion with endo-ß-glucanase

Cell walls from excised coleoptiles and from different plant parts of *R. repens* were suspended in 100 µl of water and 20 µl of a preparation of *B. subtilis* endo-ß-glucanase (22) in 20 mM sodium acetate, 20 mM NaCl, pH 5.5, was added, and the samples were incubated for 3 h at 37ºC. The products released from digestion of purified ß-glucan are mainly cellobiosyl- and cellotriosyl-(1→3)-ß-D-glucose, with smaller amounts of cellotetraosyl- and cellopentaosyl-(1→3)-ß-D-glucose. Enzyme reactions were stopped by heating for 2 min in a boiling water bath followed by cooling to ambient temperature and centrifugation at 10,000 *g* for 5 min.

Chromatography

The oligosaccharides from digestion of the *in vitro* reaction products were separated on a Carbo-Pack PA1 anion exchange column equilibrated with 0.5 N NaOH and eluted with a linear gradient of sodium acetate in 0.5 N NaOH as described by Gibeaut and Carpita (22) and detected with a pulsed amperometric detector.

For gel chromatography, the alkali extracts were applied to a 2.5 x 40 cm Sepharose 4B column (Sigma, St. Louis, MO, USA) equilibrated with McIlvaine's buffer (50 mM citric acid-100 mM Na_2HPO_4), pH 5.5. Fractions (3 ml) were collected and 500 µl of each was assayed for sugar by the phenol-sulfuric acid method (21).

In order to check for the presence of ß-glucan in the decoction of *R. repens* shoots, the water-soluble extract was subjected to precipitation with ethanol (3 volumes) at 5ºC for 18 h. The material was centrifuged for 15 min at 18,000 *g*, the pellet was solubilized in 80% ethanol, stirred and centrifuged as above. This procedure was repeated once more. The precipitated fractions were concentrated under reduced pressure for complete drying, resuspended in distilled water and submitted to enzymatic hydrolysis followed by high-performance anion exchange chromatography with pulsed amperometric detection analysis.

On the basis of the results obtained with the sequential extractions of different plant parts, the hypoglycemic effects were studied only with the combined 4 M KOH fractions since these were the richest ß-glucan-containing fractions. All 4 M KOH freeze-dried fractions were pooled, solubilized in hot water and then injected intraperitoneally into Wistar rats with streptozotocin-induced diabetes.

Streptozotocin-diabetic rats

The induction of diabetes and the animal treatment procedures were based on Pepato et al. (23). Male Wistar rats weighing 180-200 g were used for this experiment. The

animals were housed in individual cages in a room with a 12/12-h light/dark cycle and ambient temperature of 22-25ºC. They were fed a commercial stock diet containing (w/w) 9% fiber, 23% protein, and 65% carbohydrate and containing adequate amounts of vitamins and mineral nutrients. The animals were used for the experiment after an acclimatization period of at least one week before the experimental sessions.

Induction of diabetes was performed by injection of streptozotocin (40 mg/kg body weight; Sigma) dissolved in citrate buffer, pH 4.5, into the dorsal vein of the penis of rats previously fasted for 14-16 h. After the injection the animals were placed in metabolic cages with free access to water and to the same type of food they had received before administration of the drug.

The experimental groups were non-diabetic control rats, streptozotocin-induced diabetic control rats, streptozotocin-induced diabetic rats treated with a pooled fraction of 4 M KOH (4 M KOH), and streptozotocin-induced diabetic rats treated with pure barley ß-glucan (DGLUC) purchased from Sigma. Plasma glucose was determined by an enzymatic method (glucose-oxidase) before and 2, 4, 6, 8, and 24 h after administration of extracts. In both treatments, 4 M KOH and DGLUC, the animals received injections of 100 mg extract per kg body weight (24,25) and non-diabetic control rats and streptozotocin-induced diabetic control rats received saline solution. All extracts were administered intraperitoneally.

Data were analyzed statistically by analysis of variance (ANOVA) and significance was assessed by the Tukey test, and P values of less than 0.05 were considered significant. Data are reported as means ± SEM for each group (N = 10).

Results and Discussion

Table 1 shows the yield of total sugars and uronic acids in the 4 M KOH fractions of different parts of the plant of *R. repens* compared with maize coleoptiles. Growing young leaves presented a considerably higher percentage of uronic acid in the 4 M KOH fraction, 52.2%, whereas expanded leaf blades, sheath and stem presented an average of 7-9% uronic acids. This suggests that, whereas the 4 M KOH fraction was possibly contaminated with a limited amount of pectin (as shown by the detection of rhamnose, Table 2), the presence of uronic acids in the other three plant part extracts might be related to the presence of glucuronoarabinoxylan (GAX).

The analysis of monosaccharides of the 4 M KOH fractions (Table 2) showed that young leaves and expanded leaf blades are composed of glucose > xylose > arabinose, with traces of galactose and rhamnose in young leaves only. On the other hand, sheath and stem are composed of xylose > glucose > arabinose with traces of galactose. In maize, our analysis confirmed the presence of a larger proportion of glucose in the 4 M KOH fraction. This has been studied in maize by Carpita (19), who found that this glucose is directly related to the presence of a ß-glucan, whereas xylose and arabinose are part of a GAX. The comparison with maize (Table 2) suggested that young leaves and expanded leaf blades contained proportionally more ß-glucan than GAX, whereas in sheath and stem (especially the latter) the proportions were inverted (Table 2).

The presence of ß-glucan in *R. repens* was confirmed by hydrolysis with a specific sequence-dependent *B. subtilis* endoglucanase (Figure 1). Because of its high specificity, this enzyme has been used for unequivocal identification of the presence of ß-glucans (13,26). Indeed, all parts of *R. repens* contained ß-glucan and although we did not quantify this polysaccharide in the present study, its proportion can be estimated on the basis of the monosaccharide analysis (Table 1).

Because glucose proportions in cell wall

polymers are higher in growing tissues (an indication of the presence of ß-glucan), we suggest that in *R. repens* this polysaccharide is also associated with growth, as proposed by Carpita and Gibeaut (12).

The 4 M fractions from different parts of the plant of *R. repens* were analyzed by gel chromatography on Sepharose 4B (Figure 2). Their profiles were compared with the polysaccharides from maize whose molecular mass is about 250 kDa (Figure 2E) (26). Our results showed that polymers from young leaves (Figure 2A), in which ß-glucan predominates (see Table 2), displayed high molecular weight, whereas sheath and stem polysaccharides were very polydisperse.

Considering that the decoction (boiling in water for 5 min) of *R. repens* used for popular treatment of diabetes includes the shoots of the plant and the hypoglycemic effect of the precipitate fractions of the aqueous extracts of *R. repens* (18), the question arises of whether ß-glucan is solubilized in the hot water extract prepared as a decoction. We examined the decoction for the presence of ß-glucan and the results are shown in Figure 3. The treatment of the soluble solids in the precipitate of the aqueous extract of *R. repens* with the specific *B. subtilis* endo-glucanase demonstrated clearly the presence of the oligosaccharides characteristic of ß-glucan. The retention times of the tri- (G_4 G_3 G) and tetrasaccharides (G_4 G_4 G_3 G; Figure 3D) were shorter, but confirmed by co-injection with authentic ß-glucan. Our results indicate that, although present in small quantities, the water-soluble ß-glucan is indeed ingested with the decoction. It is therefore possible that it might be one of the reasons for the hypoglycemic effect observed (18).

In order to test this hypothesis, an experiment was performed in which barley ß-glucan and the ß-glucan included in the 4 M KOH combined fractions of *R. repens* were injected intraperitoneally into Wistar rats with streptozotocin-induced diabetes. Figure 4 shows that injection of barley ß-glucan induced a significant decrease in plasma glucose. This decreasing effect lasted for approximately 4 h after injection and plasma glucose level returned to the previous value after 6 h. The injection of combined 4 M KOH ß-glucan-rich *R. repens* fractions had a similar effect, but the decreasing response was slower, reaching a maximum within 4 h. Unlike barley ß-glucan, the effect induced by the hemicellulose fraction of *R. repens* lasted for up to 24 h.

A possible explanation for the difference in response of the rats to the two different sources of ß-glucan might be related to the fact that the barley ß-glucan is water-soluble and had a relatively higher level of purity. On the other hand, because the combined 4 M KOH ß-glucan fractions of *R. repens* were a mixture of ß-glucan and GAX (see Table 2), the latter being less water-soluble, it is possible that the release into the blood stream

Table 1. Yield of neutral sugars and uronic acids in the 4 M KOH fractions obtained from different parts of the plant of *Rhynchelytrum repens*.

Tissues	Total sugar content	Uronic acids
Expanded leaf blade	202.87	15.92 (7.8%)
Sheath	341.76	31.56 (9.2%)
Stem	332.04	24.42 (7.4%)
Young leaves	28.52	14.90 (52.2%)
Maize (standard)	128.61	35.10 (27.3%)

Data are reported as µg/mg 4 M KOH fraction with percent uronic acids in relation to total sugars in parentheses.

Table 2. Monosaccharide composition of 4 M KOH fractions of expanded leaf blade, sheath, stem, and young leaves of *Rhynchelytrum repens* and maize (standard).

Tissues	Rha	Ara	Glc	Xyl	Gal
Expanded leaf blade	nd	12.53	44.08	42.30	1.09
Sheath	nd	12.75	35.67	48.97	2.61
Stem	nd	8.07	19.19	52.15	2.03
Young leaves	5.48	8.41	56.65	29.46	nd
Maize (standard)	nd	10.16	62.94	21.95	4.95

Data are reported as molar ratios obtained by high-performance anion exchange chromatography with pulsed amperometric detection analysis. Rha = rhamnose; Ara = arabinose; Glc = glucose; Xyl = xylose; Gal = galactose; nd = not detected.

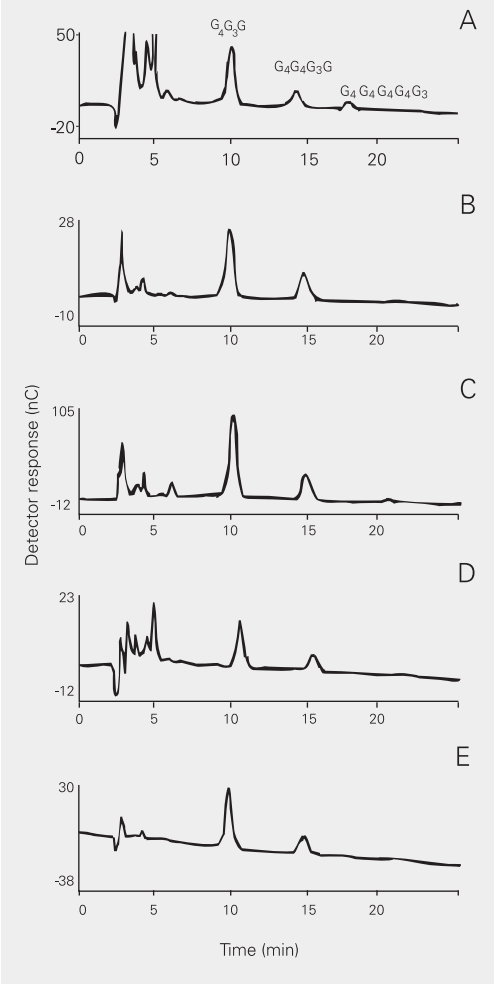

Figure 1. High-performance anion exchange chromatography with pulsed amperometric detection analysis of ß-glucan after hydrolysis of the 4 M KOH fraction with endo-glucanase from *Bacillus subtilis*. *A*, Young leaves; *B*, expanded leaf blade; *C*, sheath; *D*, stem of *Rhynchelytrum repens*; *E*, maize. G_4 G_3 G = trisaccharide, G_4 G_4 G_3 G = tetrasaccharide and G_4 G_4 G_4 G_4 G_3 = pentasaccharide. The subscripts represent the type of linkage between two glucoses (G): 4 represents a ß-1,4 linkage and 3 a ß-1,3 linkage. nC = nano Columbs.

might have been slower compared with pure ß-glucan (Figure 4). This hypothesis is supported by the facts that i) ß-glucan and GAX can interact and form intermolecular complexes amongst themselves (27) and ii) GAX has recently been shown to reduce the postprandial glucose response in healthy humans (28).

A transient hypoglycemic effect of the fractions containing high-molecular weight polymers has been reported in the extracts of several species. Takahashi et al. (24) reported the hypoglycemic activity of the non-dialyzed portion of the juice from the sugar cane stalks (*Saccharum officinarum*). These investigators reported that the main constituents isolated from the juice, saccharins C,

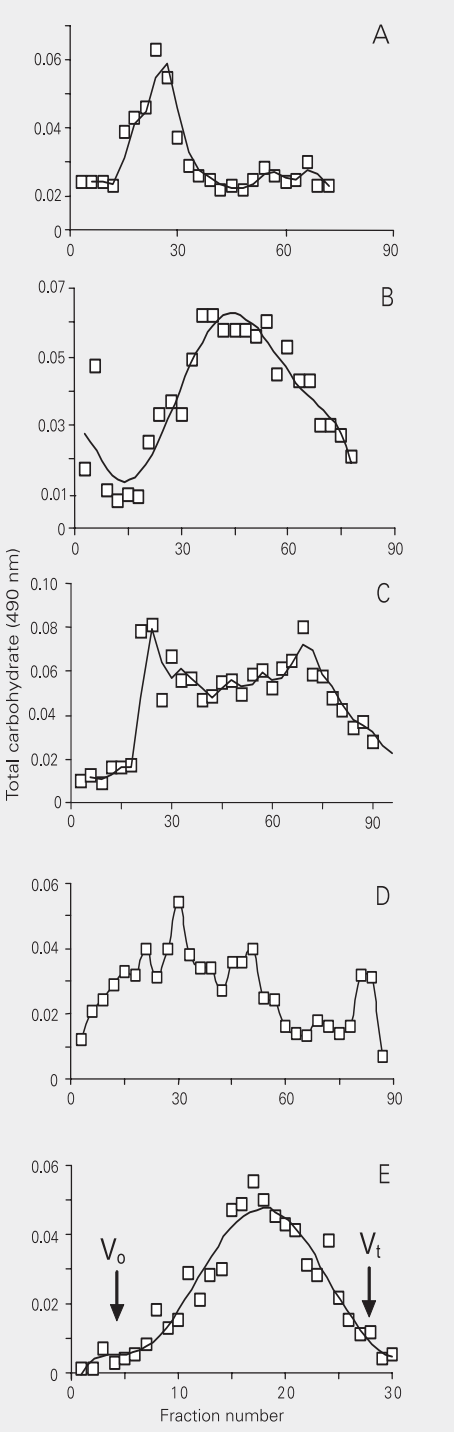

Figure 2. Gel filtration chromatography on Sepharose 4B of the 4 M KOH fraction of walls from *Rhynchelytrum repens* and maize. *A*, Young leaves; *B*, expanded leaf blade; *C*, sheath; *D*, stem of *Rhynchelytrum repens*; *E*, maize (standard). V_0 = void volume obtained using blue dextran 2000; V_t = total volume obtained using sucrose.

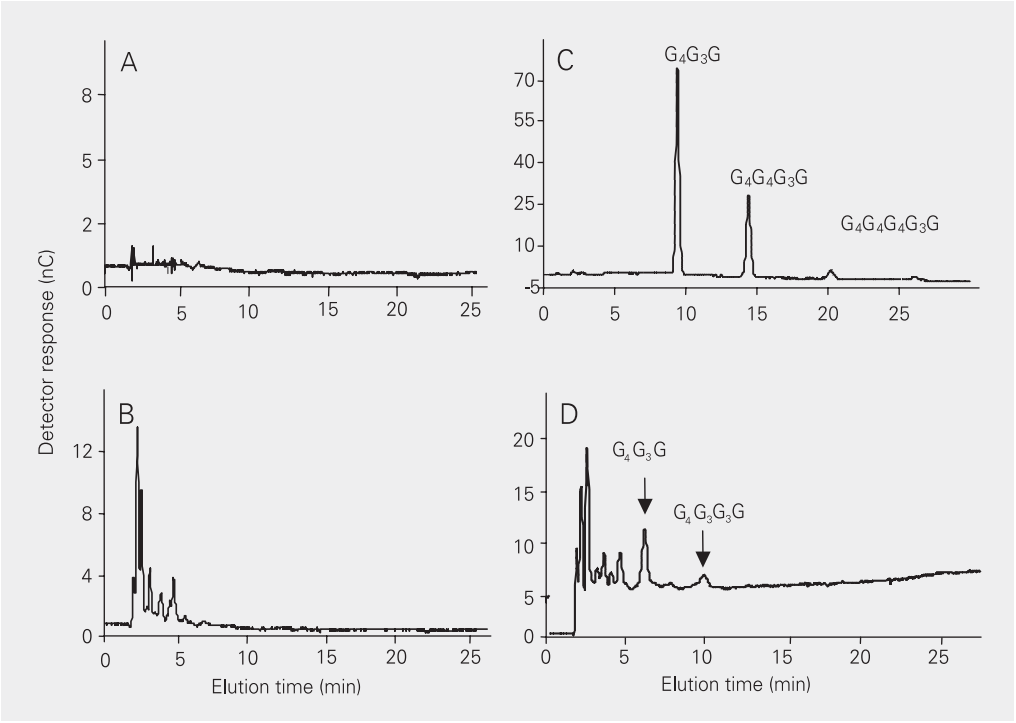

Figure 3. High-performance anion exchange chromatography with pulsed amperometric detection analysis of oligosaccharides produced by enzymatic hydrolysis with *Bacillus subtilis* cellulase. *A*, Non-hydrolyzed barley ß-glucan; *B*, non-hydrolyzed precipitate obtained from the aqueous extract of *Rhynchelytrum repens*; *C*, hydrolyzed barley ß-glucan; *D*, precipitate obtained from the aqueous extract of *Rhynchelytrum repens* after hydrolysis with *Bacillus subtilis* endo-glucanase. The arrows indicate tri- and tetrasaccharides. G_4 G_3 G = trisaccharide, G_4 G_4 G_3 G = tetrasaccharide and G_4 G_4 G_4 G_4 G_3 = pentasaccharide. The subscripts represent the type of linkage between two glucoses (G): 4 represents a ß-1,4 linkage and 3 a ß-1,3 linkage. nC = nano Columbs.

showed a transient effect of blood glucose reduction 7 h after administration but no effect after 24 h. Recent observations from our laboratory have shown that the sugar cane cell walls of different tissues of the whole plant have hemicellulose fractions composed of ß-glucan and GAX (Silva AM, Carpita NC and Buckeridge MS, unpublished data).

The general explanation for the mechanisms of action of the complex polysaccharides of plants possibly involves the stimulation of glucose utilization in the liver and peripheral tissues through the key enzymes participating in glucose metabolism, such as glucokinase, hexokinase and glucose-6-phosphatase (29-32).

R. repens contains ß-glucan in several tissues and the preparation method by decoction (boiling for a few minutes), was sufficient to solubilize some ß-glucan. Furthermore, the precipitate of the aqueous extract obtained by decoction had a hypoglycemic effect when administered intraperitoneally to rats with streptozotocin-induced diabetes (18). These results, taken together with the demonstration of the hypoglycemic effect of pure barley ß-glucan and of the ß-glucan-rich fraction of *R. repens* shoots, suggest that the cell wall polymer might be absorbed when administered intraperitoneally

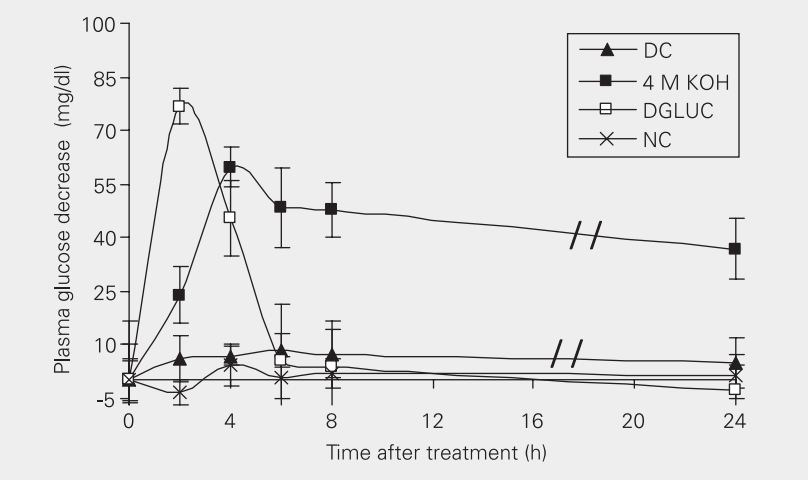

Figure 4. Anti-diabetic effect of intraperitoneal administration of ß-glucan from barley (DGLUC) and of the 4 M KOH hemicellulose fraction of *Rhynchelytrum repens* (4 M KOH) on rats with streptozotocin-induced diabetes. DC = diabetic control; NC = normal control. N = 10 for each group. $P < 0.05$ (ANOVA followed by Tukey test).

and may be involved in hypoglycemic effects observed.

These results suggest that the hemicelluloses from *R. repens* are related to the folk use of this plant for diabetes treatment. However, the use of *R. repens* must be carefully evaluated because acute effects from intraperitoneal administration can indicate a potential use, but at the same time show remarkable differences compared to oral administration. Oral administration involves other complex mechanisms of absorption and further studies are needed regarding this route of administration of the aqueous extract from *R. repens*.

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