Histochemistry and immunolocalisation of glucokin in anti-diabetic plants used in traditional Mexican medicine

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Abstract

Mexico is a megadiverse country that has 3600 to 4000 species of medicinal plants, of which approximately 800 are used to treat conditions related to diabetes mellitus (DM). DM is a chronic degenerative disease of energy metabolism that exists as two types: type 1 (DM1) and type 2 (DM2). DM is considered a public health problem that affects 7% of the Mexican population older than 20 years. DM is clinically controlled with hypoglycaemic drugs, alpha-glucosidase inhibitors, insulin secretion stimulants or the direct application of insulin. The hypoglycaemic effectiveness of specific molecules has been determined only for some medicinal plants in Mexico used to treat DM2. The presence of molecules called glucokinins, which are similar to animal insulin molecules, has been reported in some plant species; glucokinins act as both growth factors and regulators of glucose metabolism in plants. Therefore, we hypothesized that the hypoglycaemic effectiveness of some of the popularly used species for the control of DM could be due to the presence of glucokinin, as reported for Bauhinia variegata.

The goal of this work was to use histochemistry to detect, the accumulation of protein that is immunocytochemically compatible with glucokinin in slide sections of hypoglycaemic species used as remedies for DM2. The top fourteen most used medicinal plants in Mexico were selected for study via microscopic sections. Proteins were histochemically detected using naphthol blue black and Johansen’s quadruple stain, and the immunocytochemical correspondence of the proteins with glucokinin was investigated using an insulin antibody. All species studied reacted positively to proteins and glucokinin in the same structures. The presence of glucokinin in these structures and the corresponding hypoglycaemic effects are discussed in the context of the actions of other compounds.

Introduction

Globally, the prevalence of diabetes mellitus (DM) is increasing at an alarming rate. This chronic pathology severely affects human health and the quality of life. DM is a common metabolic disorder that affects approximately 2.8% of the world’s population and is anticipated to reach 5.4% by 2025. Herbal medicines have highly esteemed for a long time and thus have become a growing part of modern medicine. Diabetic populations originate in industrialised countries, but 65% of individuals with DM live in developing countries. In Mexico, approximately 10% of the population has DM, and 90% of estimated cases are type 2 DM (DM2). DM2 is the frequent in older adults and obese people. DM2 is the leading cause of death, causing 12% of total deaths. According to ethnobotanical research, Mexicans empirically uses between 150 and 269 species of plants for DM control; although there are approximately 500 species used from only a handful of families, including the Asteraceae (47 species), Fabaceae (27), Cactaceae (16), Solanaceae and Euphorbiaceae (10) and Lamiaceae (9). Plants are potential sources of hypoglycaemic drugs and are widely used in several traditional systems of medicine to prevent diabetes. Groups of chemical compounds related to the activity of these plants include polysaccharides, alkaloids, glycoproteins, terpenes, peptides, amines, steroids, phenolic compounds (flavonoids, polyphenols), coumarins, sulphur compounds, inorganic ions and glucokinins. Collip discovered in plants a compound that functions similarly to insulin but differentiated this compound from insulin by naming it glucokinin; glucokinin exists in diverse organisms in addition to plants. Since 1980, insulin-like molecules have been found in bacteria, protozoa and fungi, as well as in spinach leaves (Spinacia oleracea), water lentil (Lemna gibba) and maize (Zea mays). Because herbal recipes used to treat DM2 can be mixtures of various medicinal species, several mechanisms of action are involved in the hypoglycaemic control of plant origin. Some of the groups of chemical compounds related to the activity of these plants are polysaccharides, alkaloids, glycoproteins, terpenes, peptides, amines, steroids, phenolic compounds (flavonoids, polyphenols), lipids, coumarins, sulphur compounds and inorganic ions. The mechanisms involved in the activity of these compounds in glycaemia include competitive direct antagonism with insulin, stimulation of insulin secretion, stimulation of hepatic glycogenesis and glycolysis, adrenomimicry, blockage of the K+ channels of pancreatic beta cells, stimulation of cyclic adenosine monophosphate (cAMP) and modulation of glucose uptake from the intestine. An insulin-like protein in the inner layer of the Canavalia ensiformis seed coat that has the same molecular mass and amino acid sequences as bovine insulin was reported. Glucokinin has been detected in the seed coat in the legume Vigna unguiculata using both solid-phase adsorption-based enzyme-linked immunosorbent assay (ELISA) with human anti-insulin antibodies and reverse-phase high-performance liquid chromatography (RP-HPLC); glucokinin and human insulin showed similar patterns. Using immunohistochemistry, immunocytochemistry and transmission electron microscopy, Azvedo et al. revealed the presence of glucokinin in the leaves of Bauhinia variegata; glucokinin was found mainly in chloroplasts and associated with calcium oxalate crystals.
Glucose concentration in the blood decreased in diabetic rats when *B. variegata* was administered subcutaneously. Moreover, orally administered butanol extract from the leaves of *Baubhia forficata* significantly reduced blood glucose levels in diabetic rats. Glucokin may be involved in the biosynthesis and transport of carbohydrates. Therefore, glucokin may act in the growth and development of plants and in the production of starch in a process similar to the biosynthesis of glycogen in the liver, in which insulin is involved. Glucokin functions similarly to insulin and is present in plants; therefore, glucokin may be responsible for the hypoglycaemic effect of plants used for the control of DM2.

The goal of this work was to detect using histochemical and immunolocalisation techniques the presence of glucokin combined with accumulated protein in the parts of plant species used for the control of DM2 or those reported as hypoglycaemic.

### Materials and Methods

#### Species and organs of study

Of the species of Mexican herbal medicines used to treat DM2, we selected the following plants (alphabetically ordered by the family to which the species belongs): *Petroselinum crispum* (Mill.) Mansf., Apiaceae, 'Perejil' and 'Parsley' (leaf); *Bidens pilosa* L., Asteraceae, 'Romerito blanco', 'Mozone', 'Acachua', 'Aceitilla', 'Beggar’s Ticks', 'Blackjack', and 'Hairy beggartick' (leaf); *Brickellia cavanillesii* (Cass.) A. Gray, Asteraceae, 'Prodigiosa' (leaf); *Cynara scolymus* L., Asteraceae, 'Alcahufa', 'Alcachofera', 'Alcanfora', and 'Artichokes' (bract); *Taraxacum officinale* Weber ex F H Wigg., Asteraceae, 'Arnica de diente', 'Diente de león', 'Chicoria', and 'Dandelion' (leaf); *Parmentiera aculeata* (Kunth) L.O. Williams, Bignoniaceae, 'Cuajiote', 'Cuaxilotl', and 'Chote' (fruit); *Tecoma stans* (L.) Juss. ex Kunth, Bignoniaceae, 'Tronadora', 'Acarancillo', 'Nextamalxochitl', and 'X’k-an’lol' (leaf); *Oppuntia ficus-indica* (L.) Mill., Cactaceae, 'Nopal' (cladode); *Beta vulgaris* L., Chenopodiaceae, 'Betabel', 'Remolacha', and 'Beetroot' (leaf); *Aloe vera* (L.) Burm.f., Liliaceae, 'Sábila' (leaf); *Guazuma ulmifolia* Lam., Malvaceae, 'Guásima' and 'Cuauilote' (leaf); *Rubus adenotruchus* Schltdl., Rosaceae, 'Zarra' and 'Zarzamora' (leaf); *Buddleja cordata* Kunth, Scrophulariaceae, 'Teopozán' (leaf); *Cecropia obtusifolia* Bertol., Urticaceae, 'Guarumo' and 'Koochén' (leaf).

#### Reagents and equipment

**Fixatives**

FAA (10% formaldehyde/5% acetic acid/50% alcohol) (Merck, S.A. de C.V., Naucalpan de Juárez, Mexico). 10% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. (Electron Microscopy Science®, IACCSA, Tlalpan, Mexico).

AGF (1.5% acrolein/3% glutaraldehyde/1.5% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Science®).

**Dyes**

Naphthol blue black (NBB), Fast Green, Orange G, safranine, crystal violet (Sigma-Aldrich Co., Milwaukee, WI, USA).

**Equipment**

HM 340E Rotary Microtome (MICROM GmbH, Waldorf, Germany). Images were collected using a Canon Power Shot A640 digital camera coupled to a Zeiss Axiosstar Microscope with AxioVision (Carl Zeiss de México S.A. de C.V. Coyoacán, Mexico).

**Procedures**

** Fixation**

Plant samples were cut into 2- to 5-mm pieces and were fixed as mentioned above. Mouse pancreas served as a positive control. The pancreas was rinsed with 0.9% NaCl solution until blood was removed. The pancreas was then fixed in 10% formaldehyde and buffered with 0.1 M sodium cacodylate (pH 7.4).

After fixation, each material was washed with tap water, dehydrated in an alcohol series, infiltrated and included in FAA (10% formaldehyde/5% acetic acid/50% alcohol) (Merck, S.A. de C.V., Naucalpan de Juárez, Mexico). Plant samples were cut into 2- to 5-mm pieces and were fixed as mentioned above. Mouse pancreas served as a positive control. The pancreas was rinsed with 0.9% NaCl solution until blood was removed. The pancreas was then fixed in 10% formaldehyde and buffered with 0.1 M sodium cacodylate (pH 7.4).

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**Histochemistry**

The sections were processed by comparing similar zones between each technique using alternating slides of progressive numbering. Comparisons were made in duplicate. NBB histochemistry (1.0% NBB in 50% ethanol). for protein analysis was applied to the first and second slides of each plant organ. For the immunolocalisation technique, we used slides three and four for the positive reaction and slides five and six for the negative control. Some slides were processed using Johansen’s quadruple stain to verify the detection of protein or special structures.

**NBB stain**

For this protocol, we used NBB instead of aniline blue black, which was used by Fisher. Briefly, slides were reduced to 70% alcohol. Slides were then stained with a 1% NBB/50% alcohol solution for 5 min. After rinsing briefly in 96% alcohol followed by 100% alcohol, the slides were rinsed briefly with n-butanol. Finally, the slides were rinsed twice in xylool and then mounted on balsam.

**Johansen’s quadruple stain**

This method correlates stain affinity with specific structures as closely as possible and uses more recently developed stain solvents, such as Orange G, methyl violet 2B, Methyl Cellosolve, tertiary butyl alcohol, clove oil and glacial acetic acid. The staining is simple, even if mixtures are complex; differentiation is automatic and little personal judgement is needed. Immunolocalisation

The sections of plant organs and mouse pancreas needed for this technique were deparaffinised, rehydrated and processed according to the manufacturer’s instructions; however steps 3 (incubation with Carezyme II at 37°C for 5 min) and 14 (application of drops of CAT haematoxylin for 1 min) were omitted because Carezyme II degrades the section or detaches them from the slide, and the purple colour of the reaction of CAT haematoxylin with the cytoplasm and nuclei masks the positive reaction of the antibody. A negative control was used for each species that did not receive insulin antibodies.

Insulin immunolocalisation was performed using the Starr Trek Universal HRP Detection System® Kit, Control Number: 901-STURHP700-090314, ISO 9001 &13485 Certified. Refer to the National Committee for Clinical Laboratory Standards Quality Assurance for Immunocytochemistry approved guidelines (Vol. 19, No. 26, [European Journal of Histochemistry 2017; 61:2782]([European Journal of Histochemistry 2017; 61:2782](https://www.ejhh.com))
The sections were washed with peroxidase for 5 min to remove the endogenous peroxide and then covered with the primary antibody for 60 min. Excess antibodies were removed, after which the link reagent was applied for 10 min. Streptavidin-HRP was then applied for 10 min, and the sections were dehydrated in 100% ethanol and xylene for covering with resin.

Results

All fixation treatments worked well for both histochemical staining and glucokinin immunolocalisation, although the material fixed in FAA showed the best response of all three techniques. Histochemistry using NBB is a specific technique for proteins; in general, NBB stains blue the cell walls of the epidermis and its derived structures, such as trichomes, cellular contents, the chloroplasts of mesophyll parenchyma, and neighbouring cells of the vascular bundle, which may correspond to laticiferous or secretory canals, depending on the species. In the particular description of the positive reaction using this technique for each species, the zones of protein accumulation or strong reaction are highlighted and compared with those resulting from immunolocalisation. Only one figure based on the reactions with Johansen’s quadruple stain is presented that illustrates the association with protein (purple or violet colour in the same zone) (Figure 1L).

Immunolocalisation

The positive reaction effecting brown/reddish colour corresponds to the complex formed by the antibody against insulin from the pancreas or glucokinin from plant organs. In the mouse pancreas sections, a positive response was observed only in the beta cells of the islets of Langerhans (Figure 1A). The results based on this technique for the plant organ sections corresponded to those of the NBB histochemical technique, and the negative control of the immunolocalisation did not exhibit this reaction (Figure 1 B-D).

Sections of the organs of the species studied

P. crispum (leaf)

This section showed a strong reaction to NBB and immunocytochemistry, particularly in cells associated with vascular bundles and laticifers (Figure 1 B-D).

B. pilosa (leaf)

A positive reaction of immunolocalisation and NBB was observed in the cell walls of trichomes and vascular bundle fibres (Figure 1 E, F).

B. cavanillesii (leaf)

This section had secretory canals and laticifers that were adjacent to vascular bundles and contained protein that reacts positively with the immunolocalisation technique. There were also reactions observed in glandular trichomes (Figure 1 G,H).

Figure 1. A) Positive control mouse pancreas, islet of Langerhans. B-D) P. crispum, E, F) B. pilosa. G, H) B. cavanillesii. I, J) C. scolymus. K, L) T. officinale. L) The reaction of Johansen’s quadruple stain is shown compared with that of immunocytochemistry. The positive reaction of immunocytochemistry is shown in brown/reddish colour. The negative control of immunocytochemistry is colourless (C). The positive reaction of NBB is blue. IL, islet of Langerhans; L, laticifers; Chp, chlorophyll parenchyma; vb, vascular bundle; Sc, secretory canal; Li, laticifer with phenols; F, fibres; arrows, trichomes.
C. scolymus (bract)

A positive reaction to NBB was detected, which corresponds to the positive reaction to immunocytochemistry in chloroplasts and the cell walls of fibres located in the parenchyma. Phenolic laticifers were observed in the surrounding areas (Figure 1 I, J).

T. officinale (leaf)

Laticifers were observed that were adjacent to vascular bundles and contained abundant protein. This protein strongly positively reacted with immunolocalisation and reacted in the mesophyll (Figure 1 K, L).

P. aculeata (fruit)

A strong, positive reaction to NBB was observed in epidermal and subepidermal cells as well as in fibres of the mesocarp parenchyma, which corresponded to the weak positive reaction of immunolocalisation (Figure 2 A, B).

T. stans (leaf)

Laticifers were discovered that were adjacent to the vascular bundles and contained protein that reacted positively with immunolocalisation. An intense positive reaction of histochemistry and immunocytochemistry was also observed in some epidermal cells, in trichomes and in mesophyll cells (Figure 2 C, D).

O. ficus-indica (cladode)

A positive reaction with NBB that corresponded to immunocytochemistry was strongly present in the multilayer epidermis and in the contents of mucilage-secreting cavities. An abundance of cells with crystals was observed in epidermal and parenchyma tissue (Figure 2 E-G).

B. vulgaris (leaf)

Laticifers adjacent to the vascular bundles contained protein that reacted positively with immunolocalisation and many adjacent mesophyll cells contained crystals (Figure 2 H-K).

A. vera (leaf)

This section showed the same protein localisation with both techniques; the reaction was intense in the cell walls of the epidermis, in mesophyll chloroplasts and in the mucilage (gel) of secretory canals (Figure 3 A-D).

G. ulmifolia (leaf)

Two types of laticifers were observed that were adjacent to the vascular bundles: laticifers with orange/gold tannins and others containing protein. NBB and immunocytochemistry detected strong reactions in the mesophyll and laticifers with protein, which closely matched the sections of the negative control (Figure 3 E-H).

R. adenotrichus (leaf)

NBB and immunocytochemistry produced strong, positive reactions in laticifers, trichomes and chlorophyll parenchyma (Figure 3 I-J).

B. cordata (leaf)

Articulated laticifers were observed to be associated with vascular bundles. These laticifers reacted strongly to both NBB and immunocytochemistry; some parenchyma cells had crystals (Figure 3 K-M).

C. obtusifolia (leaf)

Both the tannin content throughout the leaf mesophyll and the observed greyish cystoliths were highlighted. NBB and immunocytochemistry strongly positively reacted in the chlorophyll parenchyma and subepidermal cells, where tannins are also located (Figure 3 N, O).

Figure 2. A, B) P. aculeata. C, D) T. stans. E-G) O. ficus-indica. H-K) B. vulgaris. E, I and K were imaged with phase contrast microscopy to highlight crystals. Ep, epidermis; Se, sub-epidermis; Cr, crystals; L, laticifers; Chp, chlorophyll parenchyma; Scv, secretory cavity; F, fibres.
Discussion

Johansen’s quadruple stain effectively corroborated the presence of protein via purple or violet colour in the same zones of reactions to NBB and immunocytochemical techniques (Figure 1L). This method brilliantly stains plant tissue more than clearly any other stain does.19 Patients with DM2 share a pathophysiology that involves pancreatic beta cells, the liver, and peripheral target tissues such as skeletal muscle and adipose tissue. Insulin resistance is a core defect in DM2 and may be a primary factor in the development of atherosclerotic cardiovascular disease and other components associated with metabolic syndrome.20 Only a few reports on the hypoglycaemic activity of medicinal plants used to treat DM2 in Mexico have confirmed the hypoglycaemic effectiveness of specific molecules in some of the mechanisms of glucose metabolism control that also help in restoring it; the following are examples of this hypoglycaemic effectiveness:

- *Petroselinum crispum* aqueous extract protects against hepatotoxicity caused by diabetes.21 The extract is effective as a hypoglycaemic treatment in rats with diabetes due to its ascorbic acid, flavonoids and essential oils.22
- *Bidens pilosa* stimulates insulin secretion, protects the islets of Langerhans and increases blood insulin levels;23 the hypoglycaemic effectiveness of this plant is due to the presence of acetylenic glycosides in the aqueous extract of the aerial portion.24,25

The effect of *B. pilosa* formulations on fasting blood glucose (FBG), fasting serum insulin, and glycosylated haemoglobin A1c (HbA1c) in diabetic subjects was evaluated. The *B. pilosa* formulations reduced FBG and HbA1c in diabetics but increased fasting serum insulin in healthy subjects. Moreover, the combination of the *B. pilosa* formulation with antidiabetic drugs resulted in better glycaemic control in diabetics. The homeostatic model assessment (HOMA) data suggested that the antidiabetic activity of this formulation occurred via improvement of beta-cell function. The safety of the *B. pilosa* formulation in healthy subjects was also tested, but no obvious side effects were observed, which indicates that *B. pilosa* is a potential antidiabetic treatment.27

In our study, *B. pilosa* samples did not result
in a strong immunocytotoxic reaction to
glucokinin; therefore, its hypoglycaemic
effect is most likely due to the reported
metabolites such as cytoplyene and related
polyynes (3-β-D-glucopyranosyl-1-
hydroxy-6(E)-tetradecene-8,10,12-triyne
and (R)-3,5,7,9,11-tridecapentayne-1,2-di
ol), which are anti-diabetics in animal
models.26,28 The data therefore reveal a new
biological action of polyynes. Interestingly,
34 polyynes have been reported in B. pilosa.
Whether all the polyynes present in this
plant have antidiabetic activities remains
unclear.26,29
- Brickellia cavanillesii has hypoglycaemic
effectiveness due to the high activity of
metabolites such as 6-hydroxyacetetyl-5-
hydroxy-2,2-dimethyl-2H-chromene,
sesquiterpen lactone (calein C), the
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glucokin was observed only in chloro-
orange/gold in Figure 3 N,O. In addition,
consisted until 18 weeks of administra-
tion; the reduction was significant and per-
vasive, easy and quick to perform. These tech-
niques could be used to screen for accumu-
lated protein in other medicinal plant spe-
cies used for the treatment of DM2 to in-
dicate the presence of glucokinin, which 
would maintain these species as potentially 
effective at controlling the deleterious 
effects of DM2 caused by high blood glu-
cose levels, which was proposed by 
Khursheed et al.86 for Spirulina platensis.

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