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Insulin-like plant proteins as potential innovative drugs to treat diabetes—The *Moringa oleifera* case study

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ABSTRACT

Various plant species have long been used in traditional medicine worldwide to treat diabetes. Among the plant-based compounds with hypoglycemic properties, studies on insulin-like proteins isolated from leaves, fruits and seeds are rarely reported in the relevant literature. Our research group has been investigating the presence of insulin-like proteins in *Moringa oleifera,* a plant species native to India, and we have obtained a leaf protein isolate and semi-purified derived fractions, as well as a seed coat protein fraction (*Mo*-SC), with hypoglycemic activity in chemically induced diabetic mice that have increased tolerance to orally administered glucose. Equally importantly, *Mo*-SC possesses insulin-like antigenic epitopes. In this context, the present review aims to highlight that prospection of insulin-like proteins in plants is of the utmost importance both for finding new drugs for the treatment of diabetes and for shedding light on the mechanisms involved in diabetes.

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Insulin-type proteins found in plants: a brief history

Various plant species are used in folk medicine in many cultures as a complementary resource for the treatment of diabetes [1]. Numerous molecules isolated from plants can reduce the plasma glucose levels and thus have potential for using as hypoglycemic agents [2]. In fact, some hypoglycemic drugs currently employed to treat type 2 diabetes are related to plant substances that were identified in experiments conducted toward confirming their associated hypoglycemic activity as they were used in the treatment of diabetes in folk medicine. For example, metformin, a drug of the biguanide class that effectively reduces glucose levels in diabetics, was originally produced from a guanidine isolated from *Galega officinalis*, a plant used as a medicinal herb in medieval Europe to treat symptoms related to diabetes and other ailments [3,4]. Small molecules with high structural diversity produced during plant secondary metabolism that promote glycemic normalization, stimulate insulin release, reduce insulin resistance and elicit insulin-mimetic effects have been isolated, including terpenes, alkaloids and flavonoids [5]. Because many drugs used to treat diabetes present such hypoglycemic mechanisms of action [6], there is a close relationship between prospection for these molecules and the development of new drugs.

Although a good deal of credit has been given to these secondary metabolites in promoting the hypoglycemic action of orally taken plant extracts, little attention has been paid to anecdotal and scientific reports that relate the antidiabetic properties of plant extracts to insulin-like plant proteins [7] and the possibility of their biotechnological use to treat diabetes. Nevertheless, speculation in this respect is not new. In 1923, soon after the discovery that insulin was present in the ethanol extract of dog pancreas, J.B. Collip (who had participated in developing this method) reported the presence of insulin-like constituents in leaves of green beans, wheat, lettuce and green onions. He also found that extracts from these plants promoted significant reductions in glucose levels in normal rabbits and in pancreatectomized dogs after subcutaneous administration [8,9]. That same year, the presence of insulin-like compounds in germinated potato and rice seeds was reported, and in 1924, data on the hypoglycemiant effect of sugar beet extracts were published [10,11].

Some 50 years later, an Indian research team reported the presence of insulin in a plant and patented the process of obtaining

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that material by ethanol-acid extraction of fruits and seeds of the bitter melon *Momordica charantia* [12]. The product obtained, called polypeptide-p, showed hypoglycemic activity in the Indian desert gerbil (*Meriones hurricane*), Indian langur monkey (*Presbytis entellus*) and diabetic humans. Despite those *in vivo* insulin-mimetic effects, polypeptide-p presented certain biochemical properties that differed from those of bovine insulin, such as the molecular weight (approximately 11 kDa), the presence of the amino acid methionine, and the absence of immunoreactivity against anti-bovine insulin antibodies [13]. Those discoveries marked the start of a new period of prospection for insulin-like compounds in plants.

Plant insulin, insulin-like protein or hypoglycemic protein: what term is most appropriate?

Table 1 summarizes some of the biochemical characteristics and other data of insulin-like proteins isolated from plants. These proteins do not necessarily present the same biochemical attributes when compared to each other or to mammalian insulin; however, they often have one thing in common: the ability to reduce blood glucose levels in laboratory animals with diabetes. Conversely, not all the isolated proteins showed the presence of insulin-like antigenic epitopes. Thus, we hypothesize that the plant proteins that are most structurally similar to mammalian insulin and are recognized by anti-insulin antibodies are more likely to activate the insulin-mimetic signaling cascade because those pathways are elicited by the specific interaction of insulin with its membrane receptors [14–16]. In view of these considerations, perhaps the term “plant insulin” would be more appropriate for such plant proteins. In turn, those that do not present many biochemical similarities with mammalian insulin might exert a hypoglycemic effect by selectively eliciting insulin-mimetic pathways. In this case, activation of pathways other than by interaction of insulin with receptors could also indirectly promote glucose reduction, if, for example, the protein or peptide stimulates β-cells to secrete insulin (Fig. 1).

In humans, two polypeptide hormones, glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (GLP-1), called incretins, are secreted by cells in the gastrointestinal tract in response to the presence of carbohydrates in meals [17]. GIP and GLP-1 help promote glycemic normalization after carbohydrate consumption not by directly stimulating the emptying of glucose from the vascular to the intracellular compartment, as insulin does,

Table 1
Identification, plant species, localization, characteristics and properties of some insulin-like proteins/peptides.

Protein/ peptide name	Species	Organ	Amino acid sequence	MW	Reactivity with human anti-insulin antibodies	*In vivo* hypoglycemic activity/ route of administration[a]	Reference
MC6.1	*Momordica charantia*	Fruit	KTNMKHMAGAAAAGAVVG	~2.5 kDa	–[b]	Yes (Normal and Streptozotocin-diabetic rats)/p.o.	[66]
MC6.2	*Momordica charantia*	Fruit	KTNMKHMAGAA	–	–	Yes (Normal and Streptozotocin-diabetic rats and NOD-mice)/ p.o.	[66]
MC6.3	*Momordica charantia*	Fruit	KTNMKHM	–	–	Yes (Normal rats)/p.o.	[66]
Polypeptide-k	*Momordica charantia*	Seed	–	~18 kDa	Not	Yes (Diabetic humans)/p.o.	[67]
MC2-1-5	*Momordica charantia*	Fruit	GHPYYSIKKS (first 10 N-terminal amino acids)	~3.4 kDa	–	Yes (Alloxan-diabetic mice)/p.o.	[73]
ILP	*Costus igneus*	Leaf	–	~5.7 kDa	Yes	Yes (Streptozotocin-diabetic mice)/p.o.	[24]
Polypeptide-p	*Momordica charantia*	Seed	–	~11.0 kDa	Not	Yes (Gerbils, langurs and diabetic humans)/s.c.	[13]
ADMc1	*Momordica charantia*	Seed	QGRQERCRHIRPREQL RSCQDFLRQQGGGR (smaller chain) QWGREQ GLEECCRQLRNVEEQCRCDALE EVAREVQSQQHGQQGSQILQHARML PSMCQIRPQRCDF (larger chain)	~12 kDa	–	Yes (Normal and alloxan-diabetic rats)/s.c.	[109]
M.Cy protein	*Momordica cymbalaria*	Fruit	GLEPTTT	~17 kDa	–	Yes (Streptozotocin-diabetic rats)/s.c., i.v. and i.p.	[76]
–	*Canavalia ensiformis*	Seed coat	1GIVEQCCASVCSLYQLENYCN21 (Smaller chain) 1FVNQHLCGSSHLVEALYLVCGERGFFYTPKA30 (Larger chain)	~6.0 kDa	Yes	Yes (Alloxan-diabetic mice)/i.v.	[22]
–	*Bauhinia variegata*	Leaf	–	~5.7 kDa	Yes	Yes (Alloxan-diabetic mice)/i.v.	[44]
ILP	*Costus igneus*	Leaf	–	~5.7 kDa	Yes	Yes (Streptozotocin-diabetic mice)/i.p	[24]
–	Spinach	Leaf	–	~6.0 kDa	Yes	–	[40]
–	*Lemna gibba* G3	Whole plant	–	~6.0 kDa	Yes	–	[40]
–	*Vigna unguiculata*	Empty pods	GIVEQXXASVXSLYQLENYXN FVNQHLXGSHLVEALYLVXGERGFFYTPKA	~6.0 kDa	Yes	–	[23]

[a] route of administration: p.o. = oral; s.c. = subcutaneous; i.v. = intravenous and i.p. = intraperitoneal.
[b] -: not mentioned.

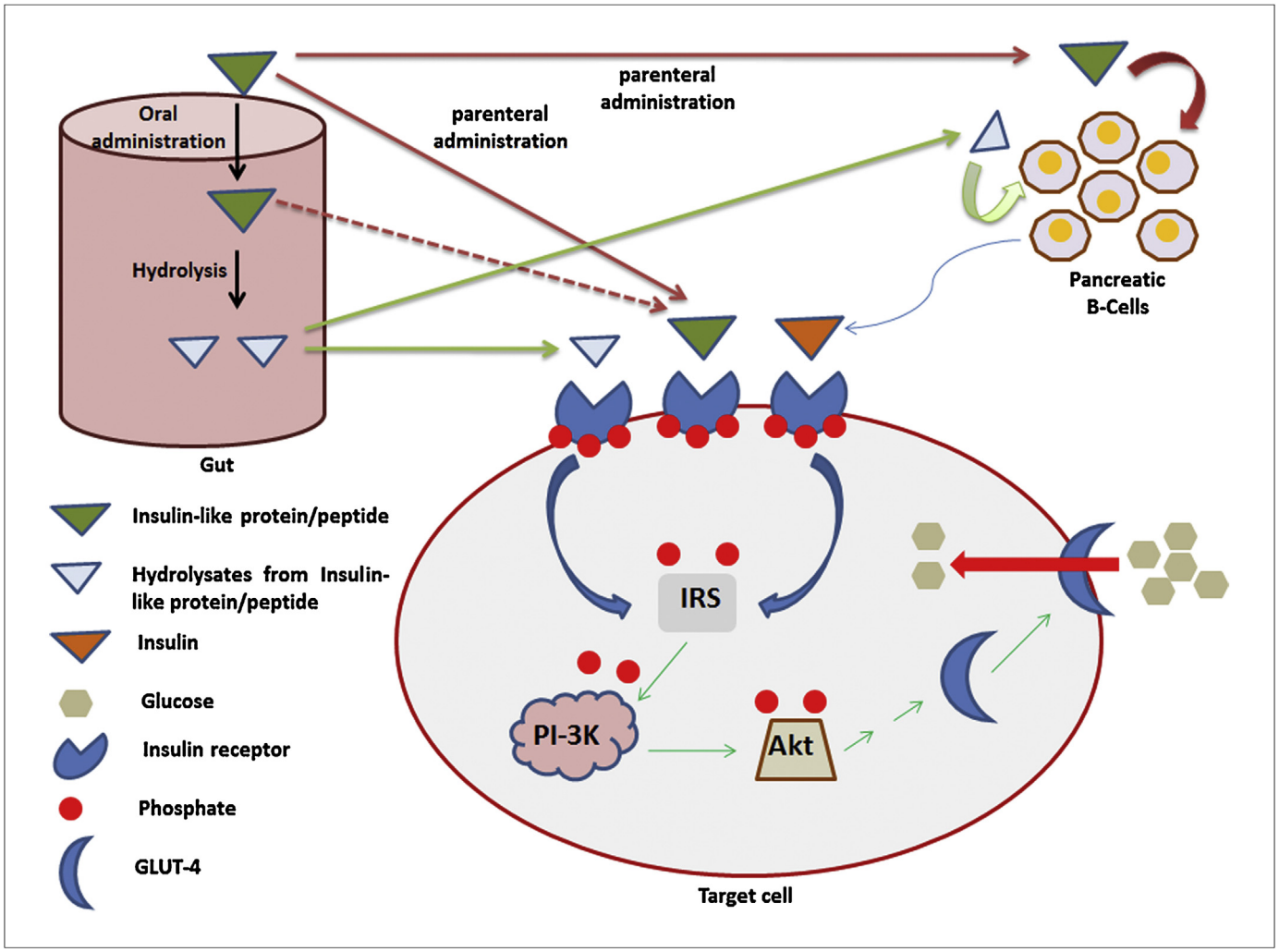

Fig. 1. Proposed mechanisms for the hypoglycemic effect of insulin-like proteins/peptides of plant origin. Proteins/peptides, after interacting with the insulin receptor, act as an insulinomimetic agent that elicits a signaling cascade that promotes blood glucose reduction. Such interaction with proteins/peptides possibly induces autophosphorylation of the insulin receptor and subsequent phosphorylation of regulatory proteins such as insulin receptor substrate (IRS), phosphatidyl inositol 3 kinase (PI-3K) and serine/threonine kinases (Akt), resulting in the stimulation of glucose transporter type 4 (GLUT-4) exocytosis and blood glucose uptake. An indirect effect is stimulation of insulin secretion, which promotes the biochemical cascades involved in reducing blood glucose. For proteins that exert a hypoglycemic effect after oral administration, they may resist the digestive hydrolysis or be partially broken down into active peptides that may be systemically absorbed to promote a hypoglycemic effect.

but by stimulating the secretion of insulin by β-cells [18]. In fact, this is the therapeutic base of the hypoglycemic treatment of patients suffering from type 2 diabetes with drugs known as dipeptidyl peptidase 4 (DPP-IV) inhibitors, which by inhibiting the lytic action of this enzyme on GIP and GLP-1, increase the half-life of these incretins, triggering the subsequent secretagogue effect of insulin [19]. For instance, a semipurified fraction containing peptides extracted from *M. charantia* fruits stimulated insulin secretion by residual β-cells in alloxan-induced diabetic mice [20]. Likewise, a protein extract obtained employing a protocol (acid-ethanol extraction followed by precipitation of proteins by acetone) often used to obtain insulin-like proteins was shown to promote the secretion of insulin in diabetic and non-diabetic rats [21].

Taking into consideration what has been described, a hypoglycemic plant protein should not necessarily be classified as an insulin-like protein or plant insulin. To be considered an insulin-like protein, the hypoglycemic plant protein should have other characteristics similar to those of mammalian insulin. This is the case for the hypoglycemic proteins purified from *C. ensiformis* and *V. unguiculata*, which contain an identical amino acid sequence and very close molecular mass to bovine insulin and are recognized by anti-insulin antibodies [22,23]. This is also the case for proteins from *C. igneus* leaves, which contain insulin-like epitopes [24], and for conglutin-γ, a glycoprotein obtained from lupin seeds that enhances glucose transport, translocation of GLUT-4 carriers and muscle-specific gene transcription by eliciting the same signaling pathways stimulated after insulin binds to its receptor [25]. Nevertheless, this classification suggestion is empirical, as the differences in the biochemical characteristics within "plant insulins" and among "plant insulins" and mammalian insulin can also represent adaptive modifications for the evolutionary success of each species [26]. Additionally, a hypoglycemic protein can also present some, but not all, of the biochemical characteristics of mammalian insulin, making it necessary to establish some prerequisites in order to classify a hypoglycemic plant protein as an insulin-like protein.

Lectins from plants: insulin-like proteins?

Lectins are proteins or glycoproteins of non-immune origin that contain at least one site that binds reversibly to carbohydrates, in certain cases with high specificity [27,28]. Some lectins interact with insulin receptors and can trigger cascade reactions, which are

normally elicited by insulin binding [29]. The lectins from *C. ensiformis* (Con A) and *Lens culinaris* (LCA) promoted increased oxidation of glucose into CO_2, similar to the action of insulin [30]. Con A and wheat germ agglutinin (WGA) mimic insulin, as they induce tyrosine aminotransferase activity [31] and activate insulin-sensitive phosphodiesterase [32]. Strengthening the hypothesis that such effects are mediated by interaction with insulin receptors, Hedo et al. [33] performed affinity chromatography and found that insulin receptors from human IM-9 lymphocytes and placenta bound to immobilized Con A and WGA covalently attached to an agarose matrix. However, Ponzio et al. [34] verified that WGA enhanced tyrosine aminotransferase activity, but did not increase autophosphorylation of the insulin receptor. This finding is incompatible with the idea that those lectins exert insulin-mimetic effects by interacting with insulin receptors, as autophosphorylation of insulin receptors happens after insulin binding, triggering a series of subsequent phosphorylation steps of intracellular regulatory proteins, culminating in the physiological effects of insulin action at the target tissues [35].

Other lectins, such as those from *Phaseolus vulgaris* (PHA) [36] and *Trichosanthes kirilowii* [37], as well as an agglutinin from *M. charantia* that binds to galactose [38], also stimulate insulin-mimetic activities *in vitro.* Moreover, a lectin purified from *Urtica pilulifera* seeds promoted a hypoglycemic effect in streptozotocin-induced diabetic rats after intraperitoneal administration [39]. These results indicate that lectins should be included as potential molecules for prospection to obtain products to treat diabetes. Additionally, there may be a parallel between the function of certain lectins and other insulin-like proteins previously described to be important in regulating plant physiology because both types of molecules are in some way related to the *in vitro*/*in vivo* activities associated with carbohydrates.

Insulin-like proteins isolated from leaves

In 1987, Collier and colleagues reported the isolation of two insulin-like proteins, one from spinach (*Spinacia oleracea*) and the other from the whole-plant extract of *Lemna gibba*, strain G3 [40]. An extraction was performed with a solution of methanol/chloroform/water/formic acid, which was just as effective as the ethanol/water/sulfuric acid solution employed by Khanna et al. [12], to extract insulin-like compounds. Collier et al. [40] also evaluated the ability of an ethanol solution acidified with hydrochloric acid to extract components from spinach leaves that showed immunoreactivity toward mammalian anti-insulin antibodies and observed similar results.

Further evidence for the existence of insulin-like proteins in plants is that both proteins from spinach and *L. gibba* were eluted in a molecular exclusion chromatography column in a similar position to that of porcine insulin, presented reactivity against the porcine anti-insulin antibody, and significantly competed with insulin labeled with radioactive iodine [^{125}I] to bind to insulin receptors of IM-9 lymphocytes. Moreover, in terms of bioactivity, these two insulin-like proteins promoted a dose-dependent increase in the aerobic glucose metabolism of adipocytes in young rats, as demonstrated by the rate of appearance and conversion of ^{14}C from glucose into CO_2. Several observations suggested that the increase in the aerobic metabolism of glucose results from stimulation of biochemical pathways related to insulin by interaction of the two insulin-like proteins from spinach and *L. gibba* with the insulin receptor because: (1) the ability of these two proteins to stimulate glucose metabolism was neutralized in the presence of porcine anti-insulin antibody, but not in the presence of guinea pig IgG; (2) the ability of Con A to stimulate glucose metabolism was not altered after addition of anti-insulin antibody or guinea pig IgG; and (3) the insulin-like proteins from spinach and *L. gibba* interacted with the insulin receptor specifically, as they did not affect, for example, binding of the growth hormone to its receptors on IM-9 lymphocytes. The insulin-like protein obtained from spinach also increased lipogenesis in adipocytes as demonstrated by incorporation of D-[^{3}H]glucose in lipids, an effect that was neutralized by the addition of anti-insulin antibodies [40]. These results further demonstrate the similarity of actions between plant insulin-like proteins and mammalian insulin characterized by increased consumption of glucose to supply energy and carbon skeletons to meet the needs of cellular anabolism (Fig. 2).

Bauhinia variegata, which belongs to the Fabaceae family, is widely used in traditional medicine to treat diabetes. It has many common names in English, including kachnar, orchid tree and variegated bauhinia. It is native to India and China and is widely cultivated in southeastern Brazil, where it is known as *pata-de-vaca,* or “cow’s foot” [41]. In addition to controlling glycemia, the tea of its leaves is also used for other medicinal purposes [42,43]. A protein with similar molecular mass to bovine insulin was purified from the leaf extract of *B. variegata* [44]. This protein, in addition to reacting with human anti-insulin antibodies, promoted a significant reduction of glycemia in alloxan-induced diabetic mice. Microscopic examination showed that this protein is located in the chloroplasts of parenchymal cells, and this observation was reinforced by the detection of an absorption peak at 670 nm, characteristic of the presence of chlorophyll. The location of this protein in chloroplasts indicates it may play a role in the plant similar to the role insulin plays in mammals: to control and promote an anabolic-catabolic steady state. In humans, the anabolic pathways regulated by insulin predominates after eating, while mobilization of reserves occurs during fasting [45,46]. In plants alternating between synthesis and storage of carbohydrates during the day and mobilization of glucose reserves at night occurs [47,48]. Based on these findings, insulin-like proteins in plants most likely participate in regulating these phases. Although there is no evidence in the literature for variation in the insulin-like protein concentrations in leaves during exposure to light or dark, it is reasonable to hypothesize that these proteins might be less expressed at night, at least in part, to favor starch mobilization toward meeting the plant energy requirements. However, such supposition needs to be experimentally tested.

The leaves of *Costus igneus*, commonly known as the insulin plant, are widely used in traditional medicine to treat diabetes [49]. Experimentally, it has been shown that the ethanol extract of its leaves promotes a significant reduction of glucose levels in alloxan-induced diabetic rats and that regular consumption of its leaves helps in controlling glucose levels in diabetic patients [50,51]. A protein isolated from *C. igneus* leaves was reactive to anti-insulin antibodies [24]. Interestingly, ethanol-containing sulfuric acid was the starting solution used to extract the active compound from the *C. igneus* leaves, essentially the same extracting solution used by researchers in studies of insulin-like proteins of other plants [12,21,23]. The insulin-like protein from *C. igneus* leaves was purified from the ethanolic extract by immunoaffinity chromatography using anti-insulin antibodies immobilized onto a sodium alginate matrix. The molecular mass of the purified protein, determined by MALDI-TOFF, was 5.7 kDa, similar to that of insulin and the insulin-like protein isolated from *B. variegata* leaves [44]. In addition, this insulin-like protein from *C. igneus* leaves had hypoglycemic activity in normal and streptozotocin-induced diabetic mice when administered orally or intraperitoneally. It also stimulated glucose uptake by RIN5f culture cells, which are responsive to insulin. However, further biophysical characterizations indicated that the *C. igneus* insulin-like protein is structurally different from recombinant human insulin. This structural difference may be related to the hypoglycemic action

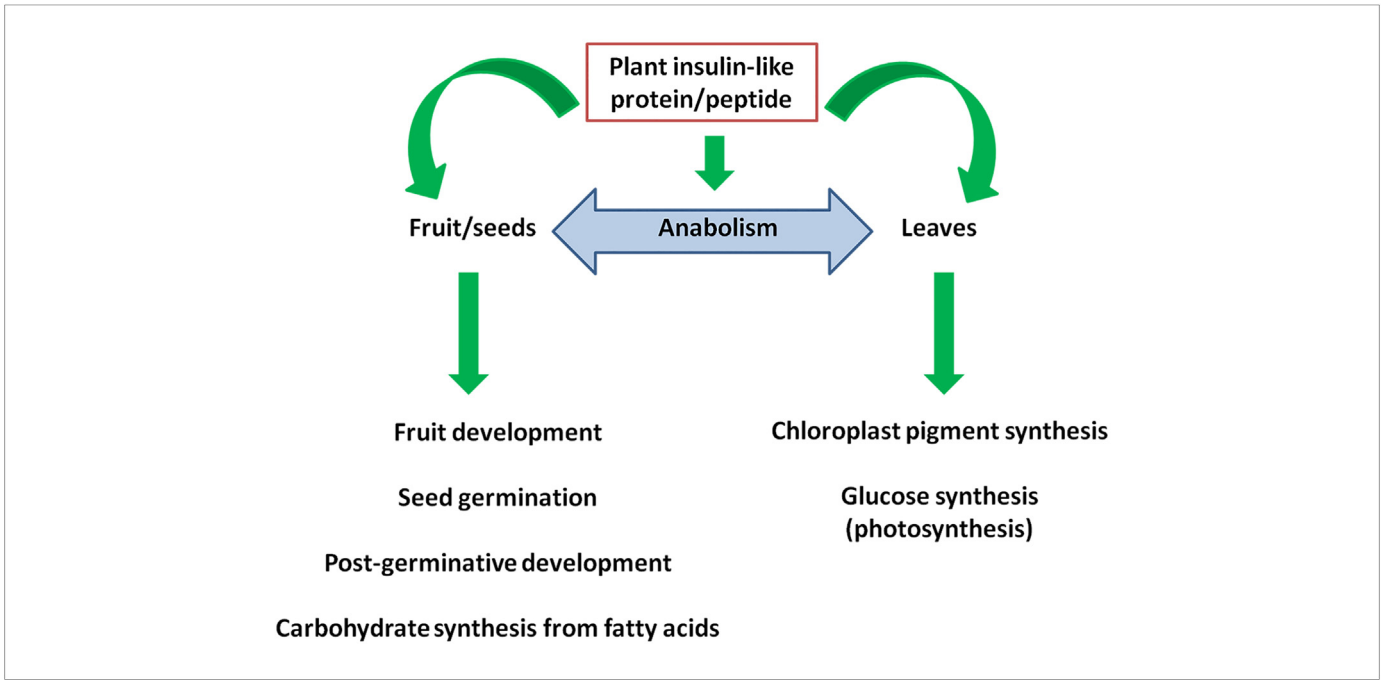

Fig. 2. Hypothetical roles of plant insulin-like proteins/peptides in the physiology of seeds, fruits and leaves.

of *C. igneus* insulin-like protein when orally administered. Alternatively orally administered proteins may naturally be complexed with other plant compounds, which could hinder proteolysis in the gut. For example, the insulin-like protein of the jack bean is purified complexed with galactorhamnan, preventing gut hydrolysis, thus maintaining the hypoglycemic effect after crossing the intestinal barrier [7].

Adding further evidence to the presence of insulin-like proteins in plants, Silva et al. [52] reported the presence of insulin-like antigens in the leaves of 42 plant species, distributed in the phyla Bryophyta (4 species), Psilophyta (1 species), Lycopodiophyta (1 species), Sphenopsida (1 species), Coniferophyta (2 species), Cycadophyta (1 species), Ginkgophyta (1 species) and Anthophyta (4 monocots and 27 dicots). This wide distribution of insulin-like proteins in the leaves of various plant species reveals that the preservation of those molecules across evolution may have helped determine the success of the plant kingdom as well as of other phyla of living things [26]. Regulation of carbohydrate metabolism is crucial to the correct and coordinated use and conservation of energy [53], which is why glycolysis in animals is closely regulated by allosteric factors such as ATP and AMP, and by hormones such as insulin [54,55]. This makes sense because individuals suffering from diabetes, regardless of whether it is because they do not secrete insulin or do not have adequate insulin signaling, develop seriously deregulated carbohydrate metabolism, accompanied by hyperglycemia and subsequent chronic complications [56]. Similarly, stunted coffee plants presented slight wilting and lower total soluble sugars in comparison to plants grown under optimal physiological photoperiods [57]. In this context, based on the hypothesis that insulin-like proteins in leaves might be associated with photosynthesis, which is a process used by plants and other organisms to convert and store light energy in the form of sugars, it is reasonable to hypothesize that insulin-like proteins have been conserved during plant evolution.

Insulin-like proteins isolated from seeds and fruits

In 1999, Oliveira et al. reported the presence of an insulin-like protein in the jack bean (*C. ensiformis*, Fabaceae family) seed coat [22]. The initial extraction of this insulin-like protein was from the flour obtained by grinding the jack bean seed coats using sodium phosphate buffer, pH 7.6. Considering that human insulin has an isoelectric point of approximately 5.4 [58], the authors predicted that the insulin-like protein of *C. ensiformis* would remain in the total extract with a negative net residual charge. The protein was adsorbed on a DEAE-cellulose matrix, which has positive groups and was eluted using a NaCl gradient. Four assays indicated that the protein in question was insulin-like: (1) the protein was similar to bovine insulin in size as determined by electrophoretic mobility in SDS-tricine-PAGE; (2) it reacted with anti-insulin antibodies in Western blot analysis; (3) it promoted significant reduction of glucose levels in alloxan-induced diabetic mice after intravenous injection; and (4) it has complete amino acid sequence similarity to bovine insulin. This study by Oliveira and colleagues [22] was the first to show the presence of an insulin-like protein in a seed coat.

Four years after the discovery of the presence of an insulin-like protein in jack bean coats, detection by Western blotting of an insulin-like protein in cowpea (*Vigna unguiculata*, also a Fabaceae family plant species), both in empty pods and seed coats, but not in the embryo, was reported by the same research group [23]. The highest concentrations of this plant insulin were measured by enzyme-linked immunosorbent assay (ELISA) using anti-human insulin antibodies during cowpea fruit development and were found to be 1.6 to 4.0 times higher 16 and 18 days after pollination, respectively, than on any other day. Thereafter, a sharp decline in insulin concentrations was observed starting 20 days after pollination (fruit opening phase). Based on these observations, they hypothesized that the higher concentration of the insulin-like protein during the initial development phase and hence in the period when the fruit is most metabolically active could be an

indication of the role of the insulin-like protein in this plant organ. Although not detected in the embryo, the presence of the insulin-like protein in the seed coat might indicate an important role in promoting accumulation of a caloric reserve in the cotyledons. Another possibility is that the protein was translocated to the seed coats. A similar phenomenon was noted in the case of a hydrophobic soy protein, synthesized in the endocarp and then deposited on the bean surface [59]. These findings show a possible role of the insulin-like antigen found in *V. unguiculata* and *C. ensiformis* in the development of the fruits and seeds of those leguminous plants.

Fruits and seeds of *M. charantia*, popularly known as bitter melon, bitter gourd or karela, have been used in many cultures to treat diabetes [60–62]. Several studies have confirmed their hypoglycemiant potential [63–65], which is associated with phenolic compounds, saponins, glycosides [60] and proteins [13,66,67]. Insulin-like molecules were extracted from decorticated *M. charantia* seeds with HCl-ethanol and fractioned [68] following a protocol developed to obtain insulin from insects and segmented worms [69]. Ethanol-acid extraction of *M. charantia* fruits and seeds and subsequent precipitation with acetone provided a mixture of proteins and peptides, named the *p*-fraction, that showed antilipolytic activity and stimulated the biosynthesis of lipids from carbon skeletons produced from glucose [70]. Such similarities of protocols to obtain insulin support the hypothesis that both the insulin structure and function were preserved during the course of biological evolution [26,71]. Ng et al. [38] also purified a galactose binding lectin (molecular weight of 124,000) from *M. charantia* seeds that displayed antilipolytic and lipogenic activities in isolated rat adipocytes.

In 2006, Yinchok-Anun et al. [21] obtained a protein fraction from *M. charantia* fruits following a protocol very similar to the one described above to obtain the *p*-fraction that also promoted increased glucose uptake by C_2C_{12} myocytes and 3T3-L1 adipocytes and that resulted in a significant reduction of plasma glucose levels in normal and streptozotocin-induced diabetic rats after subcutaneous administration. Taken together, these findings show that there are peptides/proteins from *M. charantia* fruits and seeds with insulin-like bioactivities, including the promotion of lipogenesis, the inhibition of lipolysis, the stimulation of glucose uptake by peripheral tissues that express GLUT-4 and, consequently, the reduction of glycemia [46].

Sheng et al. [72] purified a protein they called “plant insulin” from *M. charantia* seeds using molecular exclusion and reversed phase high-performance liquid chromatography. Yuan et al. [20] isolated a hypoglycemiant fraction (MC2) by ultrafiltration of the aqueous extract of *M. charantia* fruits followed by chromatography on Sephadex G-25 that was fractionated in six fractions, one of which (MC2-1) promoted a significant reduction of plasma glucose levels in alloxan-induced diabetic mice after oral administration. The main peptides in MC2-1 were those with molecular masses between 1.3 and 6.0 kDa. Subsequently, the same research group purified a hypoglycemiant peptide from MC2-1, named MC2-1-5, with a molecular mass of approximately 3.4 kDa [73]. MC2-1-5 also promoted a substantial reduction of glucose levels in alloxan-induced diabetic mice after oral administration.

Another cucurbitaceae plant classified in the genus *Momordica*, *M. cymbalaria*, has also been utilized as a medicinal herb for diabetes treatment [74]. The aqueous extract of *M. cymbalaria* fruits was shown to reduce the plasma glucose levels in alloxan-induced diabetic rats [75]. Moreover, a hypoglycemiant protein named M.Cy, with a molecular mass of 17 kDa and an isoelectric point of 5.0, reduced glycemia, but only when administered parenterally rather than orally [58,76].

The presence of insulin-like proteins in seeds appears to be physiologically important for seed development. For instance, bovine insulin accelerated the growth of *C. ensiformis* seedlings, and insulin-like antigens, receptor antigens and phosphoserine antigens were found in the seed coat of *C. ensiformis* [77]. Similar results were found by Goodman and Davis [78], who showed that insulin accelerated radicle emergence in cucumber, watermelon and sunflower, in addition to promoting an increase in acyl-CoA dehydrogenase, citrate synthase and malate dehydrogenase activities, as well as those of glyoxysomal enzymes such as isocitrate lyase and malate synthase, which are involved in converting fatty acids released from triacylglycerols into glucose. Gluconeogenesis from fatty acids seems to be crucial for seed development [79]. In a similar context, insulin is largely responsible for fetal growth in humans [80]. This set of findings supports the existence of insulin-like antigens and signaling pathways related to insulin in plants and their possible roles in promoting seed germination and development (Fig. 2), as occurs with insulin in human fetuses [81].

Moringa oleifera: a source of novel insulin-like proteins

The Moringa tree, *M. oleifera*, belongs to the family Moringaceae. It is a fast-growing perennial species native to northeastern India that has a small to medium stature and is adapted to a variety of climates [82]. Parts of this plant, such as the leaves, fruits, and seeds, have long been used in traditional medicine to treat diabetes, and ethnopharmacology studies have supported such use [82–85]. The hypoglycemic effect is quite often credited to plant secondary metabolites like phenolic compounds, terpenes and coumarins [84,86,87]. Although various types of secondary metabolites have shown hypoglycemic effect, few efforts have been made to determine whether other molecules possess this activity, especially proteins. Such lack of information and the findings of our research group on the presence of insulin-like epitopes in *M. oleifera* protein fractions led us to assess their potential hypoglycemic activity.

Indeed, insulin-like proteins were detected in the seed coat, fruits without seeds and leaves of *M. oleifera*. A protocol to obtain insulin-like proteins from plant tissues, involving acid-ethanol extraction followed by protein precipitation with acetone, was used on *M. oleifera* seed coats and cotyledons. The precipitated materials were recovered by centrifugation and subsequently exhaustively dialyzed against distilled water using a 2-kDa cutoff cellulose membrane, after which they were freeze-dried. Two protein fractions were obtained from the seed coat and cotyledon, named *Mo*-SC (*Mo*: *M. oleifera*; SC: Seed Coat) and *Mo*-COT (*Mo*: *M. oleifera*; COT: Cotyledon), respectively.

Induction of experimental diabetes in animals was performed by intraperitoneal administration of alloxan monohydrate, a pyrimidine that selectively destroys insulin-producing pancreatic β-cells. Male mice with a body weight between 30 and 50 g were fasted for 16 h, after which alloxan diluted in 0.15 M NaCl was administered at a dose of 150 mg/kg body weight [88]. Blood sampling by tail incision was taken to measure glucose levels 72 h later. Mice with blood glucose $\geq$ 300 mg/dL were considered diabetic and used in the experiments. The blood glucose levels were measured with the aid of a portable glucometer (Accu-Chek Active, Roche, USA). The intraperitoneal administration of *Mo*-SC (100 mg/kg body weight) to alloxan-induced diabetic mice promoted a significant reduction in plasma glucose levels 3 h after treatment (Fig. 3) and improved glucose tolerance when given orally (Oral Glucose Tolerance Test – OGTT), as indicated by the smaller areas under the curve (AUC) in comparison to the diabetic control group (Fig. 4). Later, it was observed that *Mo*-SC also exerted a hypoglycemic effect when administered orally (Fig. 5). The hypoglycemic effect promoted by oral administration was observed only 5 h later and, despite the significant difference

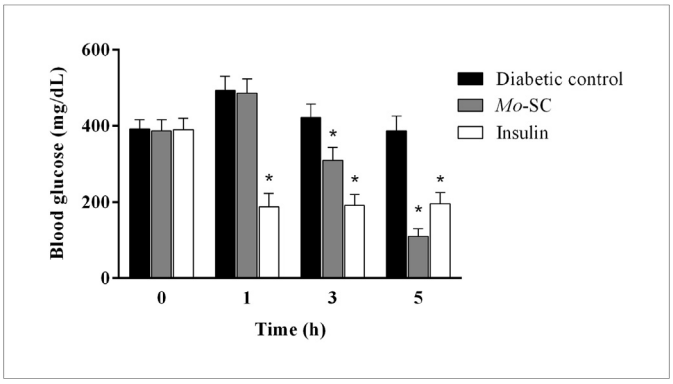

Fig. 3. Effect of intraperitoneal administration of a single dose of *Mo*-SC (100 mg/kg body weight) on the blood glucose levels of chemically induced diabetic mice (n = 12). Human recombinant insulin (0.7 IU/kg body weight) was used as a positive control. *Represents a significant difference ($P < 0.05$) compared with untreated chemically induced diabetic mice (control).

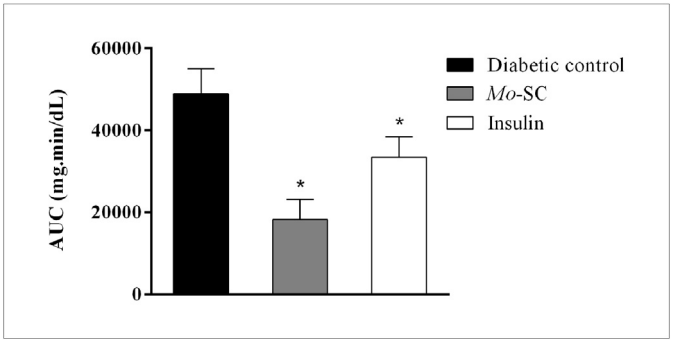

Fig. 4. Area under the glucose curve (AUC) obtained after the oral glucose tolerance test was performed on chemically induced diabetic mice (n = 12) treated with *Mo*-SC (100 mg/kg body weight) or human recombinant insulin (0.7 IU/kg body weight). *Represents a significant difference ($P < 0.05$) compared with untreated chemically induced diabetic mice (control).

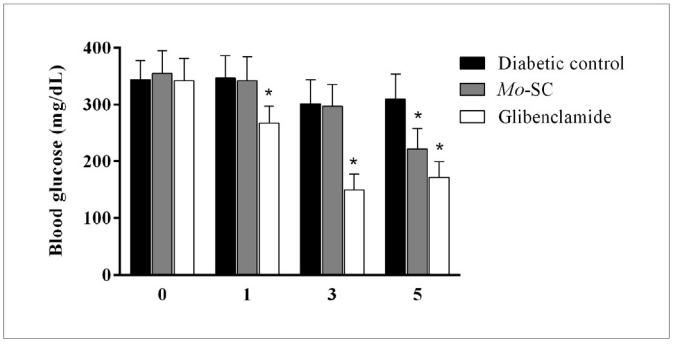

Fig. 5. Effect of the intragastric administration of a single dose of *Mo*-SC (100 mg/kg body weight) on the blood glucose levels of chemically induced diabetic mice (n = 12). Glibenclamide (50 mg/kg body weight) was used as a positive control. *Represents a significant difference ($P < 0.05$) compared with untreated chemically induced diabetic mice (control).

compared to control group, this reduction was less intense compared to the one elicited by intraperitoneal administration. Thus, the following hypotheses were raised in an attempted to explain the lower hypoglycemic effect observed by the oral route: (1) the peptides/proteins present in *Mo*-SC were possibly hydrolyzed; (2) the hypoglycemic components present in *Mo*-SC were not completely absorbed; (3) if absorbed, they might have been

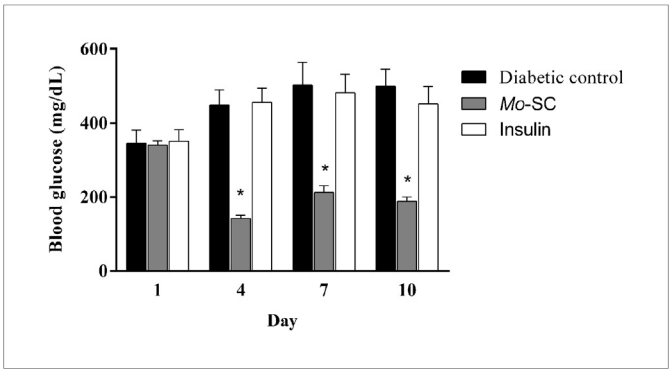

Fig. 6. Effect of the daily intraperitoneal administration of *Mo*-SC (100 mg/kg body weight) for ten consecutive days on the blood glucose levels of chemically induced diabetic mice (n = 12). Human recombinant insulin (0.7 IU/kg body weight) was used as a positive control. *Represents a significant difference ($P < 0.05$) compared with untreated chemically induced diabetic mice (control).

systemically metabolized and their concentrations were consequently reduced in the blood.

A repeated-dose test showed that *Mo*-SC had a slow and long-term glucose-lowering effect (Fig. 6). Animals were treated once a day for 10 consecutive days, using intraperitoneal administration of 0.15 M NaCl solution (diabetic control), human recombinant insulin (0.7 IU/kg body weight) or *Mo*-SC (100 mg/kg body weight). The blood glucose levels were measured in 3-day intervals, every 24 h after the last administration. The blood glucose levels in the group treated with *Mo*-SC were significantly lower for the duration of the experiment compared to those in the control group. However, after 24 h of insulin administration, the blood glucose levels of the insulin-treated animals did not differ from that of the diabetic control (saline-treated). Although this result was expected once insulin has a short-term effect because it is rapidly degraded [89], to rule out the possibility that the applied insulin was inactive the blood glucose level was also measured 5 h after its intraperitoneal administration at days 6 and 10. This trial showed that the insulin batch used lowered the blood glucose levels by around 80% in the diabetic mice indicating that the hormone was fully active. Plasma insulin has a rapid half-life (range 226–314 s) as previously observed in obese and lean Zucker rats [89]. On the other hand, some types of commercially available human recombinant insulin show slow and long lasting hypoglycemic action. This pharmacokinetic action, also observed for *Mo*-SC reduces the risks of hypoglycemia most likely due to slow turnover, which allows a long-term glucose lowering action. Moreover, the results presented by *Mo*-SC are consistent with those of a lectin from *U. pilulifera* that showed a hypoglycemic effect in streptozotocin-induced diabetic rats after 30 days of daily intraperitoneal administration [39]. The long-lasting hypoglycemic effect verified for some insulin-like plant proteins can be related, at least in part, to their structural stability. Plant proteins are usually less susceptible to hydrolysis than animal proteins [90]. Indeed, *Mo*-SC was poorly hydrolyzed by pepsin even 4 h after incubation, as demonstrated by SDS-PAGE analysis (data not shown). Conversely, peptide fragments were released upon partial digestion of insulin with pepsin [91]. The high structural stability of various proteins purified from *M. oleifera* seeds to enzyme hydrolysis might be associated to the presence of several cysteine residues in their structures, which can be involved in the formation of a higher number of disulfide bridges than insulin [92–94]. For instance, *Mo*-CBP_3, one of the proteins purified from *M. oleifera* seeds, has 4 disulfide bridges compared to 3 present in insulin. Therefore, take into account the role of the proteasomal enzymes in the general protein turnover [95], the higher resistance to proteolysis could

explain the prolonged hypoglycemic effect of *Mo*-SC. Another factor that could explain the shorter *in vivo* turnover of *Mo*-SC in mice is the involvement of the insulin-degrading enzyme (IDE), a thiol-sensitive zinc-metalloprotease that rapidly breaks down the B-chain of insulin [96], which is essential for insulin clearance. To the best of our knowledge, there is no information about the similarities between any insulin-like proteins purified with the B chain of human and other mammal insulins. Therefore, it is plausible also to speculate that *Mo*-SC is not a substrate for IDE, rendering this *M. oleifera* protein active for longer time as compared with insulin.

The electrophoretic profile of *Mo*-SC in SDS-tricine-PAGE revealed the presence of protein bands with a molecular weight near 5.6 kDa, similar to that of human insulin. In addition, dot-blot analysis, utilizing human anti-insulin antibodies, indicated that *Mo*-SC contains insulin-like proteins. On the other hand, *Mo*-COT did not cross-react with anti-insulin antibodies, neither presented a hypoglycemic effect in alloxan-induced diabetic mice (Alves BGT and Vasconcelos IM, unpublished data), suggesting the absence of insulin-like proteins. This corroborates the findings of Venâncio et al. [23], who observed the presence of insulin-like proteins in cowpea seed coats but not in cowpea embryos. Although *Mo*-SC used in our study had been exhaustively dialyzed to exclude compounds below 2 kDa molecular weight one could not discard the possibility of non-protein contaminants as polyphenols in the preparation used. Polyphenols could form complexes with proteins [97,98] and thus with *Mo*-SC, contributing with the long-term glucose-lowering effect of the *Mo*-SC preparation as long-lasting hypoglycemic effects have also been attributed to polyphenols [99]. Other non-protein components of *M. olefeira* also have antidiabetic actions [100–102].

A protein fraction from *M. oleifera* leaves also had a hypoglycemic effect in alloxan-induced diabetic mice, ameliorated polydipsia (excessive thirst, a symptom often associated to hyperglycemia), and cross-reacted with human anti-insulin antibodies as shown by dot-blot analysis. The fractionation of this protein preparation from *M. oleifera* leaves by sequential chromatography on DEAE-cellulose and Sephacryl S-200 produced a semi-purified fraction that reacted strongly with anti-insulin antibodies and had an apparent molecular weight of 6 kDa. Similarly, a protein preparation obtained from *M. oleifera* fruits without seeds also contained insulin-like antigens (Paula PC and Vasconcelos IM, unpublished data). Thus, our unpublished data reveal that *M. oleifera* should be included in the group of plants that process insulin-like proteins and, accordingly, has the potential to provide novel proteins to be developed as alternative drugs for the treatment of diabetes.

What is the importance of insulin-like proteins from plants in the context of medical biotechnology?

Biotechnology has contributed to the discovery, production and processing of hypoglycemic drugs. Currently, many compounds with different action mechanisms are available for oral treatment of individuals with type 2 diabetes [103]. There are also different types of recombinant human insulin produced by recombinant DNA technology, which differ mainly in relation to the start, duration and presence of hypoglycemic action peaks [104,105].

Given the existence of several oral hypoglycemic drugs on the market and of various insulin analogues produced by recombinant DNA technology, it could be argued that there is no advantage or justification for further prospecting insulin-like proteins in plants with the aim of possibly using them to treat diabetes. However, certain contexts do support research on hypoglycemic proteins in plants. For example, some oral hypoglycemiants cause side effects [106,107]. Recombinant human insulin itself causes hypoglycemia as an intrinsic side effect in individuals with type 1 diabetes submitted to intensive subcutaneous application of this drug [108]. This occurs because the insulin doses administered are not necessarily the same as the quantity of the hormone that would be released under certain circumstances (insulin sensitivity induced by physical exercise, dietary consumption, quantity and type of carbohydrate ingested, presence of pathologies and other factors). Therefore, a hypoglycemic event can arise when additional insulin is administered [109]. Thus, if an insulin-like protein identified lowers the glucose levels gradually, this is advantageous as it would minimize the risk of hypoglycemic episodes.

Biotechnological aspects related to the handling of insulin-like plant proteins have also been described. By determining the amino acid sequence of polypeptide-p [72], an insulin-like protein initially obtained from *M. charantia* seeds [12], Wang et al. [110] were able to clone the gene encoding this protein using reverse transcription polymerase chain reaction (RT-PCR) and other techniques. The recombinant protein, tagged with 6 histidine residues, was expressed in *Escherichia coli* BL21 (DE3), allowing purification after affinity chromatography on a nickel-nitrilotriacetic acid column. This recombinant polypeptide-p significantly reduced the plasma glucose levels of alloxan-induced diabetic mice, the same result attained by the recombinant human insulin novolin. In addition, a recombinant peptide (pQE8-MC) produced in *E. coli* after cloning and expression of the gene of an 18 amino acid hypoglycemic peptide from *M. charantia*, named MC6.1, that had been previously purified by Nag et al. [66], promoted a significant reduction of plasma glucose levels in alloxan-induced diabetic mice 4 h after intravenous administration of a 1 mg/kg dose, in comparison with the diabetic control group [111]. A multimeric peptide, named TrxA-$MC6_{10}$, was also produced and expressed in *E. coli* from 10 tandem repetitions of the gene that codes the hypoglycemic peptide MC6.1 that, when cleaved by HCl, promoted a significant decline in plasma glucose levels in alloxan-induced diabetic mice [112]. Expression of this gene in tandem repetitions improved the yield of the recombinant MC6.1, which had not been satisfactory in a previous study carried out by Liu et al. [111]. In addition to this, the hypoglycemic peptide MC6.2, derived from MC6.1 and composed of 11 amino acid residues, was chemically produced by a microwave peptide synthesizer and used in an assay in which it was intraperitoneally administered daily for 20 days to streptozotocin-induced diabetic mice [113]. This daily administration resulted in lower blood glucose levels measured every day after the treatment in comparison with the control group and also improved some symptoms related to hyperglycemia. Some analogues of the MC6.2 peptide, produced by substitution of the 1 and 8 amino acid residues by glycine or the 1, 3, 9, and 11 amino acid residues by methionine by solid-phase synthesis, also induced a hypoglycemic effect in streptozotocin-induced diabetic mice [114]. Additionally, a recent patent describes a method for obtaining a new hypoglycemic protein, ADMc1, purified from *M. charantia* seeds. ADMc1 and its recombinant version (rADMc1), produced in *Pichia pastoris*, proved to be able to normalize the glycemia in alloxan-induced diabetic rats [115].

Interestingly, hydrolyzed proteins obtained by submitting the *M. charantia* fruit extract to the hydrolytic action of alcalase promoted a hypoglycemic effect in alloxan-induced diabetic mice [73]. This result explains the observation that some peptides purified from *M. charantia* reduced the glucose levels after oral administration [66,73].

Obviously, it would not be feasible to employ a strategy of replacing recombinant human insulin with plant insulin in diabetic individuals, due to the importance of insulin for the preservation of various functions [116,117]. Nevertheless, individuals with type 2 diabetes who normally take oral hypoglycemiants but at some

moments require exogenous insulin doses to improve glucose control, given the progressive nature of this clinical type of diabetes [118], could benefit from using hypoglycemic proteins from plants because they promote a gradual reduction of glycemia [119]. In theory, this would reduce the risk of hypoglycemic events in these patients. Even individuals with type 1 diabetes might obtain some benefit from using these plant proteins: if a given hypoglycemic plant protein works to inhibit enzymes that digest carbohydrates, this might result in a lower daily need for recombinant human insulin, minimizing the risk of hypoglycemia.

In older adults with diabetes, whose glycemic targets are more flexible, for the purpose of avoiding more rigorous treatment and possibly inducing hypoglycemia (in the elderly this can lead to falls causing incapacitating fractures), therapy with insulin-like plant protein that do not suddenly reduce the glucose level would be a good alternative [120,121]. However, to the best of our knowledge, no pre-clinical studies and clinical trials have been carried out aimed specifically at evaluating the safety or the pharmacokinetic and pharmacodynamic action profile of these molecules. It should also be noted that because these plant insulin antigens come from species that are phylogenetically distant from humans, allergic reactions might occur. Cases of allergic reactions to bovine and porcine insulin, used initially to treat diabetics, were not uncommon [122]. This and other aspects should be investigated in an effort to produce safe drugs whose benefits outweigh the risks.

The method by which insulin-like plant proteins could be formulated for therapeutic use is uncertain, as the production and release of drugs involve technical aspects, such as industrial-scale production and clinical studies that go beyond the simple experimental procedures to obtain proteins. To produce adequate quantities of vegetative parts of *M. oleifera* with a hypoglycemic effect, *in vitro* plant tissue cultures may be preferable to avoid the use of great extensions of lands that could be used for food production. Alternatively, recombinant insulin-like proteins derived from *M. oleifera*, as well as from other plant species, could be produced in view of the advance of this biotechnological approach and its associated progressively low cost in comparison to field-grown plants. These proteins could be administered subcutaneously or even orally in the form of capsules and/or tablets, as previous trials have shown that they are at least partially resistant to gut proteolysis and maintain effectiveness in lowering glucose levels.

Conclusions

Few studies have been conducted on the insulin-like activity of plant proteins. Nevertheless, it is evident that these molecules have great potential as alternatives or complementary agents to treat diabetes, particularly taking into consideration the increased number of people affected by diabetes worldwide. In this context, *M. oleifera* represents a promising source of proteins/peptides with relevant *in vivo* insulin-mimetic effects, as first demonstrated by our research group, and serves as an example to encourage studies on several other plant species.

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