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IN VITRO SCREENING METHODS FOR ANTI DIABETIC ACTIVITY: A COMPREHENSIVE REVIEW

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Abstract: The clinical state known as diabetes is characterized by hyperglycaemia brought on by an absolute or relative insulin shortage. The incidence and prevalence of diabetes mellitus have increased significantly in recent decades. The pharmaceutical industry frequently uses *in vitro* techniques for large-scale production. Because they are more affordable and easier to develop, they are favoured over animal approaches. Reducing the amount of glucose produced and absorbed in the gastrointestinal tract by inhibiting enzymes that break down carbohydrates, such as α-amylase, α-glucosidase, and α-amylase inhibition, is one method of treating diabetes. The postprandial rise in blood glucose following a mixed carbohydrate diet can be considerably reduced by inhibiting the amylase and glucosidase enzymes involved in the digestion of carbohydrates. As a result, inhibition of these enzymes can be a crucial tactic for blood glucose control. We have covered several *in vitro* tests in this study, including the α-amylase, α-glucosidase, PTP-1B, insulin secretion, and glucose uptake assays. *In-vivo* methods are too costly for evaluating hits. To carry out the necessary tasks and achieve the corresponding goals, it is now essential to design and execute appropriate *in-vitro* screening techniques.

Keywords: Anti-diabetic, α-amylase, α-glucosidase, PTP-1B, insulin secretion assay, glucose uptake assay.

INTRODUCTION

Diabetes, a metabolic disease affecting protein, fat, and carbohydrates, affects a sizable portion of the global population [1]. Diabetes mellitus is a collection of metabolic disorders that are characterized by persistently high blood sugar levels. These disorders can be caused by abnormalities in insulin secretion, action, or both. The signs and symptoms of diabetes mellitus include increased urination, thirst, ketonemia and ketonuria. These symptoms are brought on by irregularities in the metabolism of fat, protein, and carbohydrates. Due to microvascular consequences (retinopathy, neuropathy, and nephropathy) and macrovascular problems (heart attack, stroke, and peripheral vascular disease), diabetes mellitus has significantly increased morbidity and mortality [2]. The primary causes of diabetes include age, urbanization, and the fast increase in unhealthy lifestyles. Following the ingestion of food and the onset of digestion, there is an increase in glucose levels and other hormones, including glucagon-like peptide (GLP-1), which is released in the intestines. As insulin acts to lower glucose levels, glucagon-like peptide 1 (GLP-1) is an incretin that functions by suppressing glucagon production, which raises glucose levels, and stimulating insulin production. This happens to offset the rise in glucose; it also lowers apatite and promotes satiety by informing the brain when a person is satisfied [3]. Additionally, eating causes the production of pancreatic hormones such as glucagon, insulin, and amylin. While glucagon acts on the liver to elevate glucose levels, insulin and amylin both function to lower glucose levels and block glucagon.

Types of Diabetes mellitus

Type 1

"Juvenile Diabetes Mellitus" (Insulin Dependent Diabetes Mellitus), and Type 2, or "Adult type" (Non-Insulin Dependent Diabetes Mellitus), are the two primary kinds of diabetes mellitus based on the aetiology.

Diabetes type 1 is a chronic illness that impairs the body's capacity to use glucose from food as fuel. Type 1 diabetes typically manifests early in life and is identified in childhood. The autoimmune system's attack on the pancreatic cells that make insulin, a hormone that aids in converting glucose into energy for the body's cells, is the initial stage of the disease. Insulin injections must be administered regularly for the survival of people with type 1 diabetes. Our scientists are pursuing research to reduce the amount of insulin injections required to maintain good glycaemic control and to prevent low blood glucose (hypoglycaemic) episodes. This research is based on over a century of experience in identifying and producing medicines for individuals with diabetes. A remedy is what we ultimately want. We are making progress.

Information regarding type 1 diabetes:

More than 1.2 meters: Type 1 diabetes affects children and teenagers worldwide. 5–10% of diabetics have type 1 diabetes. 50% or higher those with type 1 diabetes are younger than 15 years old. 36 million worldwide, patients use our diabetes treatment products [4].

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Type 2:

A complicated chronic illness known as type 2 diabetes is brought on by the body's inability to produce enough insulin or use it efficiently. Individuals with type 2 diabetes require medical intervention to maintain appropriate insulin and blood sugar levels. The pancreas secretes the hormone insulin, which regulates blood glucose levels. When there is insufficient insulin in the body, glucose from food cannot be absorbed. This results in an increase in blood glucose levels, which over time can harm blood vessels and lessen the blood's ability to carry oxygen and nutrients to the body's organs and nerves. Treatment for type 2 diabetics may be necessary to improve glucose metabolism and assist avoid long-term.

Diabetes Type 2 facts:

90% of people with type 2 diabetes accounts for all instances of diabetes worldwide. 483m+ Around the world, type 2 diabetes affects individuals. 2-4 times compared to someone without type 2 diabetes, more likely to experience a heart attack or stroke. Fifty percent of persons with type 2 diabetes are undiagnosed. [5]

Assay based on targets

The digestive enzyme amylase is responsible for converting starch into glucose, maltose, malt triose, and dextran. It plays a significant part in diabetes, cancer, and pancreatitis. The enzyme has several industrial and biotechnological uses and derived from three primary sources: plants, animals, and microbes. Human pancreatic and salivary enzymes are alpha amylases.

One metalloenzyme that works in the presence of calcium is alpha amylase. Out of all the digestive enzymes found in the human digestive system, this one is the most significant. The α-1,4 glycosidic bonds between starch, amylopectin, amylose, glycogen, and other maltodextrins are catalysed by it [6,7]. Alpha-amylases from plants, fungus, and bacteria are used in a variety of biotechnology sectors, including food, detergents, textiles, fermentation, and paper [8].

1. Alpha-amylase Inhibitor

According to the investigations, several molecules possess alpha-amylase inhibitory properties. Polysaccharides the alpha-amylase enzyme's work will change them into monosaccharides that the body can absorb. Therefore, by blocking the enzyme that facilitates the breakdown and subsequent blood absorption of monosaccharides, particularly glucose. Patients with diabetes can be avoided [9]. Therefore, it is an excellent therapeutic method or drug design target for the control of type 2 diabetes mellitus. They are often divided into two groups: Non proteinaceous inhibitors and proteinaceous inhibitors, sometimes known as peptide-based inhibitors.

- Amylases in humans

The human pancreas and salivary glands are the primary sites for the synthesis and release of the enzyme alpha-amylase. It regulates blood glucose levels and is essential to the metabolism of starch and glycogen [10].

- Amylase Inhibitory Test

Using the technique developed by Karthik *et al*., the inhibitory activity of alpha amylase in lichens at varying doses against the fungal diastase was ascertained. Phosphate buffer (pH 6.8) was used to prepare the enzyme (0.5%). In summary, 500 µl of various broccoli sprout extract concentrations and 500µl of amylase-containing 0.1M phosphate buffer (pH 6.8) were incubated for 10 minutes at 25°C. Each tube was preincubated, then 500 µl of a 1% starch solution in 0.1M phosphate buffer (pH 6.8) was added, and the tubes were incubated for an additional 10 minutes at 25°C. The addition of 1 millilitre of the dinitro salicylic acid reagent stopped the process. The identical procedure was used for the control, except buffer was used in place of the extract. After ten minutes in a water bath that was boiling, the test tubes were cooled [11]. After adding 10 millilitres of distilled water to each tube, the absorbance (A) was calculated at 540 nanometres. Using the following formula, the percentage (%) inhibition was determined:

% Inhibition= A540Control - A540 Extract / A540Control x 100

2. PTP-1B test

An important component of insulin cascade signalling is the colorimetric-based phosphoprotein tyrosine 1B (PTP1B) test [12]. Thus, PTP1B analysis generally entails dephosphorylating charged insulin receptors. Inactive glucose uptake is the result [13]. Insulin resistance can be minimized by PTP1B inhibitors. Semi-synthetic inhibitors have IC50 values of 1 mM to 12 µM, while natural inhibitors have IC50 values of 1.5 µM to 30 µM [14]. The popular P-nitrophenyl phosphate (pNPP) is the substrate typically used to measure the PTP1B enzyme's action. To conduct this assay, combine 130 µl and 40 µl of 4 µM pNPP of the provided buffer (20 µl of the test, Tris-HCl) chemical solution, DTT, EDTA, β-mercaptothion, and 20 µl of an enzyme (1 µg/ml) at 37°C [15, 16].

When pNPP is enzymatically dephosphorylated, the amount of p-nitrophenolate produced should be measured during the enzymatic reaction by utilizing a microplate reader spectrophotometer to calculate the absorbance at 405 nm. A good positive control for inhibition is ursolic acid [17]. One benefit of this approach is its sensitivity [18].

3. Assay for alpha-glucosidase

The brush edge of the small intestine contains alpha glucosidase. It can prevent postprandial hyperglycaemia and aid in the digestion of dietary carbs [19]. The alpha-glucosidase assay comprises a reaction mixture of glucose, 0.2 M sodium carbonate solution, and a 20 mM p-nitrophenol (PNP) in p-nitrophenyl-α-glucopyranoside (PNPG) solution. It is necessary to dissolve the various sample/extract amounts in a pH 6.3 potassium phosphate buffer containing 100 mM. Acarbose is used in place of the sample or extract in the positive control tube, while the control tube just includes DMSO, enzyme, and substrate. As a blank, acarbose and sample extract are combined in the absence of enzymes. For five to six minutes, the reaction mixture must be incubated at 30°C. After the incubation period is over, 2.0 ml of Na2CO3 solution is added to stop the reaction. The absorbance rate at 400 nm, measured with a spectrophotometer, is directly proportional to the enzyme's activity [20, 21].

Phenotypic assays:

1. Assay for glucose uptake

Various cell types primarily use glucose as an energy source, and glucose homeostasis depends on the control of glucose uptake by various tissues. Elevated blood glucose levels lead to problems in diabetes. Therefore, it is imperative to search for antidiabetic effects that result in increased uptake of glucose, thereby lowering blood glucose levels. Through the mechanism of increased glucose

absorption, this assay is particularly effective for evaluating the antidiabetic efficacy of drugs or crude extracts. Adipose tissue, muscle cells, and other tissues include the glucose transporter 4 (GLUT4), which oversees the insulin-dependent absorption of glucose within these cells. Numerous glucose uptake assay types are available, and they can be either radiolabelled or not [22, 23]. The discussion of these tests follows.

Researchers have employed the radio isotopic test in recent years because, in contrast to other assays, it does not require an extremely sensitive detector for detection. Utilizing glucose analogues that are radiolabelled to mimic the effects of glucose and provide a measurable quantity of glucose absorbed by the cells, this experiment can be performed. These analogues come in two varieties: phosphorylated and non-phosphorylated. The former group comprises 3-methylglucose (3MG) and 2-deoxy-D-glucose (2DG). The 2DG glucose uptake assay is based on the finding that both glucose and 2DG are subjected to similar cellular processes, wherein their phosphorylation is catalysed by the enzyme hexokinase [24]. The cell ferments 2DG concentrates to form 2-deoxy glucose 6-phosphate (DG6P) because it cannot convert to an analogue of fructose 6-phosphate. 3MG is a helpful technique for determining the early rate of glucose transfer without interference from the later stages of glucose metabolism. However, due to its rapid equilibration over the cell membrane, it requires a little incubation period. Due to the phosphorylation of 2DG, which results in the stable analogue DG6P building up within the cell, the 2DG test is preferred over the 3MG. In 3MG compared to 2DG, equilibration and radiolabeled isotope efflux will occur more quickly. Assays using radiolabelled analogues have an acceptable signal-to-noise ratio and a higher degree of selectivity compared to those using enzyme or fluorescence labelling. The expense and handling of radiolabelled substances are significant drawbacks [25].

6-[N-(7-nitrobemz-2-oxa-1,3-diazol-4-yl) amino] analogues are non-radiolabeled. Both 2-[N-(7-nitrobemz-2-oxa-1,3-diazol-4-yl) amino] and 2-deoxyglucose (6-NBDG)2-NBDG, or 2-deoxyglucose, [26]. The main component of this assay is glucose-6-phosphate dehydrogenase (G6PDH), which catalyses the reduction of nicotinamide adenine dinucleotide phosphate (NADPH) from NADP+ and the creation of from 2-deoxy glucose 6-phosphate (DG6P), 2-deoxy-6-phosphogluconate is produced. Diaphorase converts resazurin to resorufin while it converts NADPH to NADP+. The fluorophore that is left over after the reaction is measured, called resorufin, is theoretically equal to the amount of DG6P. This method works well for estimating chemicals that might have a role in controlling the absorption of glucose as well as for high-throughput screening [27]. The main benefits of this assay are that it takes less time than the radio isotopic assay and is inexpensive, repeatable, and nonradioactive. The past year has seen a decrease in the utilization of these analogues due to issues with their fluorescence measuring. But thanks to advancements in photomultiplier technology, researchers are now utilizing them once more. Fluorescence microscopy can be used to directly image cells, and the test can also be utilized suitable for high-throughput screening in a microplate format [28]. Restrictions include a reduced sensitivity and signal-to-noise ratio compared to radio isotopic tests [29].

2. Assay for insulin secretion

Since it doesn't move in the direction of a particular target, the insulin secretion assay is not target-based. The primary goal of antidiabetic medications is to enhance the secretion of insulin [30]. The main cause of type 2 diabetes is decreased insulin production. A variety of bioassays are available to determine the amount of insulin secreted. Primarily, two types of assays are utilized, which are ELISA-based and luciferase-based [31].

Since ELISA requires less time and is more accurate compared to alternative options immunoassay procedures (such as Western blot analysis and radioimmunoassay), it is the recommended method. ELISA detects secreted insulin by combining many antibodies. For optimal sensitivity, a monoclonal antibody with the main antibody needs to be an insulin-specific epitope that binds to insulin. The secondary antibody is a monoclonal or polyclonal antibody that binds to the primary antibody. The enzyme is typically coupled to a second antibody. Materials are changed by enzymes into fluorescent or bioluminescent products that can be identified with the right tools. The first step in an ELISA-based insulin secretion assay is to seed many pancreatic β-cells in a 96-well plate with a growth medium. Under the necessary circumstances, cells develop (e.g., 5% CO_2 at 37°C). This stage necessitates starving the cells, therefore after they once stuck to the plate, they ought to be washed with Compared to normal medium, Krebs-Ringer bicarbonate (KRB) buffer has a lower glucose concentration. As a starvation buffer, KRB buffer (pH 7.4) with d-glucose, bovine serum albumin, MgSO4, KH_2, PO_4, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and NaHCO3 is utilized [32]. Since the time of starvation is one of the most important parts of this assay, it should be optimized. Cells can die during longer starving periods, while shorter intervals will produce erratic outcomes. For this experiment, a starvation period of 60 to 90 minutes is typically utilized. After the fasting period is ended, the material or extract should be treated with a KRB buffer that contains more glucose than standard media.

To allow the treated cells to release insulin, place them in an incubator with CO_2. Insulin levels may drop to baseline if the incubation period is exceeded. To determine the precise amount of insulin released, the medium containing the released insulin is collected after this procedure. Considering this, the ELISA technique is applied in compliance with the manufacturer's guidelines. ELISA kits come in a range of varieties, such as fast Insulin ELISA, insulin in rats and mice, and insulin in humans. The availability of insulin, when choosing the appropriate kit, it is important to examine the method and the sensitivity of the kit [33].

CONCLUSION:

In conclusion, the *in vitro* screening of herbal extracts for anti-diabetic activity has revealed promising potential in addressing key aspects of diabetes management. Through rigorous experimentation, several extracts have demonstrated efficacy in enhancing glucose uptake, improving insulin sensitivity, and supporting pancreatic β-cell function. These findings highlight the therapeutic promise of herbal remedies as adjuncts or alternatives to conventional diabetic treatments. Further research is warranted to elucidate underlying mechanisms, optimize dosage, and assess safety for clinical application. By harnessing the pharmacological diversity of herbal extracts, we can advance towards more holistic and effective approaches in combating diabetes.

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