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DIFFERENT IN VITRO - TECHNIQUES FOR SCREENING ANTIDIABETIC ACTIVITY

Life Sciences

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ABSTRACT

Diabetes mellitus has become a major health problem worldwide. In 2017 ,425 million adults were diabetic globally and these figures are predicted to reach 629 millions by 2045. Ayurveda is one of the oldest medical systems, which consists of thousands of medical concepts, drugs and hypotheses. Unfortunately, due to lack of scientific validation in various concepts, Ayurveda is trailing behind. Hence, evidence based research is highly needed for global recognition and acceptance of Ayurveda. Reverse Pharmacology refers to reversing the routine clinic practice to the laboratory examination for the proper validation of a traditional medicinal systems. Reverse Pharmacology approach can help in reducing failure rates of clinical implications of the herbs or their formulations which are already described in Ayurveda. Human studies are ultimately necessary because the product is tested by the intended route in the eventual beneficiary of an effective treatment. But many times it is neither feasible nor ethical to conduct initial trials in humans due to lack of scientific evidence about the safety or efficacy of the drug. In vitro tests can play an important role in the evaluation of anti diabetic activity of medicinal plants as initial screening tools or as follow-up to human or animal studies. In this regard, a substantial number of in vitro techniques have been introduced till date for investigating the mechanism of anti diabetic effect. This article focusses on various In – vitro techniques used for screening of Anti diabetic activity.

KEYWORDS

Anti diabetic, Ayurveda, In vitro techniques, reverse pharmacology

INTRODUCTION:

Diabetes mellitus is defined as a chronic disorder characterized by elevated plasma glucose concentration resulting from insufficient amount of insulin and insulin resistance, or both along with disturbance in carbohydrate, fat and protein metabolism which finally results in hyperglycemia. Diabetes mellitus has become a major health problem worldwide. In 2017,425 million adults were diabetic globally and these figures are predicted to reach 629 millions by 2045[1]. In India, diabetes is quickly becoming an epidemic with an additional 62 million recently diagnosed with the disease. [2] There are many synthetic drugs available for the management of diabetes but these drugs are often found to be associated with side effects , also the chronic nature of the disease and the associated complications require regular treatment and incur huge financial expenditure on the family and the healthcare system. This has influenced researchers to focus towards natural products and alternative medicines that are effective, inexpensive, affordable and safe to consume. Ayurveda , traditional system of medicines is known to humans since time immemorial . Ayurveda is one of the oldest medical systems, which comprises thousands of medical concepts, drugs and hypotheses. Interestingly, Ayurveda has ability to treat many chronic diseases such as cancer, Diabetes, arthritis, and asthma which are untreatable in modern medicine. Unfortunately, due to lack of scientific validation in various concepts, Ayurveda is trailing behind. Hence, evidence based research is need of time for global recognition and acceptance of Ayurveda. Reverse Pharmacology refers to reversing the routine clinic practice to the laboratory examination for the proper validation of a traditional medicinal systems. Reverse Pharmacology approach can help in reducing failure rates of clinical implications of the herbs or their formulations which are already described in Ayurveda [3,4]. The main aim of this study is to present all the available in vitro techniques for carrying diabetes research which can prove helpful in studying various Ayurvedic drugs.

In Vitro Techniques

Pancreatic a-amylase is a key enzyme in the digestive system and catalyses the initial step in hydrolysis of starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose, and a number of a-(l-6) and a-(1 - 4) oligoglucans. These are then acted on by a-glucosidases and further degraded to glucose which on absorption enters the blood-stream. Degradation of this dietary starch proceeds rapidly and leads to elevated PPHG (post-prandial hyperglycemia). It has been shown that activity of HPA (human pancreatic a-amylase) in the small intestine correlates to an increase in post-prandial glucose levels, the control of which is therefore an important aspect in treatment of type 2 diabetes. Hence, retardation of starch digestion by inhibition of enzymes such as a-amylase plays a key role in the control of diabetes. Inhibitors of pancreatic a-amylase delay carbohydrate digestion causing a reduction in the rate of glucose absorption and lowering the post-prandial serum glucose levels. Some inhibitors currently in clinical use are acarbose and miglitol which inhibit glycosidases such as a-glucosidase and amylase while others such as voglibose inhibit a glucosidase. However, many of these synthetic hypoglycemic agents have their limitations, are non-specific and produce serious side effects. The main side effects of these inhibitors are gastrointestinal viz., bloating, abdominal discomfort, diarrhoea and flatulence. Herbal medicines are gaining more attention in the treatment of diabetes as they are free from side effects and cost effective when compared to synthetic hypoglycemic agents [5]

The principle of conducting this analysis is same but different chemicals are used for this. Here are some examples which are quoted from different studies carried out :

In α Vitro Amylase Inhibition :

1. It can be studied by adding the test sample which is allowed to react with α- amylase enzyme and Incubated, add starch solution. After incubation dinitrosalicylic acid reagent was added to both control and test. Keep this mixture in boiling water bath for few minutes. The absorbance was taken at 540 nm using spectrophotometer and the percentage of inhibition of α-amylase enzyme was calculated . A starch solution was prepared with potato in sodium phosphate buffer, sodium chloride and kept in a boiling water batch for few min. The α-amylase solution was prepared by mixing α-amylase in the same buffer. The colorimetric reagent was prepared by mixing equal volume of sodium potassium tartrate tetra hydrate solution and 3,5-dinitro salicylic acid (DNS) solution. Starch solution was mixed with test sample with various concentration or acarbose and α-amylase solution was added and incubated at 25°C to react with the starch solution. DNS reagent was added to the above solution, and the contents were heated for 15

min on a boiling water bath. The final volume was made up with distilled water, and the absorbance was measured at 540 nm using spectrophotometer. The percentage inhibition and IC50 value was calculated[6]

2. α-amylase inhibitory activity was studied using enzyme-starch system. Sample (1%) was mixed by stirring with 25 mL of potato starch (4%) in a beaker; 100 mg of α-amylase was added to the starch solution, stirred vigorously and incubated at 37 °C for 60 min. After the incubation period 0.1 M NaOH (2 mL)was added to terminate enzyme activity. The mixture was centrifuged for 15 min (3,000 × g) and glucose content was determined in the supernatant.[7]

3. α- Amylase Inhibition method – (Nickavar and Yousefiana, 2009).
1ml of substrate-potato starch (1% w/v), 1ml of drug solution (GLINIL drug/ethanol extract of both plant leaf and stem) of 4 different concentrations such as 250, 500, 750 and 1000µg/ml. 1ml of α-amylase enzyme (1% w/v) and 2ml of acetate buffer (0.1 M, 7.2pH) was added. The mixture was incubated for 1hr.then 0.1 ml iodine-iodide indicator (635mg iodine and 1gm potassium iodide in 250ml distilled water) was added in the mixture. Absorbance was taken at 565nm in UV-Visible spectroscopy. The percentage increase in glucose uptake by yeast cells and % of α amylase inhibition are calculated .

4. α-amylase inhibition activity was measured according to Akoro et al. (2017) with slight modifications using a microplate reader (BioTek instruments, USA) [22]. 15µl PBS (pH6.8) was added in all wells of a 96-well plate. 25µl of 0.14U/ml enzyme and 1-4mg/ml samples were pre-incubated at 37°C for 10min. The reaction was started with the addition of 40µl starch (2mg/ml) and incubated for 30min at 37°C. 20µl 1M HCL was added to stop the reaction, and 90µl iodine reagent was added. The change in color was observed, and absorbance was measured at 620nm after incubation. The control contained all the reagents except inhibitor/sample. Acarbose was used as the standard or positive control. The IC50 values were derived from the percentage inhibition plot. IC50 defines as the concentration at which inhibitor shows 50% of its inhibition activity.[9]

Inhibition Of α-glucosidase Activity

1.The α-glucosidase enzyme inhibition activity was performed by incubating α-glucosidase enzyme solution with phosphate buffer which contains test samples of different connections at 37°C for 1 hr in maltose solution. The reaction mixture was kept in boiling water few min and cooled. Glucose reagent was added and its absorbance was measured at 540 nm to estimate the amount of liberated glucose from maltose by the action of α-glucosidase enzyme. The percentage of inhibition and IC50 was calculated [10]

2. α-glucosidase inhibitory activity α-glucosidase inhibitory activity was studied according to the method of Honda and Hara (1993), wherein crude enzyme solution containing α-glucosidase and sucrase prepared from the rat small intestine was used (Ahmed et al. 2009). The enzyme solution (10 µL) and 50 µL of sample emulsion (10 mg mL -1) were incubated together with 200 µL maleate buffer (pH 6.0) for 10 min at 37 °C. The enzyme reaction was started by adding 200 µL of p-nitrophenylα-D-glucopyranoside solution (2 mM) and incubated at 37 °C. After 30 min, the reaction was terminated by treating the mixture in a boiling water bath for 5 min and after the addition of 1.0 mL of disodium hydrogen phosphate solution (0.1 M), the absorption of the liberated p-nitrophenol was read at 400 nm. An untreated enzyme solution was used as the control.[11]

3.The α-glucosidase inhibitory activity was determined by measuring the release of 4-nitrophenol from p-nitrophenyl α-D glucopyranoside. The assay mixtures for these experiments contained 0.3 ml of 10 mM p-nitrophenyl α-D-glucopyranoside, 1.0 ml of potassium phosphate (0.1M, pH: 6.8), 0.2 ml of enzyme solution and 0.2 ml of inhibitor Meshashringi and Acarbose at different concentration (20-100 µg/ml), separately, all in a final volume of 1.7 ml. Following an incubation time of 30 min at 37º C, the reaction was terminated by the addition of 2.0 ml of 100 mM sodium carbonate. The liberated p-nitrophenol was determined at 400 nm using spectrophotometer[12]

4. The inhibitory activity was determined by incubating a solution of starch substrate (2% w/v maltose or sucrose) 1 ml with 0.2 M Tris buffer pH 8.0 and various concentration of plant extract for 5 min at 37 °C. The reaction was initiated by adding 1 ml of alpha-glucosidase enzyme (1U/ml) to it followed by incubation for 40 min at 35 °C. Then the reaction was terminated by the addition of 2 ml of 6N HCl. Then the intensity of the colour was measured at 540 nm. [13]

III. Glucose Uptake Assay by Yeast Cell.

1% of yeast suspension was prepared by dissolving commercial baker's yeast in distilled water and was incubated overnight at room temperature. The yeast suspension was centrifuged at 4200rpm for 5min on the next day. The pallet was washed with distilled water repeatedly till the clear supernatant was attained. 10% w/v of the yeast cell suspension was prepared by using a clear supernatant. Samples (mixture, granules, and metformin) of different concentrations were prepared in DMSO and incubated with 1ml of 5mM glucose for 10min at 37°C. 100µl yeast suspension was added in the reaction mixture to start the reaction. After 60min of incubation at 37°C, centrifugation was done at 3800 rpm for 5min and the amount of glucose in the supernatant was measured at 520nm by UV-Vis spectrophotometer (JASCOV-530, JAPAN). The effective concentration (EC50) was obtained from the percentage activity curve. The percentage increase in glucose uptake was calculated [14]

Glucose Uptake Assay Using 3T3-L1 Cell Lines

3T3-L1 cells were cultured in 6-well plates at an initial density of 2*105 cells/2 mL overnight. Cells were then washed with Dulbecco's phosphate-buffered saline (D-PBS) and treated with the extract (100 µg/mL) or the control - metformin (100 µM). The extract or control were prepared in glucose-free DMEM-containing 100 µM of 2-NBDG. After the treatment for 2 h, the cells were washed with D-PBS, trypsinized, and pellet was re-suspended in 0.5–1 mL of D-PBS. Cellular uptake of 2-NBDG was measured using flow cytometry[15]

Studies Using Isolated Pancreatic Islet Cell Lines:

These pathways can be studied with isolated pancreatic cells from experimental animals that can be obtained by collagenase digestion technique, followed by adequate separation and transference to appropriated culture medium . It is known that insulin secretion occurs when pancreatic cells utilize glucose to generate adenosine triphosphate (ATP) from adenosine diphosphate (ADP). The resulting increase in cytoplasmic ATP/ADP ratio closes ATP-sensitive potassium channels, causing depolarization of the plasma membrane, which activates voltage dependent Ca2+ channels. This results in elevation of the intracellular Ca2+ concentration which triggers insulin secretion [16]

DISCUSSION

Diabetes mellitus is a metabolic disorder and the key factor for its control is insulin. Lack of insulin in the body of an organism affects carbohydrate, fat and protein metabolism .The control over diabetes mellitus without side effects is a major challenge to medical community. Synthetic inhibitor causes side effect such as abdominal pain, diarrhoea and soft faeces in the colon. The inhibition of alpha-amylase and alpha-glucosidase would slow down the degradation of carbohydrate, which causes a decrease in the absorption of glucose; as a result the elevation of postprandial blood glucose level gets reduced[17]. The mechanism of glucose transport across the yeast cell membrane has been used as in vitro screening method for hypoglycemic effect of various compounds/ medicinal plants in different studies. Cellular uptake of glucose from blood is crucial in reducing Diabetes Mellitus. This is generally mediated by GLUT4 in the cell. Glucose transport by insulin-stimulated by the PKB/Akt pathway has been reported in rat adipocytes and L6 muscle cells.[18].Cell line studies on these cells are carried out for glucose uptake assay and also for evaluation of cytotoxicity of that particular drug. Many herbal extracts have been reported for their anti-diabetic activities and are currently being used in Ayurveda for the treatment of diabetes. However, such medicinal plants have not gained much importance as proper medicines due to lack of scientific evidence. This review has presented different In vitro techniques used in studies for screening anti diabetic activity of Herbal drugs. These techniques are used most commonly and extensively and can be conducted in laboratory settings.

CONCLUSION:

Above mentioned techniques can be used for screening of antidiabetic activity of herbal drugs . The animal models and in vitro techniques are essential for developing a new drug for the treatment of diabetes. More animal models, software, advanced techniques and cell line studies

have to be developed for advances in diabetes research.

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