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AN E-MANUAL ON

IN-VITRO ANTI-DIABETIC ASSAY FOR PLANTS AND PLANT-BASED PRODUCTS

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INTRODUCTION

Diabetes mellitus (DM) is a severe, persistent, and complicated metabolic condition with numerous etiologies and severe short- and long-term effects (Soumya and Srilatha, 2011). Type 2 diabetes, characterized by hyperglycemia and abnormal carbohydrate metabolism, is a leading cause of morbidity and mortality worldwide and a major economic burden (Upadhyay et al., 2018). People with diabetes and its complications live in both industrialized and developing nations, creating a significant socio-economic challenge globally.

According to the IDF Diabetes Atlas 2021, Approximately 537 million adults (20-79 years) are living with diabetes in 2021. The total number of people living with diabetes is projected to rise to 643 million by 2030 and 783 million by 2045. Every 3 in 4 adults with diabetes live in low- and middle-income countries. Moreover, almost 1 in 2 (240 million) adults living with diabetes are undiagnosed. Not only the adults, but more than 1.2 million children and adolescents (0-19 years) are also living with type 1 diabetes. Every 1 in 6 live births (21 million) are affected by diabetes during pregnancy. IDF aso reported that about 541 million adults are at increased risk of developing type 2 diabetes. By the year 2035, nearly 592 million people are predicted to die of diabetes in the world (Tao et al., 2015). India is referred to as diabetes capital in the world. Pradeepa and Mohan (2021) reported that 77 million individuals had diabetes in India during 2019, which is expected to rise to over 134 million by 2045. Approximately 57% of these individuals remain undiagnosed.

Although synthetic oral hypoglycemic medications and insulin are the primary methods for managing diabetes, they do not completely halt the progression of its complications and actually make it worse because they also exhibit noticeable side effects. This is the driving factor behind the search for alternative sources of antidiabetic agent (Rao et al., 2010). The results of treating diabetic patients are still far from perfect, despite the major advancements made in the last three decades in the treatment of diabetes with oral antidiabetic drugs. The use of those oral hypoglycemic medicines has been linked to a number of drawbacks, including drug resistance, adverse side effects, and even toxicity (Salehi et al., 2019).

The primary source for finding prospective lead candidates in the development of new drugs is natural compounds, particularly those of plant origin (Sharifi-Rad et al., 2011; Sharifi-Rad et al., 2018; Salehi et al., 2018). Plant-based remedies are the mainstay of all accessible treatments, especially in rural regions, due to their ease of accessibility, low cost, and least adverse effects (Arya et al., 2011). Despite the availability of antidiabetic medications on the market, numerous plants have long been revered as a primary source of effective anti-diabetic medications. For the treatment of diabetes, herbal remedies and

phytoceuticals with low toxicity and no adverse effects are well-known therapeutic choices worldwide (Gupta and De, 2012). Diabetes is treated with medicinal plants, especially in developing nations where the cost of conventional medications is a major load on the populace (Arumugam et al., 2013). Today, the use of plant-based medicines for treating diabetes is widely recommended (Kooti et al., 2015), as these plants include a variety of phytoconstituents such flavonoids, terpen oids, saponins, carotenoids, alkaloids, and glycosides that may have antidiabetic properties (Afrisham et al., 2015). The combined action of biologically active substances (i.e., polyphenols, carotenoids, lignans, coumarins, glucosinolates, etc.) leads to the potentially advantageous properties of each plant matrix, as noted by Durazzo et al. (2018). Herbal remedies that maintain low blood sugar, lower blood pressure, increase the antioxidant defense mechanism and regulate insulin in our body offer a safer alternative for managing type 2 diabetes (Patel et al., 2012).

In our body, the carbohydrates digestion is aided by two enzymes α-amylase and α-glucosidase. Long chain carbohydrates are broken down by α-amylase, while starch and disaccharides are converted to glucose by α-glucosidase. Inhibiting α-amylase and α-glucosidase enzyme is one of the therapeutic strategies for treating postprandial hyperglycemia (Bhandari et al., 2007). Synthetic medications like acarbose and miglitol have potent inhibitory effects on α-amylase and α-glucosidase, but they can also cause many adverse side effects (Rupasinghe et al., 2016). Numerous research and reviews have revealed that phytochemicals such phenolics have inhibitory effects on α-amylase and α-glucosidase enzyme as well as the ability to treat issues associated with diabetes and obesity (Ademiluyi and Oboh, 2013; Ibrahim et al., 2014; Striegel et al., 2015; Buchholz and Melzig, 2016 and Rupasinghe et al., 2017).

In India, plants have been an excellent source of medicine since ancient times. The use of plants in the treatment of many human ailments is mentioned in Ayurveda and other Indian literature. There are over 45000 plant species reported from India, and thousands of them are said to have therapeutic properties. However, in addition to such ethnobotanical information, the scientific validation of ethnopharmacological claims is also important. Recent studies on plants used traditionally to treat diabetes or described in ancient literature have revealed anti-diabetic properties (Grover et al., 2002).

As the α-amylase and α-glucosidase inhibitors are the potential targets in the development of plant-derived anti-diabetic medicines, α-amylase and α-glucosidase inhibitory assay are two primary in-vitro bioassays mostly followed for evaluation anti-diabetic activity in plants and plant-based products. These two assays have been validated at ICAR-NEH Quality Analysis Laboratory using a good number of plant samples and standards. In this e-manual, the step-by-step procedure for these two validated *in-vitro* assays has been described in simple and easily understandable language.

α-AMYLASE ENZYME INHIBITORY ASSAY

Introduction

The assay was performed based on the principle where α-amylase degrades starch (by randomly hydrolysing all the α-1→4 glucosidic bonds) into disaccharide maltose, trisaccharide maltotriose and oligosaccharides known as dextrins. Maltose reduces 3, 5-dinitrosalicylic acid (DNSA) to form a brown coloured product having λ_{max} 540 nm. In presence of inhibitor the activity of alpha-amylase enzyme will be inhibited and there will be no coloured product formation.

Equipment and Materials required

- Spectrophotometer with absorbance wavelength of 540 nm.
- Cuvette 1 cm path length
- pH meter
- 15 mL test tube
- 15 mL tube rack
- Cyclomixture
- Water bath/Incubator
- 100mL volumetric flask
- 10 ml pipette
- Measuring cylinder (100, 250 and 500 mL)
- Beaker (250, 500 and 1000mL)
- Micropipette (10, 20 100, 200 and 1000µL)
- Pipette tips (10, 200 and 1000µL)
- Tissue paper, weighing paper, spatula
- Graph paper, pen, pencil, eraser, scale etc.

Chemicals and Reagents required

- Test sample (plant extract)
- 0.2M $NaPO_4$ buffer pH 6.9 containing 67mM sodium chloride (Stock buffer solution)
- 0.02M $NaPO_4$ buffer pH 6.9 containing 6.7mM sodium chloride (Working buffer solution)
- 1% soluble starch solution

- 60% Sodium potassium tartarate tetrahydrate in 0.8M NaOH.
- 96 mM 3,5- dinitrosalicylic acid (DNSA)
- **Colour reagent**: Warm 100mL of 60 % sodium potassium tartarate tetrahydrate in 0.8M NaOH in a beaker then add 100mL 96mM DNSA with stirring till completely dissolved.
- **α-Amylase**: Dissolve 50mg α-Amylase enzyme (porcine pancreas) in a 100mL ice cold 0.02M $NaPO_4$ buffer pH 6.9 containing 6.7mM sodium chloride.
- **Acarbose:** Dissolve 50mg acarbose in 50ml working buffer (stock). Dilute 1ml stock acarbose into 40ml with buffer (working).

Sample Preparation

- Weigh 10g sample (shade dried powder or fresh if any) in a conical flask.
- Add 50mL methanol (1:10; w/v) kept shaking for 24 hrs.
- Transfer into 50mL tubes (2 tubes) and centrifuge @10000rpm for 10 minutes.
- Filter the supernatant in Whatman No. 1 filter paper and collect the supernatant into a clean beaker.
- Repeat the extraction of the residue for 24 hrs and 48 hrs. and pull all the supernatant in the beaker.
- Concentrate the sample in a rotary evaporator at 45^0C or oven dried at 50^0C.
- Kept the extract in a clean storage bottle at -20°C
- Dissolve the sample (10mg/ml, w/v) in 0.02M phosphate buffer pH 6.9

Assay Procedure

- Pipette out 0.5mL sample (3 to 5 different concentrations) that was dissolved in the working buffer in a series of test tubes.
- Pipette out 0.05,0.1,0.2,0.3,0.4 and 0.5ml acarbose in a series of tubes.
- Make up all the tubes to 0.5ml with working buffer. For the control reaction, add 0.5mL working buffer instead of the sample.
- Add 0.5mL enzyme solution for test sample whereas for the reagent blank, add 0.5mL buffer or heat killed enzyme then mixed properly.
- Incubate the reaction mixture for 20 minutes at 37°C.
- Add 0.5 mL 1% starch solution in it.
- Incubate the reaction mixture exactly for 3 minutes at 37°C.

- After 3 minutes, add 1mL DNSA colour reagent, mixed properly and capped it with cotton plug.
- Boiled for 15 minutes in a water bath then
- Cooled in ice and room temperature for 3 minutes.
- Add 7mL distilled water in all the tubes.
- Read the absorbance at 540 nm in a spectrophotometer.
- Prepare the following datasheet.

S.N.	sample (mL)	Buffer	1% starch (mL)	Enzyme	Incubation for 3 min.	Colour reagent (mL)	Boiling water bath for 15 min followed by 3 min cooling	Water (mL)	Absorbance at 540nm			
									R1	R2	R3	Mean
1.	0(Blank)	0.5	0.5	0.5(Heat killed)		1.0		7.0				
2.	0(Control)	0.5	0.5	0.5		1.0		7.0				
3.	0.1	0.4	0.5	0.5		1.0		7.0				
4.	0.2	0.3	0.5	0.5		1.0		7.0				
5.	0.3	0.2	0.5	0.5		1.0		7.0				

S.N.	Acarbose (mL)	Buffer	1% starch (mL)	Enzyme	Incubation for 3 min.	Colour reagent (mL)	Boiling water bath for 15 min followed by 3 min cooling	Water (mL)	Absorbance at 540nm			
									R1	R2	R3	Mean
6.	0.05	0.45	0.5	0.5		1.0		7.0				
7.	0.1	0.4	0.5	0.5		1.0		7.0				
8	0.2	0.3	0.5	0.5		1.0		7.0				
9	0.3	0.2	0.5	0.5		1.0		7.0				

Calculation

Calculate the % inhibition from the following equation.

$$\% \text{ Inhibition} = \frac{(A\ control - A sample)}{A\ control} \text{X } 100$$

Tips

- Enzyme and starch solution should be prepared freshly.
- Slightly warm the starch solution for better dissolution but do not boil, keep shaking at 37°C till used.
- Keep the enzyme solution on ice till used.
- If the sample is coloured, each concentration of the sample should provide the sample blank. For this, add 0.5 ml sample instead of the buffer only after addition of DNSA reagent (for blank sample). It will minimize the background colour given by the coloured sample.

α-GLUCOSIDASE ENZYME INHIBITORY ASSAY

Introduction

The assay was performed based on the principle where α-glucosidase hydrolyzed 4-nitrophenyl-α-D-glucopyranoside into 4-nitrophenol and α-D-glucopyranoside. The 4-nitrophenol (yellow colour) release can be measured at 405nm. In presence of inhibitor the activity of α-glucosidase enzyme will be inhibited and there will be no coloured product formation.

Equipment and Materials required

- Spectrophotometer with absorbance wavelength of 540 nm.
- Cuvette 1 cm path length
- pH meter
- 15 mL test tube
- 15 mL tube rack
- Cyclomixture
- Water bath/Incubator
- 100mL volumetric flask
- 10 ml pipette
- Measuring cylinder (100, 250 and 500 mL)
- Beaker (250, 500 and 1000mL)
- Micropipette (10, 20 100, 200 and 1000µL)
- Pipette tips (10, 200 and 1000µL)
- Tissue paper, weighing paper, spatula
- Graph paper, pen, pencil, eraser, scale etc.

Chemicals and Reagents required

- Test sample (plant extract)
- 0.2M $NaPO_4$ buffer pH 6.9 (Stock buffer solution)
- 0.05M $NaPO_4$ buffer pH 6.9 (Working buffer solution)
- 5mM 4-nitrophenyl-α-D-glucopyranoside solution(substrate): Dissolve 150.6mg 4-nitrophenyl-α-D-glucopyranoside in a 100mL ice-cold 0.05M $NaPO_4$ buffer.

- **α-glucosidase:** Prepared the stock solution as 100U/ml in ice-cold buffer (0.05M NaPO4 buffer pH 6.9). For working, immediately dissolved the enzyme in ice cold to make it 0.5U/ml.
- **Acarbose:** Dissolved 40mg acarbose in 10 ml buffer.

Sample Preparation

- Weigh 10g sample (shade dried powder or fresh if any) in a conical flask.
- Add 50mL methanol (1:10; w/v) kept shaking for 24 hrs.
- Transfer into 50mL tubes (2 tubes) and centrifuge @10000rpm for 10 minutes.
- Filter the supernatant in Whatman No. 1 filter paper and collect the supernatant into a clean beaker.
- Repeat the extraction of the residue for 24 hrs and 48 hrs. and pull all the supernatant in the beaker.
- Concentrate the sample in a rotary evaporator at 45^0C or oven dried at 50^0C.
- Kept the extract in a clean storage bottle at -20°C
- Dissolve the sample (10mg/ml, w/v) in 0.02M phosphate buffer pH 6.9

Assay Procedure

- Pipette out 0.3 mL sample (3 to 5 different concentrations) that was dissolved in the working buffer in a series of test tubes.
- Pipette out 0.05,1.0,0.2.and 0.3 ml acarbose in a series of tubes.
- For the control reaction, add 0.3mL working buffer instead of the sample.
- Add 0.6 mL enzyme solution for the test sample whereas for the reagent blank, add 0.6 mL buffer or heat killed enzyme then mixed properly.
- Incubate the reaction mixture for 20 minutes at 37°C.
- Add 0.3 mL 5mM 4-nitrophenyl-α-D-glucopyranoside solution in it.
- Incubate the reaction mixture exactly for 5 minutes at 37°C.
- After 5minutes, add 0.6ml sodium carbonate (20%).
- Add 3ml distilled water.
- Read at 405nm against reagent blank

S.N.	Sample (mL)	Buffer	Enzyme	Incubation for 20 min at 37°C	Substrate (mL)	Incubation for 5 min at 37°C	Na_2CO_3 (mL)	Water	Absorbance at 405nm			
									R1	R2	R3	Mean
1.	0(Blank)	0.3	0.6 (heat killed)		1.0		0.6	3.0				
2.	0(Control)	0.3	0.6		1.0		0.6	3.0				
3.	0.1	0.2	0.6		1.0		0.6	3.0				
4.	0.2	0.1	0.6		1.0		0.6	3.0				
5.	0.3	0.0	0.6		1.0		0.6	3.0				

S.N.	Acarbose (mL)	Buffer	Enzyme	Incubation for 20 min at 37°C	Substrate (mL)	Incubation for 5 min at 37°C	Na_2CO_3 (mL)	Water	Absorbance at 405nm			
									R1	R2	R3	Mean
6.	0.05	0.25	0.6		1.0		0.6	3.0				
7.	0.1	0.2	0.6		1.0		0.6	3.0				
8.	0.2	0.1	0.6		1.0		0.6	3.0				
9.	0.3	0.0	0.6		1.0		0.6	3.0				

Calculation

Calculate the % inhibition from the following equation.

$$\% \text{ Inhibition} = \frac{(A\ control - Asample)}{A\ control} \text{X } 100$$

Tips

- Enzyme and substrate solution should be prepared freshly.
- Keep the enzyme and substrate solution on ice till used.
- If the sample is coloured, each concentration of the sample should provide the sample blank. For this, add the substrate only after the addition of sodium carbonate (for blank sample). It will minimize the background colour given by the coloured sample

REFERENCES

Ademiluyi AO, Oboh G. 2013. Soybean phenolic-rich extracts inhibit key-enzymes linked to type 2 diabetes (α-amylase and α-glucosidase) and hypertension (angiotensin I converting enzyme) in vitro. *Exp Toxicol Pathol*. 65:305–9. doi: 10.1016/j.etp.2011.09.005

Afrisham R, Aberomand M, Ghaffari M, Siahpoosh A, Jamalan M. 2015. Inhibitory effect of Heracleum persicum and *Ziziphus jujuba* on activity of alpha-amylase. *J Bot.* 824683. https://doi.org/10.1155/2015/824683

Arumugam G, Manjula P, Paari NA. 2013. Review: Anti diabetic medicinal plants used for diabetes mellitus. *J Acute Dis*. 2:196–200. doi: 10.1016/S2221-6189(13)60126-2.

Arya V, Gupta V, Ranjeet K. 2011. A review on fruits having anti-diabetic potential. *J Chem Pharm Res.* 3:204–212.

Bernfeld P, Colowick S, Kalpan N. 1955. Amylases: alpha and beta methods. *Meth Enzymol.* 1:149-158. https://doi.org/10.1016/0076-6879(55)01021-5

Bhandari MR, Jong-Anurakkun N, Hong G, Kawabata J. 2008. α-Glucosidase and α-amylase inhibitory activities of nepalese medicinal herb pakhanbhed (Bergenia ciliata, Haw.). *Food Chem*. 106:247–52. doi: 10.1016/j.foodchem.2007.05.077.

Buchholz T, Melzig MF. 2016. Medicinal plants traditionally used for treatment of obesity and diabetes mellitus - screening for pancreatic lipase and α-amylase inhibition. *Phytother Res*. 30:260–6. doi: 10.1002/ptr.5525.

Durazzo A, D'Addezio L, Camilli E, Piccinelli R, Turrini A, Marletta L, Marconi S, Lucarini M, Lisciani S, Gabrielli P. 2018. From plant compounds to botanicals and back: A current snapshot. *Molecules*. 23:1844. doi: 10.3390/molecules23081844.

Grover JK, Yadav S, Vats V. 2002. Medicinal plants of India with anti-diabetic potential, *J Ethnopharmacol*. 81 (1): 81-100.

Gupta P, De A. 2012. Diabetes mellitus and its herbal treatment. *Int J Res Pharm Biomed Sci*. 3:706–721.

Ibrahim MA, Koorbanally NA, Islam MS. 2014. Antioxidative activity and inhibition of key enzymes linked to type-2 diabetes (α-glucosidase and α-amylase) by Khaya Senegalensis. *Acta Pharmaceutica*. 64:311–24. doi: 10.2478/acph-2014-0025.

IDF. 2021. IDF Diabetes Atlas Tenth Edition 2021. International Diabetes Federation, Belgium. https://diabetesatlas.org/

Kooti W, Moradi M, Akbari S, Sharafi-Ahvazi N, AsadiSamani M, Ashtary-Larky D. 2015. Therapeutic and pharmacological potential of *Foeniculum vulgare* Mill: A review. *J HerbMed Pharm.* 4:1–9.

Li DQ, Qian ZM, Li SP. 2010. Inhibition of three selected beverage extracts on α-glucosidase and rapid identification of their active compounds using HPLC-DAD-MS/MS and biochemical detection. *J Agric Food Chem*. 58:6608–13. doi: 10.1021/jf100853c.

Matsui TC, Yoshimoto C, Osajima K, Oki T, Osajima Y. 1996. In vitro survey of alpha glucosidase inhibitory food components. *Biosci Biotech Biochem*. 60:2019-22.

Parmar I, Rupasinghe H. 2015. Antioxidant capacity and anti-diabetic activity of wild berry stem infusions. *Euro J Med Plants*. 8:11–28. doi: 10.9734/EJMP/2015/17115.

Patel DK, Kumar R, Laloo D, Hemalatha S. 2012. Diabetes mellitus: an overview on its pharmacological aspects and reported medicinal plants having antidiabetic activity. *Asian Pac J Trop Biomed*. 2:411–20. doi: 10.1016/S2221-1691(12)60067-7.

Pistia-Brueggeman G, Hollingsworth RI. 2001. A preparation and screening strategy for glycosidase inhibitors. *Tetrahedron*. 57(42):8773–8.

Pradeepa R, Mohan V. 2021. Epidemiology of type 2 diabetes in India. *Indian J Ophthalmol*. 69(11):2932-2938.

Rao M, Sreenivasulu M, Chengaiah B, Reddy K, Chetty M. 2010. Herbal medicines for diabetes mellitus: A review. *Int J Pharm Tech Res*. 2:1883–1892.

Rupasinghe HPV, Balasuriya N, Wang Y. 2017. Prevention of type 2 diabetes by polyphenols of fruits. In: Kaïs H, Al-Gubory KH, Laher I, editors. *Nutritional antioxidant therapies: treatments and perspectives*. Springer. pp. 447–66. doi: 10.1007/978-3-319-67625-8_17.

Rupasinghe HPV, Sekhon-Loodu S, Mantso T, Panayiotidis MI. 2016. Phytochemicals in regulating fatty acid β-oxidation: Potential underlying mechanisms and their involvement in obesity and weight loss. *Pharmacol Ther.* 165:153–63. doi: 10.1016/j.pharmthera.2016.06.005.

Salehi B, Ata A, V Anil Kumar N, Sharopov F, Ramírez-Alarcón K, Ruiz-Ortega A, Abdulmajid Ayatollahi S, Tsouh Fokou PV, Kobarfard F, Amiruddin Zakaria Z, Iriti M, Taheri Y, Martorell M, Sureda A, Setzer WN, Durazzo A, Lucarini M, Santini A, Capasso R, Ostrander EA; Atta-ur-Rahman, Choudhary MI, Cho WC, Sharifi-Rad J. 2019. Antidiabetic potential of medicinal plants and their active components. *Biomolecules*. 9(10). doi: 10.3390/biom9100551. PMID: 31575072; PMCID: PMC6843349.

Salehi B, Kumar NVA, Şener B, Sharifi-Rad M, Kılıç M, Mahady GB, Vlaisavljevic S, Iriti M, Kobarfard F, Setzer WN. 2018. Medicinal plants used in the treatment of human immunodeficiency virus. *Int J Mol Sci*. 19:1459. doi: 10.3390/ijms19051459.

Sharifi-Rad M, Nazaruk J, Polito L, Morais-Braga MFB, Rocha JE, Coutinho HDM, Salehi B, Tabanelli G, Montanari C, del Mar Contreras M. Yousaf Z, Setzer WN, Verma DR, Martorell M, Sureda A, Sharifi-Rad J. 2018. Matricaria genus as a source of antimicrobial agents: From farm to pharmacy and food applications. *Microbiol Res.* 215:76–88. doi: 10.1016/j.micres.2018.06.010.

Sharifi-Rad M, Salehi B, Sharifi-Rad J, Setzer WN, Iriti M. 2018. *Pulicaria vulgaris* Gaertn. essential oil: An alternative or complementary treatment for leishmaniasis. *Cell Mol Biol.* 64:18–21. doi: 10.14715/cmb/2018.64.8.3.

Soumya D, Srilatha B. 2011. Late-stage complications of diabetes and insulin resistance. *J Diabetes Metab*. 2:1000167.

Striegel L, Kang B, Pilkenton SJ, Rychlik M, Apostolidis E. 2015. Effect of black tea and black tea pomace polyphenols on α-glucosidase and α-amylase inhibition, relevant to type 2 diabetes prevention. *Front Nutr*. 2:3. doi: 10.3389/fnut.2015.00003.

Sudha P, Zinjarde SS, Bhargava SY, Kumar AR. 2011. Potent α-amylase inhibitory activity of Indian ayurvedic medicinal plants. BMC. *Complement Altern Med*. 11:5. doi: 10.1186/1472-6882-11-5

Tao Z, Shi A, Zhao J. 2015. Epidemiological perspectives of diabetes. *Cell Biochem Biophys.* 73:181–5.

Upadhyay J, Polyzos SA, Perakakis N, Thakkar B, Paschou SA, Katsiki N, Underwood P, Park KH, Seufert J. 2018. Pharmacotherapy of type 2 diabetes: an update. *Metabolism*. 78:13–42. doi: 10.1016/j.metabol.2017.08.010