INVITRO PHARMACOLOGICAL SCREENING OF FRESH LEAF JUICE OF ANNONA SQUAMOSA

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ABSTRACT

In accordance with the 3Rs as outlined by ICH, which outline the replacement, reduction, and refinement of the use of animals in research, large-scale in-vitro assays have been developed and validated for early-stage screening aimed toward filtering out molecules with the potential for Pharmacological and toxicological screening.

In order to explore potential preventive and curative effects, the current study aims to screen for potential alpha amylase and alpha -glucosidase inhibitory activities, glucose uptake potential in yeast, glucose adsorption activity, nitric oxide and DPPH scavenging activity from the widely used leaves of plant, annona squamosa Linn. Different concentrations of Annona squamosa Linn. fresh leaf juice (20, 40, 60, 80, and 100 g/ml) are examined for various in-vitro experiments using the corresponding target chemicals.a prominent and statistically significant dose-dependent Similar to the commercial medication Acarbose, inhibition of the enzymes was seen. With its considerable glucose

absorption capacity and complementing glucose adsorption activity, FLJAS has the potential to lower postprandial hyperglycemia.Furthermore, as oxidative stress plays a vital role in treatment objectives and is established by the NO and DPPH assays, antioxidant potential can slow the progression of disease.The study's findings show that Annona squamosa leaf juice has potential as a treatment for postprandial hyperglycemia.

Keywords: In-vitro pharmacological screening, Annona squamosa, postprandial hyperglycemia, anti-diabetic, α -amylase, α -glucosidase, IC 50 are some of the terms that are commonly used.

1) INTRODUCTION

Diabetes, a disease of modern society that has a negative impact on people's quality of life everywhere, requires extensive research to determine how to slow the disease's progression if it cannot be fully reversed. The World Health Organization (WHO) stated that "a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity" (WHO, 2006) is how it defines health in its broadest definition. Physical, mental, and social well-being—collectively referred to as the "health triangle"—are combined to achieve health maintenance and promotion (Georgia, 1998).

Natural medicine therapy has a lengthy history, dating back to the ayurvedic medical system and extending to the Chinese, European, and other traditional medical systems. However, when modern medicine advanced in the early 19th century, the use of herbal remedies swiftly fell out of favor.

Chronic hyperglycemia, disturbances in the metabolism of carbohydrates, fats, and proteins as a result of improper insulin secretion and/or ineffective insulin action, and possibly abnormally high levels of other counter-regulatory hormones like growth hormone, sympathomimetic amines, and corticosteroids, are the hallmarks of diabetes mellitus, a complex metabolic syndrome.

Chronic hyperglycemia is linked to multi-organ dysfunction syndrome, which causes long-term harm, malfunction, and failure and causes consequences such as diabetes retinopathy, nephropathy, neuropathy, and macro-vascular issues.

The conversion of dietary carbohydrates into glucose by the actions of digestive tract enzymes such alpha-glucosidase and alpha-amylase causes postprandial hyperglycemia. It has been suggested that postprandial hyperglycemia is a separate risk factor for cardiovascular diseases22. Therefore, it is indicated that managing postprandial hyperglycemia is crucial for managing diabetes and avoiding cardiovascular problems.The pace of hydrolytic oligosaccharide cleavage is slowed down by the intestinal -glucosidase enzyme inhibition, and the process of carbohydrate digestion moves to the lower portion of the small intestine. The overall rate of glucose absorption into the blood is delayed by this broadening of the digestive process. This has turned out to be one of the most effective methods for lowering the postprandial rise in blood sugar and hence inhibitors of alpha-amylase and α-glucosidase, which are particularly useful in postprandial glycemic control, are good candidates for prevention of diabetic complications,in turn help avoiding the onset of late diabetic complications.

Natural remedies from medicinal plants are found to be safe and effective. Many plant species have been used in folkloric medicine to treat various ailments. Even today compounds from plants continue to play a major role in primary health care as therapeutic remedies in many developing countries. Plants are a rich source of secondary metabolites with interesting biological activities. In general, these secondary metabolites are an important source with a variety of structural arrangements and properties. Distinguished examples of these compounds include flavonoids, phenols, phenolic glycosides, saponins and cyanogenic glycosides. Natural products from microbial sources have been the primary source of antibiotics, but with the increasing recognition of herbal medicine as an alternative form of health care, the screening of medicinal plants for active compounds has become very significant because these may serve as sources of antibiotic prototypes.

Custard apple, sometimes referred to as Annona squamosa Linn. (Family: Annonaceae), is a woody, semi-deciduous tree that is cultivated all over India on rocky terrain with shallow and well-drained soils.The leaf extract of Annona squamosa has been shown to have hypoglycemic and anti-diabetic effects.

2) MATERIALS AND METHODS

2.1)Annona squamosa fresh leaf juice preparation:

Annona squamosa fresh leaf juice is prepared by pressing the juice through a muslin cloth after the leaves have been carefully cleaned to eliminate any adhering contaminants.The juice was centrifuged at 5000 rpm for 15 minutes at room temperature to obtain the clear supernatant. Using a measuring cylinder, the volume of the extracted juice was measured in order to determine the juice yield percentage.

2.2) A-Amylase inhibition assay by DNSA method:

This experiment was conducted with a modified McCue and Shetty (2004) technique. 250 microlitres of extract (1.25-10 mg/ml) and 250 microlitres of 0.02 M sodium phosphate buffer (pH 6.9) were combined in a tube. the addition of 250 l of 0.02 M sodium phosphate buffer (pH 6.9) containing a 0.5 mg/ml **α** -amylase solution. This solution had a preincubation period of 10 minutes at 25°C, 250 microlitres of 1% starch solution in a buffer of 0.02 M sodium phosphate, and 10 minutes of further incubation at 25°C. By adding 500 l of the dinitrosalicylic acid (DNS) reagent, the process was stopped. The tubes were then cooled to room temperature after being incubated in boiling water for 5 minutes. A spectrophotometer was used to measure the absorbance at 540 nm after the reaction mixture had been diluted with 5 ml of distilled water. Using the same method but substituting distilled water for the extract, a control was created. The alpha amylase inhibitory activity was calculated as percentage inhibition:

Percentage of inhibition = Abs control –Abs extract/Abs control x 100

2.3)∝-Glucosidase inhibitary Assay:

Using α -glucosidase from Saccharomyces cerevisiae, the impact of the plant extracts on glucosidase activity was assessed using the method outlined by Kim et al. (2005). P-nitrophenyl glucopyranoside (pNPG) was used as the substrate, and a 20 mM phosphate buffer solution with a pH of 6.9 was used to prepare it. 100 microlitres α -glucosidase (1.0 U/mL) was pre-incubated with 50 L of the various extract strengths (ethanol and water) for 10 min. The reaction was then started by adding 50 L of 3.0 mM (pNPG) as a substrate that was dissolved in 20 mM phosphate buffer (pH 6.9). After 20 minutes at 37°C, the reaction mixture was halted by adding 2 mL of 0.1

M Na_2CO_3. The yellow paranitrophenol produced from pNPG was used to measure the α - glucosidase activity.

Percentages of the blank control were used to express the results. Inhibition percentage is determined as

Inhibition percentage = (Absorbance of control – Absorbance of extract) × 100 Absorbance of control

2.4) Glucose uptake studies by Yeast cells

Materials:

Glucose 25 mM was used as the substrate. The positive control was metronidazole (Flagyl, Pfizer).

Commercial baker's yeast was purchased from a nearby bakery and used to analyze glucose uptake while being affected by FBPRD and the common medication metronidazole. Repeated centrifugation at 3000 rpm for 5 minutes in distilled water was used to repeatedly wash the yeast until the supernatant liquids were clear. In distilled water, a 10% (v/v) yeast suspension was made. To 1 mL of 25 mM glucose solution, different doses of FBPRD (25,50,100,200,400, and 800 g/mL) were added to 1 mL of a 25 mM glucose solution, and the mixture was then let to sit for 10 minutes at 37 °C. 100microlitres of yeast suspension was added to the reaction to start it off, and it was then vortexed and incubated at 37 °C for an additional 60 min. After 60 minutes, the tubes were centrifuged (2,500 g, 5 min), and the amount of glucose was determined spectrophotometrically (540 nm) by the DNSA method in the supernatant (Cirillo, 1962). As a conventional treatment, metronidazole was employed.Three duplicates of each experiment were performed.The following formula was used to compute the percentage increase in yeast cells' absorption of glucose:

Increase in glucose uptake (%) = absorbance (Sample) – Absorbance(Control) f X 100

Absorbance (Sample)

2.5) Glucose Adsorption Assay

The Ou et al. technique was used to assess the extract's ability to absorb glucose. 100 mL of a glucose solution with five different concentrations (5, 10, 15, 20, and 25 mM) were mixed with approximately 10 ml of FBPRD. Each of these combinations was thoroughly combined, agitated, then incubated for six hours at 37 degrees Celsius in a shaker water bath, respectively.

After incubation, the mixture was centrifuged at 4800 rpm for 20 minutes, and then, using a glucose oxidase peroxidase testing kit, the glucose content in the supernatant was ascertained. The formula G1-G6, where G1 is the glucose concentration in the initial solution and G6 is the glucose concentration after 6 hours, was used to calculate the quantity of bound glucose.

Glucose adsorbed = Intial glucose concentration -Glucose concentration after 6hours

2.6) Nitric oxide radical scavenging activity in vitro:

Reagents

a. sodium phosphate buffer was created using 0.272g of Na_2HPO_4 and 1.1g of NaH_2PO_4 in 150ml of water. NaoH solution was used to maintain the pH at 7.4 and 200ml of the solution was created.

b. 10mM Nitroprusside sodium

c.0.5g of the Griess reagent was dissolved in 10ml of distilled water as the next method.

d. Group and concentration preparation of the plant extract.

In-vitro Assay Method: The Griess-Ilosvoy reaction can be used to calculate the nitric oxide-scavenging activity (Garrat, 1964). At physiological pH (7.2), sodium nitroprusside is thought to break down in the solution and produce NO. Nitrogen dioxide (NO_2) combines with oxygen in an aerobic environment to produce stable compounds (nitrate and nitrite). The Griess reagent can be used to determine the amounts of Nitric oxide scavengers compete with oxygen, which lowers the formation of nitrite ions. Different amounts of leaf extract (20-100 g/ml) were treated with sodium nitroprusside (5 mM) in standard phosphate buffer saline (0.025 M, pH 7.4), and tubes were incubated at 29 for 3 hours. The control experiment was carried out exactly the same way but without the test chemicals and with the same amount of buffer. Samples that had been incubating for three hours were diluted with 2 ml of Griess reagent. On the spectrophotometer, the absorbance of the color that resulted from the diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthylethylenediamine hydrochloride was seen at 550 nm. Ascorbic acid, which was used in the same way as the leaf extract, was the standard. Scavenging for nitric oxide radicals Graphed in comparison to the standard and calculated as the percentage inhibition using the formula.

% inhibition = [O.D of control - O.D. of Test/O.D. of control] X 100

2.7)DPPH radical scavenging activity:

4.3 mg of DPPH (1,1-Diphenyl-2-picrylhydrazyl) was added to 3.3 ml of methanol to create the DPPH solution, which was then shielded from light by placing aluminum foil over the test tubes. 3 ml of methanol was added to 150 micro litres of DPPH solution, and an instantaneous absorbance measurement at 517 nm was made as a control reading. In order to make the volume consistently 150 microlitres , 50 pi of various concentrations of the plant sample extracts (40, 80, 120, 160, 200, 240, 280, 320, and 360 ug/ml) as well as the standard chemical (Ascorbic Acid) were taken.After additional dilution with methanol to a final volume of 3 ml for each sample, 150 micro litres of DPPH was added to each tube. After 15 minutes, an absorbance reading at 517 nm was acquired using methanol as a blank on a UV-visible spectrometer. For each extract and standard preparation, the IC50 values were determined. The following formula was used to determine the DPPH freeradical scavenging activity:

$$\% \text{ scavenging} = \frac{\text{Absorbance of control - Absorbance of test sample}}{\text{Absorbance of control X 100}}$$

3) RESULTS AND DISCUSSION

Diabetes mellitus is an often life threatening chronic disorder with increasing incidence throughout the world. In recent years, there is a steady rise in the rate of incidence of Diabetes mellitus and estimated that 1 in 5 may be diabetic by 2025 (Romila et al., 2010). Medicinal plants are ties of most effective plants were in part explained by the ability of the phytoconstituents to increase glucose transport and metabolism in muscle and/ or to stimulate insulin secretion (Edwards et al., 1987). The α-amylase and α-glucosidase inhibitory activity of the FLJAS are depicted in table 1 & 4. Acarbose belongs to the α- glucosidase inhibitor class of the oral hypoglycaemics and is known to inhibit both alpha amylase and alpha glucosidase. For this reason, acarbose was used as positive control in both assays. From the data obtained, it was found FLJAS showed significant inhibitory activity

4) CONCLUSION

According to historical records, several types of plant foods contain pharmacologically active chemicals at levels high enough to provide a drug-like effect when ingested in moderation. Several in-vitro pharmacological tests on fresh Annona squamosa leaf juice produced statistically significant results for in-vitro anti-diabetic assays.These investigations, however, are insufficient to support the claims, hence a thorough, exhaustive battery of pharmacological,

photochemical, and bioanalytical tests, followed by observational studies in humans, must be conducted.

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***3.1) In- vitro*assay of α-amylase inhibition by FLJAS(DNSA method)**

S.No.	**Concentration of FLJAS (μg/ml)**	**% α-amylase inhibition**
1	20	21.52±0.160
2	40	27.59±0.025
3	60	36.23±0.070
4	80	45.17±0.010
5	100	56.91±0.0290

***3.2) In- vitro*assay of α-amylase inhibition by Acarbose(DNSA method)**

S.No.	**Concentration of Acarbose (μg/ml)**	**% α-amylase inhibition**
1	20	32.347±0.06
2	40	40.270±0.116
3	60	48.287±0.183
4	80	52.450±0.275
5	100	58.753±0.068

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***3.3) In- vitro*assay of α-glucosidase inhibition by FLJAS**

S.No.	**Concentration of FLJAS (μg/ml)**	**% α-glucosidase inhibition**
1	20	13.42±0.091
2	40	23.71±0.039
3	60	35.63±0.030
4	80	40.38±0.031
5	100	52.16±0.107

The values expressed are the Mean± SEM of 3 observations (n=3)

3.4)In- vitro**assay of α-glucosidase inhibition by Acarbose**

S.No.	**Concentration of Acarbose (µg/ml)**	**% α-glucosidase inhibition**
1	20	18.61±0.05
2	40	32.57±0.090
3	60	54.45±0.265
4	80	68.21±0.165
5	100	79.14±0.114

The values expressed are the Mean± SEM of 3 observations (n=3)

3.5) Effect of FLJAS treatment on glucose uptake in yeast

S. No.	**Concentration of FLJAS (µg/ml)**	**% increase in glucose uptake by yeast cells**
1	50	15.52±0.460
2	100	24.45±0.125
3	200	30.73±0.370
4	400	36.17±0.410
5	800	42.71±0.22

The values expressed were the Mean ± SEM of 3 observations (n=3)

3.6) Effect of Metronidazole treatment on glucose uptake in yeast

S. No.	Concentration of Metronidazole(µg/ml)	% increase glucose uptake by Yeast cells
1	50	16.67±0.25
2	100	31.65±0.524
3	200	49.73±0.237
4	400	54.17±0.810
5	800	56.71±0.029

3.7)Effect of FLJAS incubation on *in -vitro* glucose adsorption capacity

S. No.	Concentration of Glucose (Mm/L)	Glucose Adsorption Capacity(mg/dL)
1	5	6
2	10	8
3	15	16
4	20	19
5	25	19

3.8) Effect of FLJAS vs Ascorbic acid on nitric oxide radical scavenging activity

S. No.	Concentration of test sample	NO scavenging by FLJAS (µmoles of nitrate/mg of protein)	NO scavenging by ASCORBIC ACID (µmoles of nitrate/mg of protein)
1	(20 µg/ml)	21.02±0.112	10.34± 1.23
2	(40 µg/ml)	19.60±0.090	8.34±2.34
3	(60 µg/ml)	17.54±0.158	5.23± 3.24

4	(80 µg/ml)	13.31±0.602	3.23±0.24
5	(100 µg/ml)	13.66±0.235	2.21±1.23

The values expressed were the Mean ± SEM of 3 observations (n=3)

3.9)DPPH scavenging assay by FLJAS & Ascorbic acid :

S. No.	Concentration of sample (µg/mL)	% DPPH scavenging by ASCORBIC ACID	% DPPH scavenging by FLJAS
1	40	43.35±0.45	34.36±3.21
2	80	51.52 ±0.32	46.25± 2.35
3	120	58.25±1.23	48.76± 2.56
4	160	70.85 ±2.21	54.65±5.43
5	200	78.41±3.25	64.52±2.34
6	240	86.43±4.26	71.65±0.65
7	280	96.43±4.32	82.34±2.54
8	320	98.29±2.54	84.32± 3.45
9	360	98.63 5.63±	84.76± 2.65