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Alpha amylase inhibitory activities of extract of *Annona squamosa* Linn. Leaves in streptozotocin induced diabetic rats

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KEYWORDS

Annona squamosa; anti-diabetic; α-amylase inhibition; postprandial hyperglycemia; streptozotocin.

ABSTRACT:

The leaves of Annona squamosa (AS) are reported to have anti-diabetic property, but its active principle and mechanism of action is not known. For further investigations, different extracts (hexane, chloroform, butanol, hexane washed methanol, total methanol extract and water decoction) of AS leaves were prepared, characterized and tested for their inhibitory property against activity of pancreatic α-amylase under in vitro and in vivo conditions. Hexane extract showed the lowest IC50 0.925 mg/mL and further tested for streptozotocin induced diabetes. Its hypoglycemic response was correlated with inhibitory activity with respect to α-amylase in pancreatic homogenate by using different doses and compared with standard drugs i.e., acarbose and glimepiride. It significantly reduced the raised glucose levels to 41.18±2.46 % at 100 mg/kg WB and 78.10±1.57% at 400 mg/kg WB. It also raised the serum insulin from 8.56±1.42 (experimental control) to 16.26±1.20 µU/mL at 400 mg/kg BW and simultaneously inhibited the activity of α-amylase to tune of 76.69±2.52% at 100 mg/kg WB and 86.67±2.30% at 400 mg/kg BW. In glimepiride treated rats, there was significant rise in serum insulin without inhibition of α-amylase and in case of acarbose treated rats; there was significant inhibition of α-amylase, without significant rise in serum insulin. Thus, it enhances the insulin secretion (acts as secretagogue) and also inhibits the pancreatic α-amylase in small intestine. Therefore, it could be used for the management of postprandial hyperglycemia in diabetes.

1. INTRODUCTION

Disorders of carbohydrate metabolism and its abnormal uptake may cause severe health problems such as unregulated diabetes and obesity. Diabetes mellitus (DM) is a metabolic disorder resulting from deficiency in insulin secretion and regulation of insulin or both. Hence disturbances in the metabolism of carbohydrate, fat and protein occurred; which may ultimately lead to several complications in diabetes mellitus such as retinopathy[1], nephropathy[2], neuropathy[3], microangiopathy[4] and increased risk of cardiovascular disease[5-7]. The therapeutic strategies for DM-2 include the reduction in demand for insulin, stimulation of insulin secretion and enhancement of the action of insulin at the target tissues. The inhibition of digestion and uptake of oligo- and disaccharides play an important role in the management of postprandial hyperglycemia[8]. The commonly used drugs available in market for diabetic patients are insulin and its preparations such as sulfonylureas[9-11], meglitinides[11], biguanides[11-13], glucosidase inhibitors[14], aldose reductase inhibitors[15], thiazolidinediones[16], carbamoylmethyl benzoic acid[11,17], insulin-like growth factors[18,19]. The glucosidase inhibitors are mainly used to manage the uncontrolled postprandial hyperglycemia, which is one of the main factors for manifestation of diabetic complications. Thus inhibition of these enzymes can definitely decrease the postprandial hyperglycemia and could be a key tactic in the

Journal of Chemical Health Risks
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JCHR (2023) 13(6), 3452-3461 | ISSN:2251-6727

management of diabetes mellitus[11,20]. Pancreatic α-amylase (E.C. 3.2.1.1) 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 number of α-(l-6) and α-(1-4)-oligoglucans. These products are then acted upon by α-glucosidases and further degraded to glucose for final absorption[21]. The α-glucosidase (E.C. 3.2.1.20) is involved in the hydrolysis of glycosidic bonds from the non-reducing end of the partially digested oligosaccharides in the small intestine resulting in the monosaccharides for absorption[22]. Commercially available α-glucosidase inhibitors such as acarbose, miglitol[11,23,24] and voglibose[11,23-24] are widely used to treat patients with type 2 diabetes, but they are associated with some side effects like abdominal bloating and discomfort, diarrhea and flatulence[25]. Even, number of research evidences in public domain suggesting hyperinsulinemia associated with insulin resistance is an independent risk factor in the development of BPH26 and the pathogenesis of prostate carcinoma[27]. Research published from India have shown that vitamin D deficiency is highly prevalent among women with DM-228, levels of sialic acid residues in platelet proteins in diabetes[29]. Association of OLR1 gene polymorphism with Met S probably influences BMI in Indian population[30].

The use of medicinal plants as alternatives or supplementary medicines to allopathic drugs is in current practice throughout the world, especially for the management of life style mediated metabolic disorders[31]. The plant products are gaining more attention of the scientific communities because of their multi-targeted drug action and minimal side effects[32-35]. Since diabetes is a multi-etiological disease, use of herbal formulations would be more convincing, being natural cocktail of variety of secondary metabolites, having different targets in signaling pathways of diabetes manifestation. Besides exhibiting hypoglycemic property, these herbal preparations often show antioxidant and anti-inflammatory potential.

The fruits of custard apple (Annona squamosa), commonly known as sitaphal, are edible in India. Leaves of the plant have medicinal potential with nutritional composition, phytochemical and health-promoting biological properties[36]. These are the source of environmental neurotoxins[37] & found to have anti-diabetic[38-41] anti-oxidant[39,41], hypoglycaemic[39], antihyperlipidemic[38], anti-inflammatory[38], antibacterial and antifungal[36], anti-fertility[42], anti-tumor[43], anti-hyperthyroidism[44] activities etc. The leaves of plant have shown the presence of various types of phytochemicals such as steroids[45], alkaloids46, saponins, terpenes[47], tannins[48], phenolic substances[49], volatile oils[50,5] and mucilage[52], but its effect on intestinal enzymes, responsible for carbohydrate metabolism is not known. Earlier we have reported its inhibitory effect of α-glucosidase[53] and here we are reporting its role on activity of intestinal α-amylase.

2. MATERIALS AND METHODS

2.1 Chemicals

2-Chloro-4-nitrophenol α-D-maltotrioside (CNPG3) and streptozotocin (STZ) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Glucose assay kit was purchased from Accurex. Sodium dihydrogen orthophosphate dihydrate (NaH2PO4.2H2O) and disodium hydrogen phosphate dihydrate (Na2HPO4.2H2O) were procured from Himedia, India. All other laboratory chemicals used were of analytical reagent (AR) grade.

Healthy male rats of CF strain, weighing 100-150 g were obtained from the central animal house facility of Institute of Medical Sciences, BHU. These animals were acclimatized in our departmental animal house for 7 days with free access to food (standard laboratory chow) and tap water. The experimental protocol was approved by the Animal Ethics Committee of Institute.

2.2 Collection of plant material and preparation of extracts

The leaves of Annona squamosa were collected from Botanical Garden of Institute of Medical Sciences, BHU and their authenticity was reconfirmed on pharmacognostical parameters and direct comparison with sample preserved in the department. After authentication, the leaves were washed under running tap water to remove adhering material, air dried in the shade and pulverized in a mechanical grinder. Leaves were separately extracted with n-hexane and methanol in continuous Soxhlet apparatus 20 hr each. The fresh water decoction (ASWD) was separately prepared by boiling the

Journal of Chemical Health Risks
www.jchr.org
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leaf-powder in double-distilled water and standardized as g of leaf/mL of decoction. The chloroform (ASCE), n-butanol (ASBE) and n-hexane washed methanol extract (ASMHx) were prepared by fractionation of methanol extract with the help of separating funnel. The solvent free extract was prepared by distillation and desiccation until constant weight was attained. The percentage yields of all the extracts were calculated. For in vitro enzymatic assay, all extracts were dissolved in DMSO and diluted with 0.9% saline. The water decoction was prepared in water in mg/mL. Similarly, the drug vector was also prepared in which the pure DMSO was diluted with normal saline in same ratio. Earlier results have indicated that all these extracts showed hypoglycemic response and n-hexane extract was most potent among all these extracts[27], so ASHE is used for further investigation purpose.

2.3 Assessment of α-amylase assay

2.3.1 In-vitro study

Preparation of enzyme source

For preparation of tissue homogenate, a small piece of pancreas of normal rat was taken out in pre-cooled PBS and cleaned tissue was dried on blotting paper, weighed and homogenized in glass-teflon homogenizer. It was centrifuged at 5,000 g for 30 min and its supernatant was used as enzyme source. Final volume of homogenate was maintained to get 20% (w/v).

Assay by $CNPG_3$ method

The spectrophotometric assay method was used with slight modification[53-55]. Briefly, the 180 µL of tissue homogenate was mixed with 360 µl of 40 mM phosphate buffer (pH 6.9), test sample/positive control of various concentrations, incubated at 37°C for 15 min and then 720 µL of CNPG3 was added for further incubated at 37°C for 10 min. Finally, absorbance was measured at 405 nm against blank. A control reaction was carried out without the test sample.

2.3.2 In-vivo study: Induction of diabetes in rats by streptozotocin (STZ)

Rats weighing between 100 to 150 g were randomly selected for inducing diabetes. The intra-peritoneal injection of streptozotocin (50 mg/kg, dissolved in chilled citrate buffer-pH 4.5) was given to overnight fasted rats. After 5 days, blood glucose was measured by GOD/POD method. The rats having blood glucose, higher than 200 mg/dl, were considered as diabetic[56,57].

2.4 Estimation of glucose and insulin level and assessment α-amylase assay

The normal and diabetic rats were randomly divided in to various groups (n=6) and treated with ASHE/ Acarbose /Glimepiride /drug-vector for 7 days. On 7th day, the blood glucose was measured by GOD/POD and serum insulin was measured by Immulite 1000 (Siemens) solid-phase, two-site chemiluminescent immunometric assay kit. Treated animals were sacrificed and pancreatic homogenate were incubated for assay of α-amylase activity and further experiment was performed as section The design of group classification is given below:

Groups	Treatment plan
Normal control (drug vehicle)	20% tween 20@5mL/kg BW
Experimental control (diabetic rats with drug vehicle)	20% tween 20@5mL/kg BW
ASHE extract	100 mg/kg BW
ASHE extract	400 mg/kg BW
Glimepiride (Standard anti-diabetic drug)	1 mg/kg BW
Acarbose (Standard anti-diabetic drug)	10 mg/kg BW

Journal of Chemical Health Risks
www.jchr.org
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2.5 Statistical analysis

Data are expressed as mean ± SEM. Analysis of variance (ANOVA) followed by post hoc analysis (Dunnett's test) were used for data analysis using the SPSS statistical package, version 16.0. Differences at $P < 0.05$ were considered significant.

3. RESULTS

3.1 Inhibition of α-amylase of different extracts of AS leaves

The in vitro α-amylase inhibitory studies demonstrated that all extracts of AS leaves had α- amylase inhibitory activity when tested in concentration range from 0.1 mg/mL to 1.5 mg/mL. The inhibition was concentration dependent. Based on % inhibition, the ASHE showed the highest degree of inhibition. It was found to be 77.31% at concentration of 1.5 mg/mL. However, at concentration of 0.1 mg/mL, the inhibition was found only up to 16.64%. The potency of ASBE extract varied from 9.75-70.65% at various concentrations 0.1 mg/mL to1.5mg/mL. The ASCE and ASWD showed almost equal potency of 66.88% and 64.71% respectively at concentration of 1.5 mg/mL. The ASMHx extract inhibited the α-amylase activity up to 58.68% and ASME had least inhibition in range of 48.32% (Figure 1).

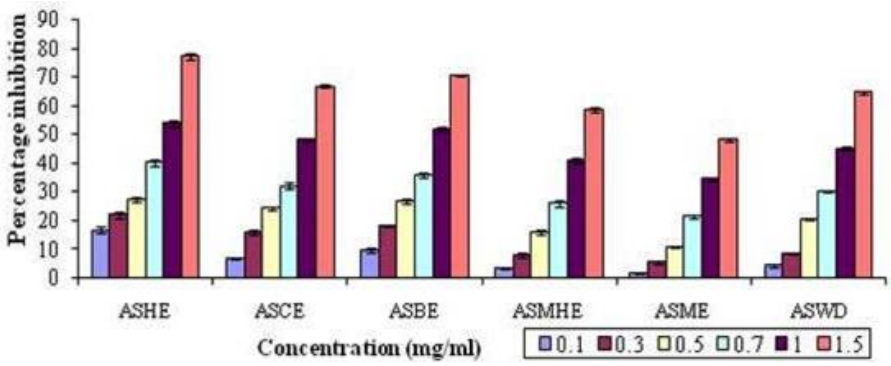

Figure 1. α-Amylase inhibitory activity of Annona squamosa L. leaves

In similar conditions, acarbose showed the significant inhibitory potency on pancreatic α-amylase in concentration dependent manner. It showed inhibition from 7.45-79.25% in concentration range of 0.1 mg/mL to1.0 mg/mL (Figure 2).

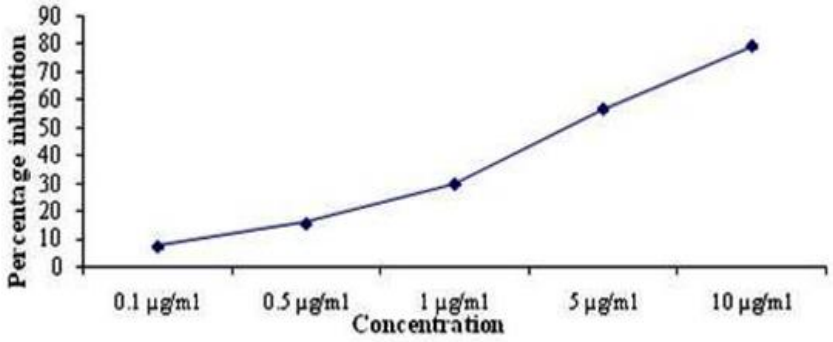

Figure 2. α-Amylase inhibitory activity of acarbose

The IC50 values of each extract and acarbose were summarized in table 1.

Journal of Chemical Health Risks
www.jchr.org
JCHR (2023) 13(6), 3452-3461 | ISSN:2251-6727

Table 1 The IC50 values of each extract and acarbose

Groups	α-amylase IC_{50} mg/ml
ASHE	0.925
ASCE	1.088
ASBE	1.007
ASMHE	1.269
ASME	1.505
ASWD	1.146
Acarbose	0.0049

3.2 Measurement of percentage inhibition in blood glucose level and raise in serum insulin in STZ induced diabetic rats

The oral treatment with ASHE extract significantly reduced the blood glucose level to the tune of 41.18±2.46 % at 100 mg/kg BW and 78.10±1.57 % at 400 mg/kg BW respectively. In similar conditions, glimepiride (1 mg/kg BW) reduced the blood glucose level by or to 45.69±2.52 % and acarbose reduced the blood glucose level to 61.66±1.52% at 10 mg/ BW dose (Table 2).

Table 2 Effect of ASHE on blood-glucose level, blood-insulin level and α-amylase activity in STZ induced diabetic rats

Parameters	Normal control	Experimental Diabetic control	ASHE 100mg/kg BW	ASHE 400 mg/kg BW	Acarbose 10 mg/kg BW	Glimepiride 1 mg/kg BW
Insulin level (µU/ml)	22.46±2.45	8.56±1.42	11.58±1.80*	16.26±1.20**	10.50±1.94	17.36±0.90**
α-amylase activity(% inhibition)	-	-	76.69±2.52**	86.67±2.30**	73.82±1.2**	36.66±0.72
Glucose level(% inhibition)	-	-	41.18±2.46**	78.10±1.57**	61.66±1.52**	45.69±2.52*

The insulin level in normal rats was found to be 22.46±2.45 (µU/mL), while in experimental control (diabetic rats) its value was found to be 8.56±1.42 (µU/mL). The ASHE treatment raised the insulin level to 11.58±1.80 (µU/mL) at 100 mg/kg BW and & 16.26±1.20 µU/mL at 400mg/kg BW respectively. In the glimepiride treated diabetic rats, insulin level was raised to 17.36±0.90 µU/mL at 1 mg/kg and in acarbose (10 mg/kg) treated diabetic rats the insulin level was raised to only 10.50±1.94 µU/mL at respectively.

Journal of Chemical Health Risks
www.jchr.org
JCHR (2023) 13(6), 3452-3461 | ISSN:2251-6727

The hexane extract significantly inhibited the activity of α amylase in concentration dependent manner. It was reduced to 76.69±2.52% at 100 and 86.67±2.30% 400 mg/kg respectively. In Acarbose (10 mg/kg BW) treated rats the inhibition was upto73.82±1.2% and in glimepiride (1 mg/kg) treated rats the inhibition was found to be 36.66±0.72 (unit if any) only (Table 2). These agents were used as positive controls.

4. DISCUSSION

The results of in vivo studies showed that all the AS extracts inhibit α-amylase activity in concentration dependent manner, but the degree of inhibition was least in methanolic extract and maximum in n-hexane extract. This suggests that the active principle of AS leaves for inhibition of α-glucosidase lies in the non-polar fraction. Earlier phytochemical investigations showed the presence of terpenes such as monoterpenes, diterpenes, triterpenes,tetraterpenes and sesquiterpenes[58-60] and flavonoids, such as flavones, flavonols, flavanones and catechins[61,62] etc. which are polyphenolic compounds and soluble in non-polar solvents, thus its inhibiting property may be associated to such compounds. We have also reported earlier63 about the highest FR scavenging potential of the n-hexane fraction.

Higher level of blood glucose (386.93±2.67 mg/dl in comparison to 115±4.53mg/dl glucose in normal rats) in STZ injected rats clearly shows the establishment of diabetic condition in these rats. It is further supported by lowering of serum insulin, it was 22.46±2.45µU/mL) in normal control rats but it was reduced to 8.56±1.42 µU/mL in STZ injected rats. When rats were treated with different concentration of ASHE for 7 days, there was significant reduction in the raised blood glucose level (Table 2) along with rise in serum insulin, suggesting its action on pancreas. In ASHE treated rats, the serum insulin was raised to 11.58±1.80 µU/mL at 100mg/kg and 16.26±1.20 µU/mL at 400 mg/kg, which are near to normal values in rats. It is similar (in which aspect) to drugs of sulfonyl urea group, which are known as secretagogue. Further, the n-hexane extract was also found to inhibit the activity of α amylase in pancreatic homogenate in concentration dependent manner (Table 2).

We have reported earlier its inhibitory activity on α-glucosidase and such type of combined inhibitory activity is also reported in other plants such as Parmeliaperlata, Illicium verum[64-65]. This suggests the multi-targeted approch of crude extracts of medicinal plants. The standard drug acarbose shows inhibitory property on both the enzymes α-amylase and α- glucosidase[66-68]. Interestingly, in similar conditions, glimepiride did not show any significant inhibitory role on α-amylase. A sub-toxic chlorpyrifos and lindane exposure induces oxidative stress in muscle cells, activates RSKs & HSP25 and induces HSP25 and thereby stimulates insulin-stimulated glucose uptake in muscle cells[69,70]. In addition, it has been suggested that involvement of MDA in the glycation of proteins provides support for the potential use of an antioxidant therapy in patients of non-diabetic nephrotic syndrome[71].

5. CONCLUSION

Thus, based on this scientific report, it could be suggested that n-hexane extract of leaves of Annona squamosa (ASHE) has hypoglycemic response. It involves two mechanisms simultaneously: first, it enhances the insulin secretion (acts as secretagogue) and second, it inhibits the activity of α-amylase in small intestine. This extract and its sub-fractions could be used for the management of postprandial hyperglycemia in type II diabetics as alternative therapies.

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Journal of Chemical Health Risks

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