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ANTIDIABETIC EFFECT OF A POLY HERBAL FORMULATION (D) ON HUMAN PANCREATICAMYLASE

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ABSTRACT

Diabetes has become a common global health problem that affects >170million people worldwide. It is one of the leading causes of death and disability. It is estimated that by 2030, the number will rise to 366 million (www.who.int). The majority of diabetes (~90%) is type 2 diabetes (T2D) caused by a combination of impaired insulin secretion from pancreatic beta cells and insulin resistance of the peripheral target tissues, especially muscle and liver., Traditional Medicines obtained from medicinal plants are used by about 40-60% of the world's population. Though there are many approaches to control diabetes and its secondary complications, herbal formulations are preferred due to lesser side effects and low cost. In this review we have studied the inhibitory effect of a polyherbal formulation consisting of ten antidiabetic *herbal plants* Gymnema*sylvestre*, Stevia*rebaudiana,* Momordicha*charantia,* syzimumcumini, Withania*somnifera*, Tinospora*cardiofolia*, Ocimum*santurm*, Moringaoliefera, Momordica *dioica* and Phyllanthus*emblica*,. The aqueous and ethanolic extract showed significant inhibition on *humanPancreaticamylase.*

KEYWORDS: Acarbose, Alpha amylase, DNS, human pancreatic amylase, Type 2 diabetes.

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INTRODUCTION

Diabetes has become a common global health problem that affects >170million people worldwide. It is one of the leading causes of death and disability. It is estimated that by 2030, the number will rise to 366 million (www.who.int). The majority of diabetes (~90%) is type 2 diabetes (T2D) caused by a combination of impaired insulin secretion from pancreatic beta cells and insulin resistance of the peripheral target tissues, especially muscle and liver. The 'top' three countries in terms of the number of T2D individuals with diabetes are India (31.7 million in 2000; 79.4 million in 2030), China (20.8 million in 2000; 42.3 million in 2030); and the US (17.7 million in 2000; 30.3 million in2030). Clearly, T2D has become an epidemic in the 21st century where India leads the world with the largest number of diabetic subjects. Diabetes is the third most dreaded disorders with more than 150 million people suffering from this disorder presently and the number is increasing annually by 33% in the US alone. DM (Diabetes mellitus) is a metabolic disorder characterized by chronic hyperglycemia or increased blood glucose levels by disturbances in carbohydrate, fat and protein metabolism resulting from absolute or relative lack of insulin secretion[1]. The frequency of this disorder is on the rise globally, is likely to hit 300 million by 2025 with India projected to have the largest number of diabetic cases [2]. Two major types of diabetes mellitus are Type-I Insulin Dependent Diabetes Mellitus (IDDM) or Juvenile onset diabetes mellitus which is caused by beta cell destruction in pancreatic islets and Type-II NonInsulinDependentDiabetes Mellitus (NIDDM) or Maturity onset diabetes mellitus caused by abnormalities in gluco-receptor of beta cells, reduced sensitivity of peripheral tissues to insulin, excess of hyperglycemic hormones like glucagon. Several synthetic medicines are in use currently such as sulfonylureas (5mg-3g), repaglinides (1.5mg-8mg) and biguanides (25mg-2g) etc1.These synthetic drugs suffers from most common adverse effects like hypoglycemia, hypersensitive (photosensitivity) nausea, vomiting, flatulence, diarrhea or constipation, headache, weight gain it B12deficiency, abdominal pain, anorexia, metallic taste, and lactic acidosis. Natural herbal drugs which have been used since ancient period for diabetes are comparatively safe and effective. But in case of diabetes most of the drugs are to be used life long hence herbal treatment will be best remedied rather than synthetic drugs. Type 2 diabetes is one of the primary threats to human health due to increasing prevalence, chronic course and disabling complications [3]. Many diverse therapeutic strategies for the treatment of Type 2 diabetes are in use. The conventional available therapies for diabetes include stimulation of endogenous insulin secretion, enhancement of the action of insulin at the target tissues, oral hypoglycemic agents, such as biguanids and sulfonylureas and the inhibition of degradation of dietary starch by glycosidases such as α-amylase and α-glucosidase[4]. 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 a number of α-(l-6) and α-(1 - 4) oligoglucans. These are then acted on by α-glucosidases and further degraded to glucose which on absorbtion enters the bloodstream. Degradation of this dietary starch proceeds rapidly and leads to elevated PPHG (post-prandial hyperglycemia). It has been shown that the activity of the HPA (human pancreatic α-amylase) in the small intestine correlates to an increase in post-prandial glucose levels, the control of which is therefore an important aspect of treatment of type 2 diabetes[5]. Hence, retardation of starch digestion by inhibition of enzymes such as α-amylase plays a key role in the control of diabetes. Inhibitors of pancreatic α-amylase delay carbohydrate digestion causing a reduction in the rate of glucose absorption and lowering the post-prandial

serum glucose levels [6]. Some inhibitors currently in clinical use are acarbose and miglitol which inhibit glycosidases such as α-glucosidase and α-amylase while others such as and voglibose inhibit α-glucosidase. However, many of these synthetic hypoglycemic agents have their limitations, are non-specific, produce serious side effects and fail to elevate diabetic complications. The main side effects of these inhibitors are gastrointestinal viz., bloating, abdominal discomfort, diarrhea and flatulence [7]. Herbal medicines are getting more importance in the treatment of diabetes as they are free from side effects and less expensive when compared to synthetic hypoglycemic agents [8,9]. In India, indigenous herbal remedies such as Ayurveda and other Indian traditional medicine have since ancient times used plants in treatment of diabetes[10]. Ethnobotanical studies of traditional herbal remedies used for diabetes have identified more than 1,200 species of plants with hypoglycemic activity [3,11]. A number of medicinal plants and their formulations are used for treating diabetes in the traditional Indian Ayurvedic system as well as in ethnomedicinal practices. Even though, these traditional practices are empirical in nature, over 200 million people in India with limited access to primary health carecenters, depend on the traditional system of medicine to cater to their health care needs[12]. However, this traditional knowledge, derived empirically, has to be supported by scientific testing. WHO (World Health Organization) (1980) has recommended the evaluation and mechanistic properties of the plants effective in such systems [13,14]. The search for new pharmacologically active agents obtained by screening natural sources such as medicinal plants or their extracts can lead to potentially and specific inhibitors for α-amylase [6]. Pharmacological properties α-glucosidase inhibitors such as acarbose that can also inhibit pancreatic α-amylase revealed that the complications of DM such as onset of renal, retina, lens and neurological changes and the development of ischemic myocardial lesions are prevented or delayed [15]. Long-term day-to-day management of diabetes, with acarbose is well tolerated and can improve glycemic control as monotherapy, as well as in combination therapy [16].

MATERIALS AND METHODS

Chemicals such as soluble starch, isopropanol, methyl-butyl-tertiary ether, petroleumether, hexane, methanol, chloroform, and ethanol were of Analytical grade. HPA (Human pancreatic alpha amylase), Aarbose was purchased from the Sigma Aldrich USA. Anti-diabetic medicinal plants that are used were selected from WHO monographs. Powders used are of high quality and proved to have anti-diabetic property. Preparation of plant extracts: Air dried plant material was crushed, powdered and extracted using a soxhlet apparatus with polar and non polar solvent. The solvent used were hot water, cold water, ethanol, isopropanol, petroleum ether, tertiary methyl-butyl- ether and chloroform. Cold water extracts were obtained by adding distilled water to the crushed material in the ratio 1:4 and kept in a rotary shaker for 24hrs at 30 degree Celsius and at 130 rpm respectively. After 24 h it was filtered, dried and the resulting extract was stored in the refrigerator. Hot water extracts were obtained by soxhlet extraction were 400ml of distilled water to plant powders Similarly medicinal powders were extracted using organic solvent in the ratio 1:3 by soxhlet extraction. Qualitative phytochemical analysis for Extracts were tested for the presence of alkaloids, tannins, flavonoids, polyphenols, saponins etc using standard procedures. [1]1:10 dilution of extracts was prepared and then subjected to phytochemical screening. Pancreatic alpha-amylase inhibition assay was performed to screen the Formulation for alpha amylase inhibition. Two methods were used Starch iodine and Dinitrosalicyclic acid assay. Starch iodine assay: Human pancreatic Alpha amylase (PPA) cleaves α-1, 4 glycosidic linkages in starch to produce reducing sugar (glucose, maltose). To determine the presence of inhibitors which decreases the activity of

HPAAssay was carried out according to Xiao et al (2006)[17] based on starch-iodine test.The total assay mixture composed of 40 µl 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride), 0.02 units of PPA solution and plant extracts of concentration from 0.1-1.5 mg ml^{-1} (w/v) were incubated at 37°C for 10 min. Then soluble starch (1%, w/v) was added to each reaction well and incubated at 37°C for 15 min. 1M HCl (20 µl) was added to stop the enzymatic reaction, followed by the addition of 100 µl of iodine reagent (5 mM I_2 and 5 mM KI). The colour change was noted and the absorbance was read at 620 nm on a microplate reader. The control reaction representing 100% enzyme activity did not contain any plant extract. To eliminate the absorbance produced by plant extract, appropriate extract controls without the enzyme were also included. The known PPA inhibitor, acarbose, was used a positive control at a concentration range of 6.5 - 32.8 µgml^{-1}. A dark-blue colour indicates the presence of starch; a yellow colour indicates the absence of starch while a brownish colour indicates partially degraded starch in the reaction mixture. In the presence of inhibitors of the extracts the starch added to the enzyme assay mixture is not degraded and gives a dark-blue colour complex whereas no colour complex develops in the absence of the inhibitor, indicating that starch is completely hydrolyzed by α-amylase.Plant extract of different concentration ranging from 0.5 - 10mg were dissolved using 1ml of DMSO (Dimethyl sulphoxide). To each of the test tube containing the plant extract add 200µl of PPA enzyme and incubate for 10minutes at 37°C. After incubation add 40µl of 1%Starch and again incubate at 37°C for 15 minutes. Then add 40µl of 1molar HCL to stop the enzymatic reaction, followed by addition of 100µl of iodine reagent. Control-1 representing 100% enzyme activity did not contain any plant extract. Absorbance was read at 620nm using UV Spectrophotometer 3, 5-dinitrosalicylic acid assay. The inhibition assay was performed using the chromogenic DNSA method. [18]The total assay mixture composed of 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride), 0.04 units of PPA solution and extracts at a concentration from 0.1-1.5 mg ml^{-1} (w/v) were incubated at 37°C for 10 min. After pre-incubation, 500 µl of 1% (v/v) starch solution in the above buffer was added to each tube and incubated at 37°C for 15 min. The reaction was terminated with 1.0 ml DNSA reagent, placed in boiling water bath for 5 min, cooled to room temperature, diluted and the absorbance measured at 540 nm. The control PPA at 0.21 Uml^{-1} represented 100% enzyme activity and did not contain any plant extract. To eliminate the absorbance produced by plant extract, appropriate extract controls with the extract in the reaction mixture except for the enzyme were also included. The known PPA inhibitor acarbose was used a positive control at a concentration range of 6.5 - 32.8 µgml^{-1}. One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of maltose from starch per min under the assay conditions. The IC_{50} values were determined from plots of percent inhibition versus log inhibitor concentration and calculated by logarithmic regression analysis from the mean inhibitory values. The IC_{50} values were defined as the concentration of the extract, containing the α-amylase inhibitor that inhibited 50% of the PPA activity. Percentage of inhibition was calculated by using the formula:

% Relative enzyme activity = (Enzyme activity of test/Enzyme activity of control) *100
% Inhibition in α-Amylase activity = (100 -% Relative enzyme activity)

DNS (3, 5 Dinitrosalicylic acid) assay

The determination of α-Amylase inhibition was carried out by quantifying the reducing sugar (glucose equivalent) liberated under the assay condition. The enzyme inhibitory activity was expressed as decrease in units of glucose liberated [3]Plant extract concentrations ranging from 0-4mg were incubated with 1ml of 1unit

PPA Enzyme for 30minnutes at 37°C.After incubation 1ml of 1% buffered starch was added and the mixture was further incubated for 10minutes at room temperature.The reaction was stopped by adding 1ml DNS reagent and the contents were heated in boiling water bath for 5minutes.Blank was prepared without plant extract and enzyme which was replaced with equal quantity of 0.1M phosphate buffer.Control representing 100% enzyme activity without plant extract was also included.The absorbance was read at 540nm using UV Spectrophotometer.The reducing sugar released from starch was estimated as glucose equivalent from standard graph.The antidiabetic property was determined through inhibition of alpha amylase which was expressed as percentage of inhibition and calculated by following equation

% Inhibition = Absorbance of control – Absorbance of test/Absorbance of control*100.

RESULTS AND DISCUSSION

Drugs that reduce post-prandial hyperglycemia by suppressing hydrolysis of starch such as HPA inhibitors have been found useful in the control of diabetes mellitus [19,20].There are many herbal formulations are identified and being used in ethanomedicine to treat diabetes and due to the lack of unremitting scientific substantiation such medicinal plants have lost their importance. In the present study, Ten indigenous antidiabetic medicinal plant polyherbal formulations were screened for the human pancreatic amylase inhibitory potential. Several studies performed in these plants state them to be hypoglycemic, but none of these plants have been studied or tested for human pancreatic α-amylase inhibitors in order to justify their hypoglycemic property. To search such potent bioactive compounds from medicinal plants the traditional extraction procedure like hot water extraction was performed to authenticate the inhibitory compound.Primary screening for α-amylase inhibition was performed based on starch-iodine colour complex formation. While the ethanol and hot water extracts showed significant (*p* value ≤ 0.05) HPA inhibitory activity (10 - 60.5%).

Standard drug	Concentrations (ug/ml)	% inhibition	Ic_{50} value
Acarbose	100	59.53	
	80	48.56	
	60	39.63	83.78
	40	28.34	
	20	22.11	
	10	19.10	

Table1
IC_{50} values of acarbose on HPA

Figure-1 shows Inhibition of HPA activity based on starch-iodine color assay by various solvent extracts of formulation-D at 0.1-1.5 mg ml^{-1} (w/v)

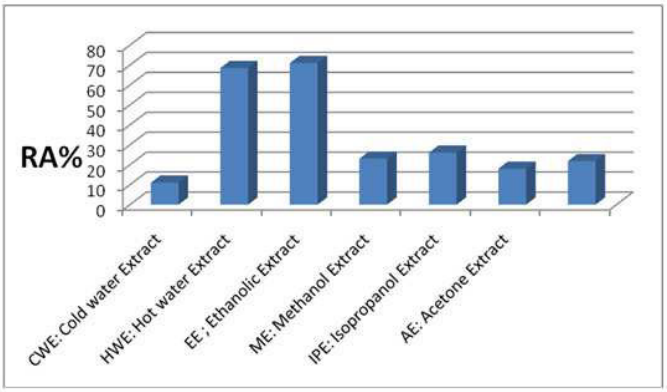

FIGURE 1
The percent relative enzyme activity (RA %) of Human pancreatic amylase (HPA) on the inhibition with different extracts. of formulation- D.

(a) Cold water extracts-CWE (b) Hot water extracts-HWE (c) Methanol extracts-ME (d) Isopropanol extracts-IPE (e) Methyl-tertiary-butyl-ether extracts-MTBE (f) Cyclohexane extracts-CHE of the listed plants. Pure Human pancreatic α-amylase serves as control. Respectively with α-amylase and starch.*In vitro* studies resulted in a significant ($p \leq 0.05$) decrease in the enzyme activities from 0.21 U to 0.09 U. It could thus be speculated that the extracts possess significant HPA inhibiting activity. Phytochemical analysis of these extracts revealed the presence of alkaloids, steroids, Tannins,flavonoids saponins and glycosides (Table-2).

Table 2
Phytochemical screening of formulation-D.

Test	CWE:	HWE	EE	ME:	IPE	AE	MTBE
Protein	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Carbohydrates (glycosides)	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Amino acids	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Alkaloids	+ve	+ve	+ve	-ve	-ve	-ve	-ve
Steroids & Terpenoids	S	T	T	S	S	S	S
Flavonoids	+ve	+ve	+ve	-ve	+ve	-ve	-ve
Phenol	+ve	+ve	+ve	-ve		+ve	-ve
Tannins	-ve	+ve	+ve	-ve	+ve	-ve	+ve

CWE: Cold water Extract, HWE: Hot water Extract, EE; Ethanolic Extract, ME: Methanol Extract, IPE; Isopropanol Extract, AE: Acetone Extract, MTBE: Methyl-tertiary-butyl Ether

CONCLUSION

Since the Indian population has long been using all the 10 plants for food and medicinal purposes, they form a part of the local pharmacopoeia. Our results suggest that one of the targets for hypoglycemia property of formulation is human pancreatic amylase inhibition. However, isolation and characterization of the compounds responsible for this inhibitory activity as well as *in vivo* studies need to be performed to confirm these observations. These phytochemical (s) /bioactive principle from the plants responsible for the HPA inhibition are currently being isolated and characterized. This extract seems to possess potential HPA inhibitory activity and could be a good candidate to carry out further *in vivo* studies.

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