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ENZYME INHIBITORS FROM *PRUNUS PERSICA* (L.) BATSCH: AN ALTERNATE APPROACH TO TREAT DIABETES

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ABSTRACT

In India, the prevalence of diabetes mellitus is alarmingly increasing and it is the need of the hour to be addressed appropriately. In this area of study, herbal remedies are considered to be the convenient method due to their traditional acceptability and availability, low costs, lesser side effects. Potent inhibitors found in some vegetables, herbs and fruits have been known to be effective for diabetes prevention. The ethanolic extract of the fruit was tested for their *in vitro* alpha amylase and glucosidase inhibitory activities to establish anti-diabetic potentials at digestive levels. Susceptibility of LDL oxidation was carried out to check the potency of the fruit to inhibit or decrease the susceptibility of LDL to its oxidized state. The inhibition to develop a free radical environment instead to pave way to bind to its active site and indulge in the formation of erythrocytes by the fruit extract was focused in haemoglobin glycosylation method. The hydrogen peroxide induced haemolysis exhibit rich antioxidative and protective effect of fruit extract to prevent diseases associated with oxidative stress. The result depicts the promising action of *Prunus persica* as an effective candidate in preventing the effects hyperglycemia.

KEY WORDS: ***Prunus persica*, diabetes mellitus, oxidative stress.**

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INTRODUCTION

Diabetes is a most common endocrine disorder characterized by disturbance of carbohydrate, fat and protein metabolism with multiple complications and premature mortality, accounting for at least 10% of total health care expenditure in many countries[1]. It is evidenced by hyperglycemia resulting from defects in insulin secretion, insulin action or both[2]. In India, between 1995 and 2025, the diabetics are projected to rise from 19 to 57 million. Current treatment for type 2 diabetes remains inadequate, prevention is preferable[3]. Modern medicines such as biguanides, sulfonylureas, and thiozolidinediones are available for the treatment of diabetes. However, they also have undesired effects associated with their uses[4]. Alternate medicines, predominantly herbal drugs are preferable for the treatment of diabetes. Common advantages of herbal drugs are effectiveness, safety and acceptability[5]. Alpha-amylase acts upon large polysaccharides (starch) at internal bonds. Suppression of the mammalian alpha-amylase enzyme in the human digestive system would delay the degradation of starch and oligosaccharides before they can be absorbed. This would decrease the absorption of glucose and consequently reduce postprandial blood glucose level[6]. Recently it has been shown that phenolics play a role in mediating alpha-amylase inhibition and therefore it has potential to contribute to the management of type 2 diabetes[7]. The enzyme alpha-glucosidase catalyses the final step of glucose absorption in the intestine during the digestive process of carbohydrates, and hence alpha glucosidase inhibitors could retard the rapid utilization of dietary carbohydrates and suppress postprandial hyperglycemia[8]. The possibility of clinical use of such inhibitors for diabetic or obese patients has been attempted by acarbose, which has been shown to effectively reduce the intestinal absorption of sugars in humans [9, 10]. During metabolism, oxygen radicals may be produced, and under insufficient antioxidant capacity, these radicals may also trigger lipid peroxidation, increasing susceptibility of LDL to oxidation. Changes in LDL size and LDL oxidizability were induced by hypertriglyceridemia. As HDL inhibits the oxidative modification of LDL,[11] its reduction in diabetic patients could to influence the susceptibility of LDL to oxidation. There is evidence that glycosylation of hemoglobin impairs nitric oxide (NO) related relaxation of human mesenteric vessels[12] and it has been shown that Hb glycosylation alters NO binding to Hb thiols, lowering NO bioavailability and impairing vasodilation in rabbit aortic rings[13]. The aim of this study to evaluate for the protective antidiabetic action of *Prunus persica* (L.) Batsch belonging to the family Rosaceae - native of China by inhibiting the formation of oxidative stress in the body.

MATERIALS AND METHODS

Plant Materials

The fruit *Prunus persica* (L.) Batsch with uniformity in size, shape and maturity with no defects were procured from The Nilgiris and Coimbatore, Tamil Nadu between the months of December and March were washed in clean potable water. Taxonomic identification and confirmation of the plants were made by Dr. G.V.S. Murthy, Botanical Survey of India, Coimbatore, Tamil Nadu and a voucher specimen BSI/SRC/5/23/2010/Tech.-2147 is deposited in the Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India for future reference.

Inhibition of α-amylase activity

The α-amylase inhibition assay was adapted by modified method[14]. The starch solution (0.5% w/v) was obtained by boiling and stirring 0.25 g of potato in 50 ml of deionized water for 15 min. The enzyme solution (0.5 unit/ml) was prepared by mixing 0.001 g of α-amylase (EC 3.2.1.1) in 100 ml of 20 mM sodium phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride. The extracts were dissolved in DMSO to give concentrations from 10 to 100 mg/ml (10, 20, 40, 60, 80, 100 mg/ml). The coloured reagent contains 96 mM of 3, 5-dinitrosalicylic acid (20 ml), 5.31 M sodium potassium tartarate in 2 M sodium hydroxide (8 ml) and deionized water (12 ml). 1 ml of fruit extract and 1 ml enzyme solution were mixed in a tube and incubated at 25°C for 30 min. To 1 ml of this mixture add 1 ml of starch solution and the tube incubated at 25°C for 3 min. Then, 1 ml of

the coloured reagent was added and the closed tube was placed into an 85°C water bath. After 15 min, the reaction mixture was removed from the water bath and cooled thereafter, diluted with 9 ml distilled water and the absorbance value was determined at 540 nm. Acarbose was used as positive control. The inhibition percentage of α-amylase was assessed by the following formula: α-amylase (%) = 1- (Control-Sample)/Control) ×100.

Inhibition of α-glucosidase activity

The enzyme inhibition activity for α-glucosidase was assessed according to the method reported with minor modifications [15]. The reaction mixture contained 50 µl of 0.1 M phosphate buffer (pH 7.0), 25 µl of 0.5 mM 4-nitrophenyl α-D glucopyranoside, 10 µl of test sample at various concentrations and 25 µl of α-glucosidase (0.2 Unit/ml). This reaction mixture was then incubated at 37°C for 30 min. The reaction was terminated by adding 100 µl of 0.2 M sodium carbonate solution. The enzymatic hydrolysis of substrate was monitored by the amount of p-nitrophenol released in the reaction mixture at 410 nm using a microplate reader. Controls were conducted in an identical manner replacing the fruit extracts with methanol. Acarbose was used as positive control. All experiments were carried out in triplicates. The inhibition percentage of α-glucosidase was assessed by the following formula: % Inhibition = {1-(sample absorbance/control absorbance)} × 100

Effect on susceptibility of LDL oxidation

The susceptibility of LDL to *in vitro* oxidation was assessed by the technique described by Regnstrom *et al*., [16]. The LDL preparation was dialysed against a 100-fold volume of 0.02 mol/l phosphate buffer (pH 7.4)/0.16 mol/l NaCl, (dialysis buffer) purged with liquid nitrogen for 15 h at 4°C in darkness. The buffer was changed three times. EDTA-free LDL was diluted in dialysis buffer to a final concentration of 25 µg/ml and oxidation initiated by addition of a freshly prepared aqueous $CuSO_4$ solution to a final concentration of 1.66 µmol/l. LDL oxidation kinetics were monitored by the change in 234 nm absorbance at 30°C on a Shimadzu spectrophotometer UV-3100. The dienes formed during LDL oxidation produce an absorbance spectrum with a distinct peak at 234 nm with essentially no inter individual variation, the initial absorbance at 234nm was taken as the baseline and the changes in absorbance in the presence of only fruit juice (50 µg) of fruit extract (test) and also in their absence (control) were recorded every 30 min for 4 h. The differences of absorbance in the tests and control tubes after the lag phase were used as the measure of LDL susceptibility to oxidation.

Inhibition of haemoglobin glycosylation activity

Asgary *et al*., method was followed for the effect on haemoglobin glycosylation[17]. Blood from normal volunteers was drawn using EDTA as an anticoagulant. The red blood cells were washed thrice with 0.14 M NaCl solution. Then 1 volume of red blood cell suspension was lysed with 2 volume of 0.01 M phosphate buffer, pH 7.4, and 0.5 volumes CCl_4. After lysing, the haemolysate was freed from debris by centrifugation, the upper layer was separated and the haemoglobin concentration has measured by the Drabkin method[18,19]. One ml of haemoglobin solution (5g/100 ml) and 1 ml of solution containing glucose (2g/100ml) and gentamycin (20 mg/100 ml) in 0.01 M phosphate buffer, pH 7.4, were incubated in the dark at room temperature. Then, the glycosylation degree of haemoglobin in the presence of different products of fruit extracts (50µg) and also in their absence was measured by the colorimetric method [19, 20].

Inhibition of H_2O_2 red cell lysis assay

The assay was carried out based on the procedure reported by Su *et al*.,[21]. The blood sample was obtained from rat and made for 0.5% erythrocyte suspension for the assay. The reaction mixture consisted of 0.1 ml of erythrocyte suspension (0.5%), 1.0ml of extract (0.1 mg ml^{-1}) and 0.1 ml of H_2O_2 (100 mM). The mixture was incubated at 37°C for 60 min and then 8.4 ml of distilled water was added to the mixture and centrifuged at 1000 rpm for 10 min. The absorbance of the supernanant was read at 415 nm. The percentage of erythrocyte hemolysis inhibition effect was calculated by the following equation:

Inhibition rate (%) = {1-(A1-A2)/A0*100}

Where, A0 is the absorbance of the supernatant without extract, A1 is the absorbance of the extract addition and A2 is the absorbance of extract solution.

STATISTICAL ANALYSIS

The results were expressed as Mean ± Standard Deviation.

RESULTS AND DISCUSSION

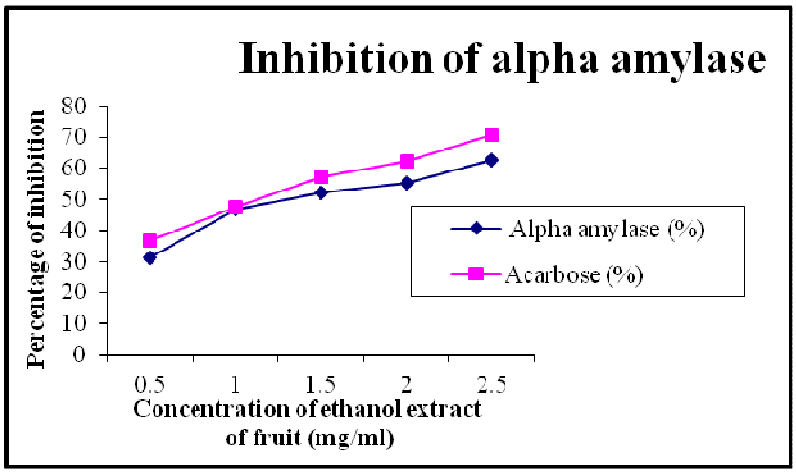
One of the most serious, chronic diseases is Diabetes mellitus. Decreasing post-prandial hyperglycemia is one of the therapeutic approaches in which retardation of absorption of glucose takes place by inhibiting carbohydrate hydrolyzing enzymes. The fruit *Prunus persica* (L.) Batsch significant for their antioxidant potential was selected for the study. The whole fruit was extracted in ethanol checked by for their inhibitory potentials against acarbose and gallic acid as positive controls.

In vitro alpha-amylase inhibition activity

The enzyme α amylase begins the process of starch digestion and breaks them into small pieces with two or three glucose units. The alpha amylase inhibitors, which interfere with enzymatic action in the small intestine, could slow the liberation of maltose from starch, resulting in delaying maltose conversion to glucose and decreasing postprandial plasma glucose levels[23]. Some phenolic compounds in sweet potato, strawberry, raspberry, olive oil, pears, coca and lentils are reported to be an effective human α amylase inhibitors [22]. Flavonoids and anthocyanin are also reported to have inhibitory activity against α-amylase[22]. In the present study, the fruit extract found to possess significant inhibitory effects on starch break-down *in vitro*. At a concentration of 0.5 mg/ml the activity of alpha amylase was 31.28% whereas in 2.5mg/ml it was 62.67%. They also showed dose-dependent increase in alpha-amylase inhibitory activity (Figure 1). The values were comparable with that of standard acarbose.

Figure 1

Inhibitory activity of ethanol extract of the fruit of Prunus persica (L.) on alpha amyalse

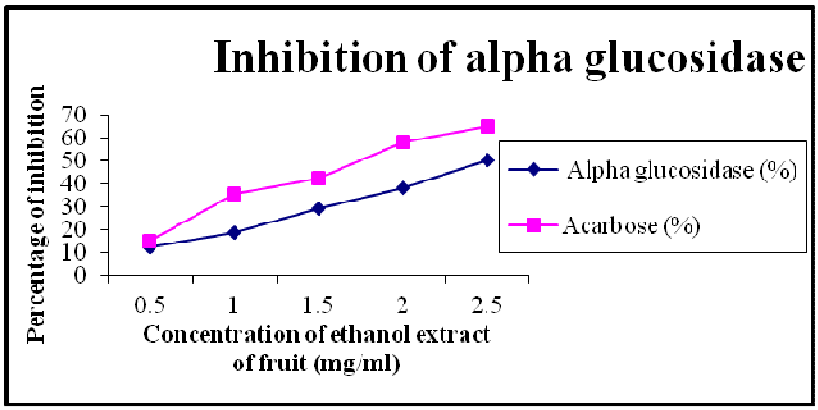


In vitro α- glucosidase inhibition activity

Inhibition of alpha glucosidase is useful in treatment of both postprandial hyperglycemia and hyperinsulinaemia, and thereby in improving sensitivity to insulin. Alpha glucosidase is a key enzyme in carbohydrate digestion in the small intestine [24, 25, 26]. It is further helpful in reducing the stress on beta cells of the pancreas. The inhibitory activity of alpha glucosidase enzyme in the small intestine is the rate limiting step in the conversion of oligosaccharide and disaccharide to monosaccharide, necessary for gastrointestinal absorption. The fruit tested

have considerably high alpha glucosidase inhibitory activity. The value indicated that the fruit extract tested was more active like Acarbose, the positive control. The values of the fruit extract ranged from 0.5 mg/ml to 2.5 mg/ml. The value resulting from acarbose was about 65.25%. At 2.5 mg/ml, *Prunus persica* (L.) Batsch inhibited about 50.23% activity of alpha glucosidase activity (Figure 2).

Figure 2
Inhibitory activity of ethanol extract of the fruit of Prunus persica (L.) on alpha glucosidase

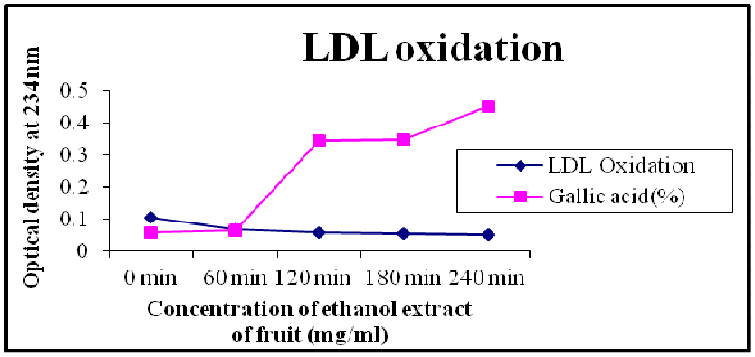


The percentage inhibition concentrations of *Prunus persica* (L.) Batsch on alpha glucosidase showed a concentration-dependent reduction. The lowest concentration of the peach extracts showed a minimum inhibition of nearly 12.25%. Therefore, the antidiabetic effect of *P.persica* (L.) Batsch might attribute to its inhibitory effect against alpha glucosidase that retards the digestion of carbohydrate to delay the postprandial rise in blood glucose. From the results, it can be concluded that ethanolic extract of fruit of *P.persica* (L.) Batsch can be an excellent choice of drug with alpha-glucosidase inhibitory activity and can thus reduce the rate of digestion and absorption of carbohydrates.

Effect on susceptibility of LDL oxidation

Diet may play an important role in health promotion and disease prevention[27]. Formation of Conjugated Diene (CD) serves as a marker of the oxidation process[28]. The oxidized LDL contributes to platelet aggregation, smooth muscle cell proliferation and LDL oxidation in patients with hypercholesterolemia, hypertension, diabetes mellitus, chronic renal failure and in smokers [29]. Malvidin, one of the important components of red grapes, was previously found to be more active antioxidant in protecting LDL against oxidation than the corresponding glucoside malvin[30]. Thus, the consumption of natural antioxidants is beneficial in preventing diabetes. Figure 3 represents the time course of LDL oxidation where several LDL concentrations were used. The decrease in absorbance observed after 0 min of incubation of 1mg/ml LDL may reflect further oxidation of conjugated dienes; infact, they represent an intermediate stage in lipid peroxidation and are transformed into other oxidation products. The formation of conjugated dienes is decreased by *P.persica* (L.) Batsch fruit extract. The results are considered to be noteworthy when compared to the findings of other studies concerning antioxidants[31].

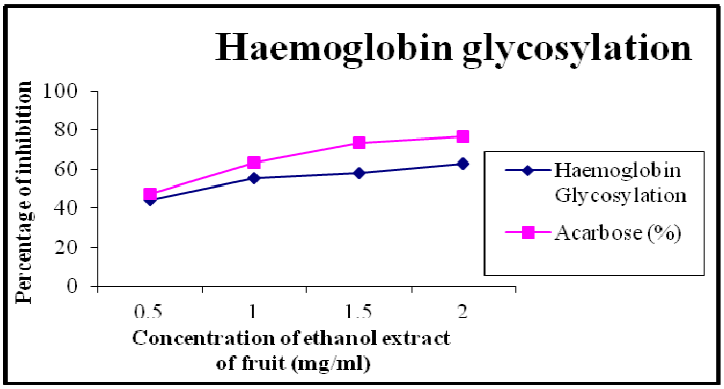
Figure 3
Inhibitory activity of ethanol extract of the fruit of Prunus persica (L.) on LDL oxidation



Effect on haemoglobin glycosylation

Evidence indicates that phenolic compounds are potent inhibitors of LDL oxidation, it is not yet clear whether these compounds are absorbed in humans in a metabolically active form. Recent studies claimed antioxidant activity of red wine *in vivo*[32,33,34]. However these studies used nonspecific methods to determine antioxidant activity. During diabetes the excess glucose present in the blood reacts with hemoglobin to form glycosylated hemoglobin (glucose bound hemoglobin). The rate of glycosylation is directly proportional to concentration of blood glucose and with improvement of glycemic control the glycosylated hemoglobin also decreases [35]**.** Hence the estimation of glycosylation of hemoglobin is a well established parameter useful in the management and prognosis of the disease[36]. The percentage inhibition of glycosylation is dose dependent, as the dose increases, inhibition also increases (Figure 4).

Figure 4
Inhibitory activity of ethanol extract of the fruit of Prunus persica (L.) on haemoglobin glycosylation



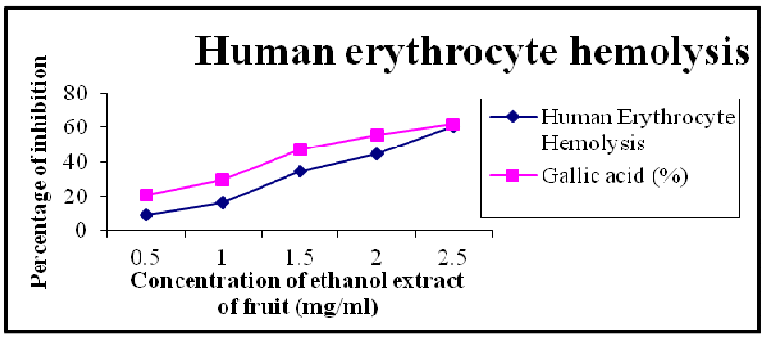
The effect of the extract on hemoglycosylation was demonstrated by the degree of glycosylation of hemoglobin in the presence of extract. Ethanol extract of *P.persica* exhibited 32.78% to 62.9% inhibition of hemoglycosylation and standard acarbose showed 40.03% to 76.74% inhibition at 0.5 to 2.5 mg/ml concentrations respectively. As the concentration of the drug increase, the formation of glucose-hemoglobin complex decreases and free haemoglobin increases, which slow the inhibition of glycosylated hemoglobin. The activity of ethanol extract of the fruit *Prunus persica* (L.) Batsch was found to be good as the standard drug acarbose.

Protective effects on hemolysis in H_2O_2-lysis test

The *in vitro* oxidative hemolysis of erythrocytes was used as a model to study the free radical-induced damage of biological membranes and the protective effect of the extracts of *P.persica* (L.) Batsch. The interference of the extract components into the membrane bilayer and the

resulting restriction on its fluidity might hinder the diffusion of H_2O_2 and its consequent damaging effects [37]. It can be concluded that the extract tested have the anti-oxidative potential for the H_2O_2- induced hemolysis. The extracts would not be toxic for the biomembrane but would play a protective role for erythrocytes. Figure 5. exhibited the effect of the fruit extract. The percentage of H_2O_2 induced hemolysis by the fruit extract was observed to be 9.21 to 60.18% to that of standard which ranged from 20.49 to 61.67% at 0.5 to 2.5 mg/ml concentration. It was found that it did not show any harmful effect on erythrocytes. The results showed a best protective activity against the erythrocytes hemolysis at a concentration of 2.5 mg/ml. There was a significant difference in hemolytic reduction based on the concentration. However, a percentage of hemolytic inhibition of the extract was not significantly higher than acarbose used as positive control in terms of hemolysis reduction.

Figure 5
Inhibitory activity of ethanol extract of the fruit of Prunus persica (L.) on H_2O_2 induced hemolysis



CONCLUSION

In vitro studies on the ethanolic extract of *Prunus persica* (L.) Batsch provide a more detailed view of antidiabetic properties and it is first of its kind. This study demonstrated that an appreciable alpha glucosidase and alpha amylase inhibitory activity together with its potent antioxidant potential can make it the future safe drug of choice in diabetes treatment. The fruit exhibited a good LDL oxidation – decreased sensitivity which in turn helps in the prevention of vascular complications in diabetics. Therefore, regular diet, improvement of glycemic control and antioxidant intake in diabetics could maintain antioxidant defense and reduce oxidative stress. The fruit extract also possess protective effects on human oxidative hemolysis. Future studies on the fruit extract might help in the isolation and characterization and identification of active compounds which are the new lead molecules for natural inhibitors. In-depth research of insulinotrophic activity will address the established molecular mechanism by which the fruit and its active compounds regulate glucose homeostasis and hence validating its role as a valid alternative for synthetic agents.

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