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Research Article

THE STUDY OF POTENTIALITY OF *PICRORHIZA KURROA* ROOT PROTEINS TO INHIBIT FREE RADICALS AND A-AMYLASE ENZYME

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ABSTRACT

Objective: To prevent oxidative damage to cells caused by free radicals that are responsible for many diseases, such as atherosclerosis, diabetes and cancer. *Picrorhiza kurroa* (*Katuki*) is an ayurvedic medicinal plant used in the current study to evaluate the effectiveness of its root proteins precipitated at 60% ammonium sulfate, and further dialyzed protein sample was investigated for scavenging free radicals and also inhibiting alpha-amylase which helps to control postprandial hyperglycemia.

Methods: The present study was done by Anti-oxidant methods 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, hydroxyl scavenging method, nitric oxide (NO) method, total anti-oxidant method , α-amylase inhibition and its kinetic studies by DNS method.

Results: The protein significantly inhibited DPPH radicals 66.19±6.08%, hydroxyl radicals 59.18±9.05%, nitric oxide radicals 45.38±19.52% and total antioxidants present is 82.91±2.81% compared with standard ascorbic acid 93.67±3.91%. The protein extract (60%) inhibited rat pancreatic α-amylase 41.62±22.3% compared with acarbose standard 91.09±1.8%. These results provide valuable information that *P. kurroa* proteins exhibits good antioxidant and anti-diabetic property.

Conclusion: *Picrorhiza kurroa* root proteins (60%) posses potent antioxidant and antidiabetic effects. Kinetic studies showed it is a non competitive inhibition. alpha-amylase inhibitor is a protein which inhibits rat pancreatic alpha-amylase specifically at a optimum temperature 37°c, pH 6.9, and time 35'.

Keywords: *Picrorhiza kurroa*, Free radicals, Anti-oxidant, Anti-diabetic, α-amylase.

INTRODUCTION

Picrorhiza kurroa is a small perennial herb also called katuki Indian name from the scrophulariaceae family. The rhizome of Picrorhiza has been traditionally used to treat worms, constipation, low fever, scorpion sting, asthma and ailments affecting the liver [1].

Current research is focused on anti-oxidants and anti-diabetic effects. Study of anti-oxidants and its importance is necessary because they are the molecules that inhibits the oxidation of other molecules. oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell [2]. In diabetes, there is increased oxidative stress in combination with reduced anti-oxidant status, this result in greater vulnerability to the damaging effects of free radical [3].

The most important active constituents of Picrorhiza are the iridoid glycoside picrosides I, II, III, and kutkoside, known collectively as kutkin. Many other active constituents have been identified, including nine cucurbitacin glycosides, apocynin, and drosin. The roots of *P. kurroa* yielded, on extraction with petroleum ether, kutchina-glucosidal bitter principle. It was finally reported to be a stable mixture of two glycosides viz., picroside-1 (6"O-cinnamoylacatolpol) and a new glucoside kutkoside, characterised as 10-O-D-mannitol, kutkiol and kutki sterol and a ketone which was found to be identical with apocynin isolated from *Apocynum canabinum*. The synthesis of apocynic and its homologs has been reported. In a preliminary study, the root and rhizomes of *P. kurroa* have been reported to contain flavonoid and devoid of saponin and alkaloid.

Epidemiological, clinical and experimental studies indicate an association between oxidative stress, low-grade inflammation and both the development of Type 2 diabetes mellitus (DM2) and its late stage complications [4].

DM is a chronic metabolic disease with the highest rates of prevalence and mortality worldwide that is caused by an absolute or relative lack of insulin and or reduced insulin activity [5]. It is characterized by hyperglycemia and long-term complications affecting the eyes, kidneys, nerves, and blood vessels, and is the most common endocrine disorder [6].

Identifying the potentiality of Picrorhiza proteins on inhibiting α-amylase enzyme helps to lower postprandial blood glucose level for Type 2 diabetic patients.

These studies could contribute significantly to the pharmacological properties, and results suggest that plant proteins may possess best biological and medicinal value.

But there are no *P. kurroa* proteins systematic studies on the anti-diabetic and anti-oxidant activity, which we now present in this communication [7]. Therefore, aim of this study is to explore potentiality of *P. kurroa* proteins on anti-oxidant and anti-diabetic effects that is carried out *in-vitro*.

MATERIALS AND METHODS

Chemicals

Napthylethylene diamine dihydrochloride, ethylenediaminetetraacetic acid (EDTA), deoxyribose, ferric chloride, ammoniumsulfate, ammonium persulfate, acrylamidesolution, ammonium molybdate, sodium nitropruside, sulfanilamide, thiobarbutiric acid, Starch, ascorbic acid, sulfuric acid, Nacl, Kcl, SDS, Na_2HPO_4, NaH_2PO_4, TEMED, 3,5-dinitrosalicylic acid (DNS), H_3PO_4, Tris-Hcl, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-trichloroanisole (TCA), H_2O_2, maltose, were of analytical grade and are obtained from SRL, Rankem and Merck.

Ammonium sulfate fractionation
The prepared crude extract was subjected to ammonium sulfate precipitation. In this step, pellet was formed after incubation for 4 hrs at 4°C. The pellet was collected by centrifugation at 3,000 rpm for 10 minutes at 0°C was air-dried and re-extracted with cold 10% TCA and centrifuged at 3,000 rpm for 10 minutes. Protein residue was collected, washed until acid-free and then air dried sample used for protein estimation [8].

Dialysis fraction
The ammonium sulfate precipitated sample was dissolved in a suitable amount of double distilled water. The sample containing salts was removed by extensive dialysis at 4°C with 6 changes for 3 days against double distilled water (pH=7.4) at 2.5 Kda molecular cutoff. The complete removal of salts was confirmed by checking with Nessler's test by 1:1 ratio of sample and Nessler's reagent according to Meloan and Kiser [9] and it was performed.

Anti-oxidant assays

DPPH assay

The dialyzed protein sample was mixed with DPPH (0.1 mm) in methanol solution. After 20 minutes, incubation at room temperature absorbance was read at 517 nm. The scavenging percentage of DPPH was calculated according to the following equation:

RSA %=(([Abs sample-Abs blank] × 100)/Abs control). Ascorbic acid standard was prepared of concentration 10 µg/0.1 ml. Blank=water (1 ml) plus sample solution (2.0 ml), negative control=DPPH solution (1.0 ml, 0.25 mm) plus methanol (2.0 ml) [10].

Nitric oxide radical scavenging assay
At physiological pH, NO generated from aqueous sodium nitroprusside solution interacts with oxygen to produce nitrite ions, which may be quantified by the griess illosvoy reaction [11].

Hydroxyl radical scavenging assay
The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with thiobarbituric acid. Hydroxyl radical was generated by the Fe3+-ascorbate-EDTA-H_2O_2 system (the Fenton reaction) [12].

Evaluation of total anti-oxidant and reducing power capacities
The total anti-oxidant activity of the extract was measured using a modified version of the method described by [13]. In which the anti-oxidant activity is expressed as the absorbance of samples measured at 695 nm. While its reducing power, as a reduction of Fe3+ to Fe2+, was measured by method of [14] that modified by [15].

Inhibition of α-amylase
α-amylase inhibitors inhibit the action of α-amylase enzyme leading to a reduction in starch hydrolysis that shows beneficial effects on glycemic index control in diabetic patients. The source of α-amylase studied was rat pancreas.

Extraction procedure of α-amylase from rat pancreas

Rat pancreas was homogenized with 10 mm ice cold phosphate buffer containing 100 mm Nacl (1:10 dilution; w/v) and the appropriate amount of protease inhibitors. The homogenate was centrifuged for 10 minutes at 10,000 rpm. And the supernatant was taken as a source of the enzyme [16].

α-amylase inhibition assay

The porcine pancreatic α-amylase was incubated with dialyzed 60% protein sample. One percent starch was used as a substrate. The plant extracts without α-amylase were used as controls, and the test reading were subtracted from the absorbance of these controls [16].

Kinetics of rat pancreatic α-amylase

Effect of pH on α-amylase activity

α-amylase activity from the rat pancreas was determined using 1% soluble starch as substrate at a pH range from 4.4 to 9 using 25 mm sodium phosphate buffer. The optimum pH for enzyme activity was determined using DNS reagent [17].

Optimum temperature

Effect of temperature on α-amylase activity: A temperature gradient was employed in order to determine the α-amylase activity. Buffer, substrate solution, and dialyzed samples were incubated at different temperatures between 10°C and 80°C. The optimum temperature was determined using DNS reagent for enzyme activity.

Optimum time for the inhibition

Different incubation time intervals was performed by pre-incubating the enzyme by adding the assay mixture containing buffer and starch (20 mm phosphate buffer pH 6.9.) at different time 10, 25, 30, 40, 50 and 60 minutes. The optimum time was determined using DNS reagent for enzyme activity.

Effect of substrate concentration on α-amylase inhibition activity

Starch concentration for α-amylase activity was varied from 5.0 to 25.0 mg/ml in presence and absence of inhibitor, and 0.2 m phosphate buffer (pH 6.9) and enzyme activity was performed. Kinetic data were transformed into Lineweaver–burk (L-B) plots. The km value was calculated by L-B plot [18].

Statistical analysis
Results were expressed as the Mean±standard error of the mean. The significance of the differences between the means of the control and the experimental results was established by analysis of variance (ANOVA single factor) with a ($p<0.05$).

RESULTS AND DISCUSSION

The DPPH method measures the stable free radical by virtue of delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerize as would be the case with most other free radicals [19].

The delocalization also gives rise to deep violet color, characterized by an absorption band in ethanol solution centered at about 520 nm. The effects of different concentrations 3.25 µg, 6.5 µg, 9.75 µg,13 µg of protein extract on DPPH radical as shown in Fig. 1 and were found to be 59.18%, 61.73%, 69.38%, 74.48% scavenging activity.

Reference standard ascorbic acid showed 89% scavenging activity at a concentration of 10 µg (Fig. 1).

These results clearly indicate proteins have good anti-oxidant activities by statistical significant value **$p<0.01$ and mean standard deviation (SD) 66.19±6.08%.

Hydroxyl radical is particularly reactive and dangerous. Hydroxyl radical damages proteins in various ways and damages membrane by initiating the oxidation of fatty acid in membrane lipids, a process termed lipid peroxidation. It also damages nucleic acid, both by causing polynucleotide strand breakage and by changing the structure of DNA bases [20].

Effects of hydroxyl scavenging activity at different protein concentration 65 µg, 130 µg, 195 µg, 260 µg, were found to be 48.84%, 56.34%, 61.12%, 70.45%. Reference standard ascorbic acid showed 97.60% at 10 µg concentration (Fig. 2).

Proteins hydroxyl scavenging activity were statistically significant **$p<0.01$ and mean SD 59.18±9.05%. NO scavenging assay is based

on the scavenging ability of the protein extract as well as ascorbic acid, which is used as a standard. The scavenging of NO was found to increase in dose-dependent manner [21]. Maximum inhibition of NO was 75.32% at 26 µg concentration, and 10 µg ascorbic acid exhibited 88.83% inhibition (Fig. 3).

Proteins exhibit statistically significant value **p<0.01 and mean SD 45.38±19.5% (Table 1). Measurement of total anti-oxidant activity in body fluids in patients with atherosclerosis, reperfusion injury, septic shock, diabetes is of important prognostic and diagnostic value [22]. The ability of protein extracts to possess total anti-oxidants was found to be 86.31% maximum at 975 µg (Fig. 4).

Reference standard showed 91.01% at 10 µg. Statistical values were 82.91±2.81% mean SD and **p<0.01. α-amylase inhibitors inhibit the action of α-amylase enzyme leading to a reduction in starch hydrolysis that shows beneficial effects on glycemic index control in diabetic patients. Different concentrations of protein extracts 16.25 µg, 32.5 µg, 81.25 µg, 162.5 µg were examined by DNS method, α-amylase inhibition found to be 20.83%, 29.16%, 37.50%, 79%. Reference standard showed 91% at a concentration of 75 µg (Fig. 5).

Table 1

Ascorbic acid (standard) 93.67±3.91%		
Antioxidant assays	**Mean±SD%**	****p value(<0.01)**
DPPH assay	66.19±6.08	0.000426
Hydroxyl radical scavenging assay	59.18± 9.05	0.000426
Nitric oxide radical scavenging assay	45.38±19.52	0.0055
Total antioxidant assay	82.91±2.81	0.0043
Acarbose (standard) 91.01±1.8%		
Antidiabetic assay	**Mean±SD%**	***p value (<0.05)**
α-amylase Inhibition assay	41.62±22.3	0.01

SD: Standard deviation

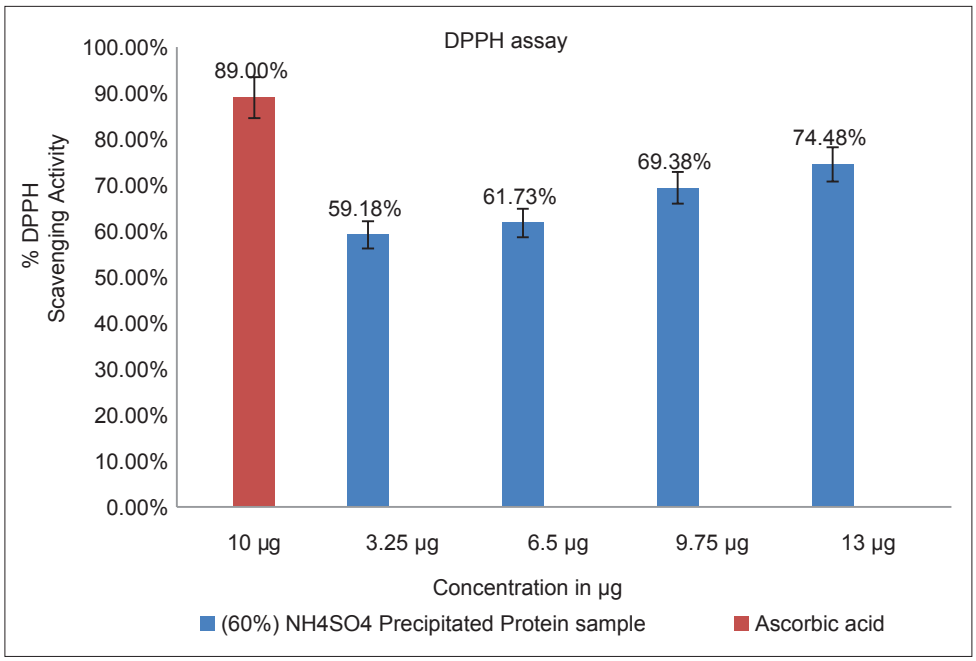

Fig. 1: 2,2-diphenyl-1-picrylhydrazyl scavenging activity of Picrorhiza kurroa

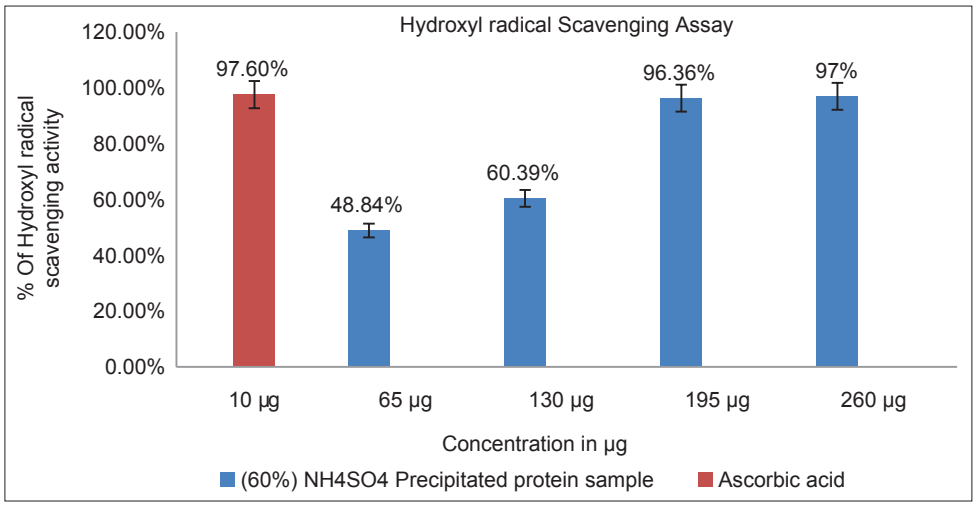

Fig. 2: Hydroxyl radical scavenging activity of Picrorhiza kurroa

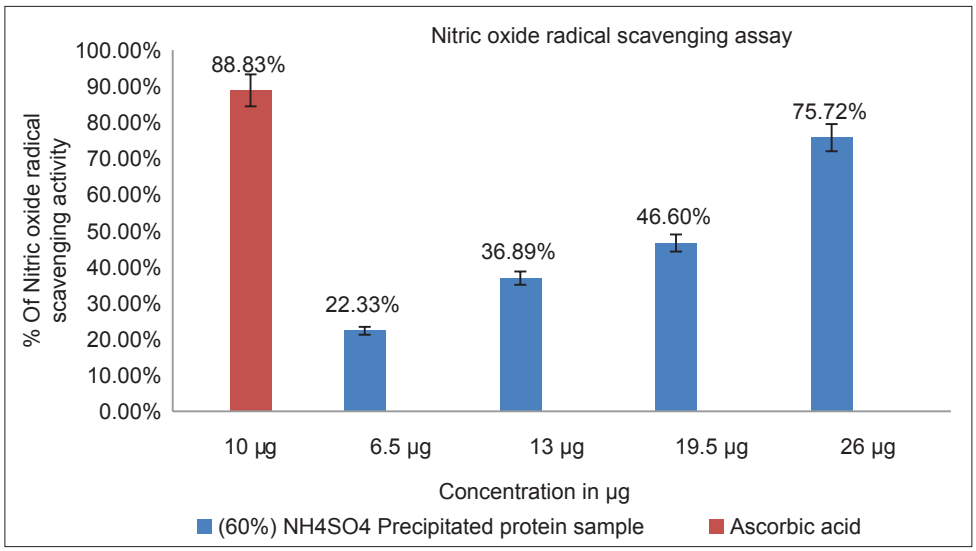

Fig. 3: Nitric oxide radical scavenging activity of Picrorhiza kurroa

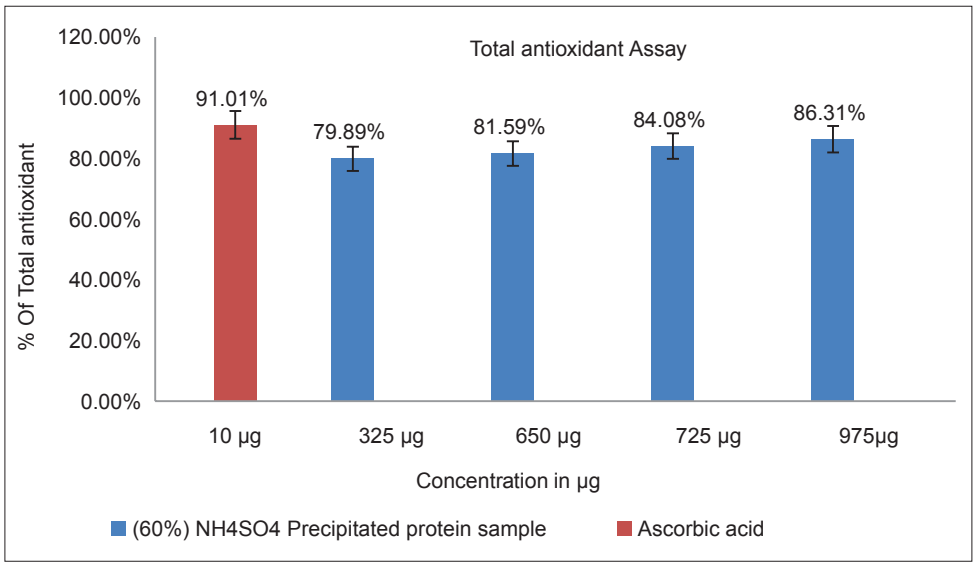

Fig. 4: Total antioxidant activity of Picrorhiza kurroa

Study of rat pancreatic α-amylase kinetics

Effect of temperature on α-amylase activity to observe the effect of temperature on α-amylase, starch hydrolyzing activity was assayed at 10-80°C. The α-amylase from rat pancreas has showed enzyme activity in a wide range of temperature but the optimum temperature was 37°C, with a progressive decline after that temperature and a complete inactivation at 80°C (Fig. 6).

Kinetic study of rat pancreatic α-amylase

A reduction in enzyme activity (28%) was observed at 70°C, whereas at 80°C the enzyme was inactivated, it may be due to the fact that, as the temperature rises, a progressive inactivation of enzyme occurs, due to thermal inactivation of proteins in their structure, or it may also be due to incorrect conformation of protein, hydrolysis of the peptide chain, destruction of amino acid, or aggregation (Saeeda Bano *et al*., 2011). The effect of pH on Pancreatic α-amylase activity determined at various pH ranging from pH 4.4 to pH 10.0 (Fig. 7).

The optimum pH for α-amylase activity was 6.9 at this pH enzyme activity is more. The change in the pH will affect the polar and non-polar intra molecular attractive and repulsive forces and alter the shape of the enzyme and the active site as well to the point where the substrate molecule could no longer fit and chemical change would be inhibited by taking place as efficiently or not at all.

Effect of time on α-amylase activity examined by monitoring the amount of product formed at several time intervals, the efficiency of enzymes and the optimum incubation time and initial velocity can be determined. Under defined conditions of enzyme concentration, substrate concentration, pH, temperature the activity of the enzyme is measured at different incubation period 7, 14, 21, 28, 35, 42 minutes. Optimum time for α-amylase activity was 35 minutes at this time it showed maximum activity (Fig. 8).

Km and Vmax are determined by the use of L-B plot. The enzyme catalyzed reaction is carried out in the presence of inhibitor as well in its absence. In the absence of inhibitor (protein extract), the value of km=−0.8 and value of Vmax=1; and in the presence of inhibitor (protein extract), km=−0.8 and Vmax=0.8 the nature of inhibition obtained was noncompetitive (Fig. 9).

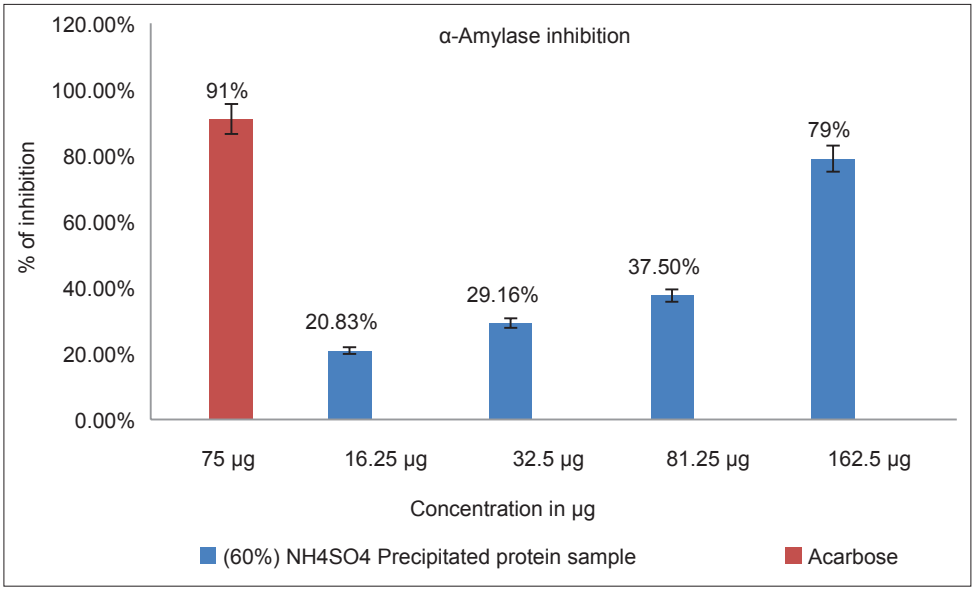

Fig. 5: α-amylase inhibition activity, enzyme source rat pancreas, Picrorhiza proteins are used as inhibitors. The statistical significant value mean standard deviation 41.62±22.3% and *p<0.05 indicates proteins can efficiently inhibit α-amylase enzyme

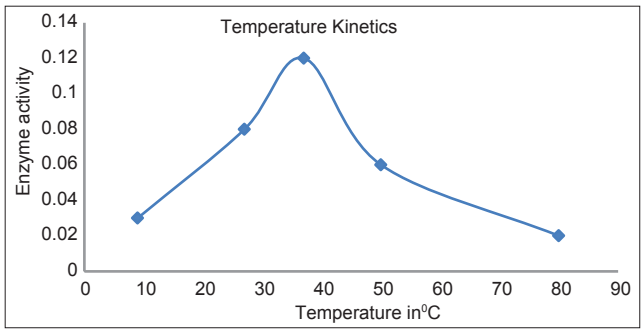

Fig. 6: Effect of temperature on α-amylase activity, the optimum temperature was found to be 37°C

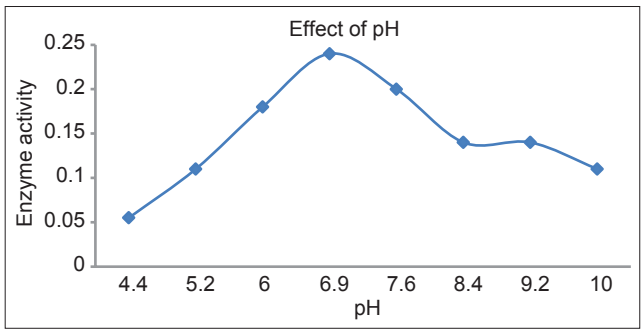

Fig. 7: Effect of pH on α-amylase activity, the optimum pH was found out to be 6.9

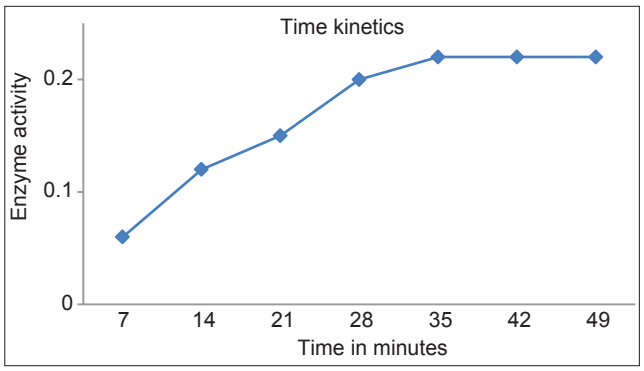

Fig. 8: Effect of time on α-amylase activity, the optimum time was found out to be 35'

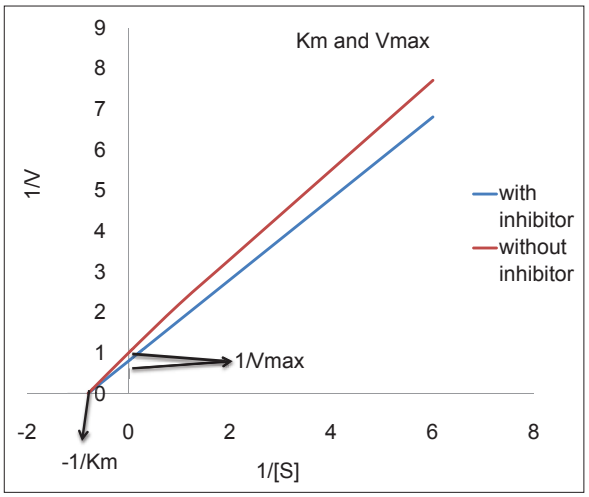

Fig. 9: Lineweaver-burk Plot showing the 1/Vmax and −1/km, in the (blue line) presence and (red) absence of inhibitor

CONCLUSION

The obtained results indicate that *P. kurroa* exhibits potent anti-oxidant activity in different *in-vitro* assays including DPPH, hydroxyl radicals, NO radicals, total anti-oxidants in the proteins. Also, proteins have the ability to inhibit α-amylase activity from rat pancreas, and this might be useful for therapeutic purposes.

Kinetic information about rat pancreatic α-amylase is useful for examining possible mechanisms for the reaction. This is true for all types of reactions; kinetic principles are used to understand both catalyzed and non-catalyzed reactions.

For α-amylase enzyme, protein extract acting as a non-competitive inhibitor value of km remains same but Vmax changes, the optimum time, temperature, pH this information is useful for understanding how metabolism is regulated and how it will occur under different conditions. Also for understanding pathological states. Diseases and disorders often involve alterations in enzymes or enzyme activities.

Understanding the way that enzymes work is critical for understanding how drugs work, because most drugs function by interacting with enzymes. In addition, the more you know about an enzymatic reaction, the more information you will have for designing new drugs. Our study indicates that proteins of *P. kurroa* have good anti-oxidant and α-amylase enzyme inhibiting capability.

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