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Moringa Oleifera Seed Protein Hydrolysates Inhibit Haemoglobin Glycosylation and ?-Glucosidase Activity in-vitro

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Abstract

In recent times, the biological activities of enzymatic digests of plant and animal proteins have been investigated and have been shown to exhibit multidirectional effects against key enzymes involved in the pathophysiology of a number of diseases. The present study evaluated the inhibitory effects of M.oleifera seed protein hydrolysates on haemoglobin glycosylation and ?-glucosidase. Proteins were hydrolyzed using the enzymes pepsin, trypsin, papain and chymotrypsin. The resulting hydrolysates were evaluated for inhibitory activities against non-enzymatic haemoglobin glycosylation as well as ?- glucosidase. Peptic and chymotrypsin hydrolysates demonstrated the best inhibitory effects against hemoglobin glycosylation, while chymotryptic and tryptic hydrolysates had better ?-glucosidase inhibitory activities. Kinetic data showed that the hydrolysates inhibited ?-glucosidase inhibitory effects by different mechanisms, such tryptic and chymotrypsin hydrolysates indicated a competitive mode of inhibition while papain and pepsin hydrolysates displayed mixed inhibition of ?-glucosidase. These results suggest that M.oleifera seed proteins contain peptides that can be harnessed to formulate peptides which could serve as novel alternatives to current therapies in the management of diabetes mellitus.

Index terms— M.oleifera, hydrolysates, pepsin, trypsin, papain, chymotrypsin, hemoglobin, ?-glucosidase, diabetes mellitus.

1 Introduction

eptide products of plant and animal proteins have in recent times have been exploited for therapeutic purposes (Olusola et al., 2018). Peptides of therapeutic value have been utilized in the treatment and management of a variety of disorders (Lien and Lowman, 2004). For the most part, emphasis has been placed on the use of peptides and protein hydrolysate preparations as possible alternatives in the management of cardiovascular diseases such as hypertension (Arise et al., 2016 a) and diabetes mellitus (Arise et al., 2016 b , Olusola and Ekun, 2019). One plant whose proteins encode potentially bioactive peptides is M. oleifera.

Moringa oleifera is naturalized in India, especially in the Western & Himalayan regions. It is also found in the tropical regions of Africa as well as the Middle East (Madubuike et al., 2015). Mune-Mune et al., **??**2016) reported that M. oleifera seeds have a relatively high protein content, over 30%, making it an excellent source of potentially therapeutic peptide products when subjected to enzymatic hydrolysis. Globulins and albumins constitute the major portion of Moringa oleifera seeds (Baptista et al., 2017), and amino acid analysis reveals that it contains high proportion of basic and acidic amino acids, moderate amount of most hydrophobic amino acids, but limiting in sulfurcontaining amino acids such as methionine and cysteine (Okereke and Akaninwor, 2013). Parts of the plant such as its leaves, seeds and roots have been evaluated for their health promoting benefits which include hypoglycemic effects (Villarruel-López et al., 2018) antimicrobial activity (Bukar et al., 2010), antioxidative potentials (Wright et al., 2017) among other properties.

Diabetes mellitus is a metabolic disorder occurring as a result of disturbances in insulin function. It is characterized by severe hyperglycemia and leads to a plethora of derangements in the metabolism of carbohydrate, proteins and lipids (Arise et al., 2016 b). These ultimately cause damage to organs, such as liver, kidney, retina, as the disease progresses. One of the adverse effects of elevated blood glucose is the formation of advanced glycated end products, and this occurs when blood glucose forms non-enzymatic covalent adducts with protein and lipids in plasma. (Ramasamy et al., 2005). These glycated products may set the stage for the onset of generation of reactive oxygen species; activating a number of proinflammatory pathways causing impaired cellular function, and these are thought to occur by signal transduction processes mediated by the receptor for advanced glycated end-products (RAGE) play key roles in pathogenesis the diabetic cataracts, diabetic neuropathy and nephropathy (Singh et al., 2014).

Current therapeutic approaches are aimed at controlling glucose levels by slowing its rate of release into the blood stream by inhibiting glucoside cleavage enzymes such as ?-amylase and ?-glucosidase; increasing insulin sensitivity by altering the activities of incretin degrading enzymes such as dipeptidylpeptidase (iv) in combination with lifestyle changes (Katzung et al., 2012). However, these chemotherapeutic approaches give rise to certain untoward side effects such as gastrointestinal discomfort and renal damage. Also, many of these drugs, owing to their high cost of procurement, increase economic burden on patients as well as their relatives . Hence, the search for newer, safe and cost-effective alternatives in the management of diabetes mellitus cannot be overemphasized, and recently attention has turned to newer sources such as peptides and hydrolysate preparations (Olusola and Ekun, 2019).

There have been reports about several biofunctional properties of various parts of the M. oleifera plants (**??**nwar et al., 2007). Previous work had demonstrated that hydrolysates obtained from its seed proteins possess ?-amylase inhibitory activities (Garza et al., 2017, Olusola et al., 2018). Hence, this study aims to evaluate the inhibitory activities of M. oleifera seed protein hydrolysates on hemoglobin glycosylation and ?-glucosidase to further justify their anti-diabetic potentials.

2 II.

3 Materials and Methods

4 a) Materials

5 i. Collection of Moringa oleifera seeds

Moringa oleifera seeds were bought from stores in Ikere-Ekiti, Ekiti State, Nigeria, and authenticated by the Department of Plant Science and Biotechnology, Adekunle Ajasin University Akungba Akoko.

ii. Chemicals and Reagents Enzymes: pepsin (from porcine stomach), trypsin (from bovine pancreas), papain (from Carica papaya), chymotrypsin (human), and alpha-amylase (from saccharomyces cerevisiae) were products of Kem Light Laboratories, India. alpha-glucosidase (Saccharomyces cerevisiae) and other reagents used were of analytical grade and were purchased from Sigma Aldrich (USA).

6 b) Methods

7 i. Isolation of Moringa oleifera seed proteins

The Moringa oleifera seeds were dried and pulverized before being kept in an air-tight container at 4 0 C.This were defatted using n-hexane as described by Wani et al., (2011).The meal was extracted four times with n-hexane(60-80 o C) using a meal/solvent ratio of 1:10(w/v). The meal was dried at 40 0 C in a vacuum oven and ground again to obtain a fine powder, termed defatted seed meal, which was then stored at -20 0 C.The protein component of the defatted meal was extracted using the method described by Alashi et al., (2014) with modifications. Defatted Moringa seed meal was suspended in 0.5M NaOH pH 12.0 at a ratio of 1:10 and stirred for one hour to facilitate alkaline solubilisation. This was then centrifuged at 18 0 C and 3000g for 10min. Two additional extractions of the residue from the centrifugation process were carried out with the same volume of 0.5M NaOH and the supernatants were pooled. The pH of the supernatant was adjusted to pH 4.0 to facilitate acid-induced protein precipitation using 5M HCl solution; the precipitate formed was recovered by centrifugation as described above. Also the pH of the supernatant formed was further adjusted to a pH of 5.5 using 0.1M NaOH. The precipitates formed were recovered by centrifugation. They were then washed with distilled water, adjusted to pH using 0.1M NaOH, freeze-dried and the protein isolate was stored at -20 0 C until required for further analysis.

ii. Preparation of Moringa oleifera seed protein Hydrolysates The protein isolate was hydrolysed using the methods described by Onuh et al.,(2015) with slight modifications. The conditions for hydrolysis were tailored for each enzyme in order to optimize its activity. Hydrolysis were done using each of pepsin (pH 2.2, 37 0 C), trypsin (pH 8.0, 37 0 C), papain (pH 6.0, 50 0 C) and chymotrypsin (pH 8.0, 37 0 C). The protein isolate (5% w/v, based on the protein content of the isolate) was dissolved in the appropriate buffer (phosphate buffer, pH 8.0 for trypsin and chymotrypsin, glycine buffer, pH 2.2 for pepsin, phosphate buffer, pH 6.0 for papain). The enzyme was added to the slurry at an enzyme-substrate ratio (E: S) of 2:100. Digestion was performed at the

specified conditions for 24 hours with continuous stirring. The enzyme was inactivated by boiling in water bath (95-100 0 C) for 15 minutes followed by centrifugation at 700g for 30 minutes. The supernatant containing target peptides were then collected. Protein content of samples were determined using biuret assay method with bovine serum albumin (BSA) as standard.

8 iii. Inhibition of Hemoglobin Glycosylation

This was investigated by estimating the degree of non-enzymatic hemoglobin glycosylation according to the method described by Venu, et al., (2016) with modifications. Glucose solution (2%), 0.06% hemoglobin and Gentamycin (0.02%) solution were prepared in phosphate buffer 0.1M, pH 7.4. 1ml each of above solution was mixed. 0.25ml, 0.50ml, 0.75ml and 1ml of hydrolysate was added to above mixture. Gallic acid was used as standard. The mixture was kept in dark at room temperature for incubation for 72hours. At 520nm, haemoglobin glycosylation was measured with a spectrophotometer and % inhibition was calculated thus:

Percentage of hemoglobin glycosylation = Abs(sample) -Abs (control)/ Abs (sample) x 100%

9 iv. Determination of ?-glucosidase Inhibition

The effect of the hydrolysates on ??-glucosidase activity were determined according to the method described by (Kim et al., 2005) with slight modifications, using ??-glucosidase from Saccharomyces cerevisiae. The substrate solution p-nitrophenyl glucopyranoside (pNPG) was prepared in 20 mM phosphate buffer, and pH 6. pre-incubated with 100??L of the different concentrations of the hydrolysates for 10 min. Then 50??L of 3.0 mM (pNPG) as a substrate dissolved in 20 mM phosphate buffer (pH 6.9) will be added to start the reaction. The reaction mixture were incubated at 37 ? C for 20 min and stopped by adding 2mL of 0.1 M Na 2 CO 3 solution. The ??-glucosidase activity was determined by measuring the yellow-colored para-nitrophenol released from pNPG at 405 nm. The results were expressed as percentage of the blank control. Percentage inhibition were calculated as:% Inhibition = (Acontrol-Asample) / Acontrol × 100 v.

10 Determination of Kinetic Parameters of ?glucosidase Inhibition

The kinetic parameters of ??-glucosidase by the hydrolysates were determined according to the modified method described by Ali et al., (2006). Briefly, 50??L of the (5 mg/mL) hydrolysate was pre-incubated with 100??Lof ??-glucosidase solution for 10 min at 25?C in one set of tubes. In another set of tubes, ??-glucosidase was pre-incubated with 50??L of phosphate buffer (pH 6.9). 50??L of pNPG at increasing concentrations (0.63-2.0 mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixtures were then incubated for 10 min at 25 °C, and 500??L of Na 2 CO 3 solution was added to stop the reaction. The amount of reducing sugars released were determined spectrophoto-metrically at 405nm using a paranitrophenol standard curve and converted to reaction velocities. A double reciprocal plot (1/V versus 1/[??]) where V is reaction velocity and [??] is substrate concentration was then plotted. The mode of inhibition of the hydrolysates on ??-glucosidase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot using Michaelis Menten kinetics.

11 vi. Statistical Analysis

Results were expressed as mean of replicates ± standard error of mean (SEM). The data were statistically analyzed using One Way Analysis of Variance (ANOVA) and Duncan's multiple range tests. Differences were considered statistically significant at $p<0.05$ using Microsoft Excel and GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA).

12 III.

13 Results

14 a) Inhibition of Haemoglobin Glycosylation

The effects of Moringa oleifera seed protein hydrolysates on non-enzymatic haemoglobin glycosylation at a concentration range of 0.25mg/ml to 1.00mg/ml are illustrated in Figure 1. The hydrolysates showed a concentration-dependent reduction in the inhibition of hemoglobin glycosylation. Also, they demonstrated significantly lower ($p<0.05$) inhibitory effects when compared to gallic acid. Peptic hydrolysates exhibited inhibitory effect of 62.583±0.621% at a final concentration of 1.00mg/ml, which was significantly ($p<0.05$) higher when compared to other hydrolysates, at the same concentration. Chymotrypsin hydrolysates, with a inhibitory extent of 53.513±0.361% had higher inhibitory effects than tryptic and papain hydrolysates (38.360±0.439% and 46.540±0.323% respectively) while tryptic hydrolysates had lowest inhibitory activity. Figure **??** depicts the IC 50 Values of Moringa oleifera seed protein hydrolysates in inhibiting hemoglobin glycosylation as compared to gallic acid. Peptic hydrolysates inhibited hemoglobin glycosylation to a 50% extent at a concentration of 0.533±0.392mg/ml, while tryptic, papain and chymotrypsin hydrolysates exhibited 50% inhibition at 0.113±0.027mg/ml, 0.599±0.026mg/ml and 0.765±0.046mg/ml respectively. With the exception of

tryptic hydrolysates, all the other hydrolysates had significantly ($p<0.05$) higher IC 50 values when compared to gallic acid. Hydrolysates derived from tryptic digestion had significantly lower ($p<0.05$) IC 50 values than other hydolysates, just as values obtained for peptic and papain hydrolysates were not significantly ($p<0.05$) different from each other, but were lower than those of chymotrypsin hydrolysates.

15 b) ?-Glucosidase Inhibitory Activity

The inhibitory activities of the hydrolysates on ?-glucosidase -catalyzed hydrolysis of p-nitrophenyl glucopyranoside at varying concentrations are presented in Figure **??**. The hydrolysates displayed increasing inhibitory activity with increasing concentration, with peptic, tryptic, papain and chymotryptic hydrolysates attaining 71.040±6.322%, 80.620±2.308%, 74.06±0.081% and 72.39±0.450% inhibition respectively at a final concentration of 1.0mg/ml. Tryptic hydrolysates demonstrated the highest inhibitory activity at all concentrations in this study($p<0.05$). At lower concentrations however, papain hydrolysates had significantly higher ($p<0.05$) inhibitory activity than peptic and chymotrypsin hydrolysates.

Figure **??** shows the IC 50 values of the four hydrolysates in inhibiting the reaction catalyzed by ?glucosidase. Peptic and tryptic hydrolysates inhibited the reaction to a 50% extent at concentrations of 0.465±0.394mg/ml and 0.151±0.027mg/ml respectively, while papain and chymotrypsin hydrolysates had IC 50 values of 3.348±0.028mg/ml and 0.085±0.013mg/ml respectively. The IC 50 values of tryptic and chymotrypsin hydrolysates were not significantly ($p<0.05$) different from each other, but they were lower than those obtained by peptic and papain hydrolysates. Also, peptic hydrolysates had a significantly ($p<0.05$) lower IC 50 value than papain hydrolysates.

Each bar represents the mean of triplicate determinations ± SEM. Bars with same letters are not significantly different at ($p < 0.05$), while bars with different letters are significantly different.

16 c) Kinetics of ?-glucosidase inhibition

The effects of M. oleifera seed protein hydrolysates on the kinetics of ?-glucosidase-catalyzed hydrolysis of p-nitrophenyl glucopyranoside, p-NPG, to p-nitrophenol are illustrated in figures 5-8. The kinetic parameters from the resulting Line-weaver Burk plots are summarized in Table 1. In the absence of inhibitory hydrolysates, the Michaelis constant, k m of ?-glucosidase for its substrate was determined to be 0.297 p-NPG, while maximum velocity, Vmax, was 270.27mM/mg/min. All hydrolysates except papain hydrolysates, caused a concentration dependent increase in the apparent k m of the enzyme. Also, all hydrolysates reduced the maximum velocity, Vmax as well as the catalytic efficiency, CE, of the ?-glucosidase reaction. Hydrolysates from chymotrypsin digestion exhibited the most reduced CE, while peptic hydrolysates displayed the most reduced Vmax, when compared to other hydrolysates.

The enzyme-inhibitor dissociation constant, k i , was lowest with chymotryps in hydrolysates (0.193mg/ml), although it was only slightly lower than 0.203mg/ml obtained for tryptic hydrolysates. Papain hydrolysates had the highest k i value of 1.278mg/ml. The mode of inhibition of peptic, tryptic and papain hydrolysates was the mixed type, while chymotrypsin hydrolysates displayed a competitive inhibition of ?glucosidase.

17 Discussion a) Inhibition of Hemoglobin Glycosylation

The formation of advanced glycation end products (AGEs) as a result of poorly controlled hyperglycemia in diabetes mellitus leads to a plethora of complications such as retinopathy, renal dysfunction atherosclerosis, among other devastating conditions (Ramasamy et al., 2005, Singh et al., 2014). These AGEs cause deleterious effects by promoting the generation of reactive oxygen species which activate a cascade of signaling pathways, leading to an increase in the production of pro-inflammatory mediators, leading to other complications such as the formation of atherosclerotic plaques and culminating in cardiovascular disease in diabetic patients **??**Han et al., 2018). Certain plant extracts have been reported to inhibit haemoglobin glycosylation in vitro (Hosseini et al., 2015), but information has been scarce on the abilities of peptides and protein hydrolysates to inhibit haemoglobin glycosylation. In this study, the hydrolysates displayed a concentration dependent reduction in their abilities to inhibit haemoglobin glycosylation in vitro, such that peptic and chymotrypsin hydrolysates had >50% inhibition at a final concentration of 1.00mg/ml, which was higher than those of other hydrolysates. This may be due to the possible influence of the nature of the peptides in the hydrolysate preparations. Chymotrypsin cleaves proteins specifically at C-terminals of aromatic amino acid residues, while pepsin being relatively non-specific, hydrolyzes proteins at C-terminals of aminoacyl residues having hydrophobic and aromatic side chains (Voet and Voet, 2011). This may give rise to residues such as Trp, Tyr, Leu, Phe, Ile found at these positions, and as such could be responsible for anti-AGE formation. This is evidenced by the recent report by Han et al., (2014) that Asn-Trp dipeptides inhibited the formation of AGEs in mice models.

18 b) ?-Glucosidase Inhibition

?-glucosidase is one of the enzymes found on the brush border membranes of the intestinal mucosa and participates in carbohydrate digestion by hydrolyzing glucose residues from oligosaccharides (Voet and Voet, 2011). Thus, the modulation of the activity of this enzyme represents on key strategy in the control of blood glucose levels in the management of 1. The Michaelis constant, k m , of ?-glucosidase for p-nitrophenyl glucopyranoside in the absence

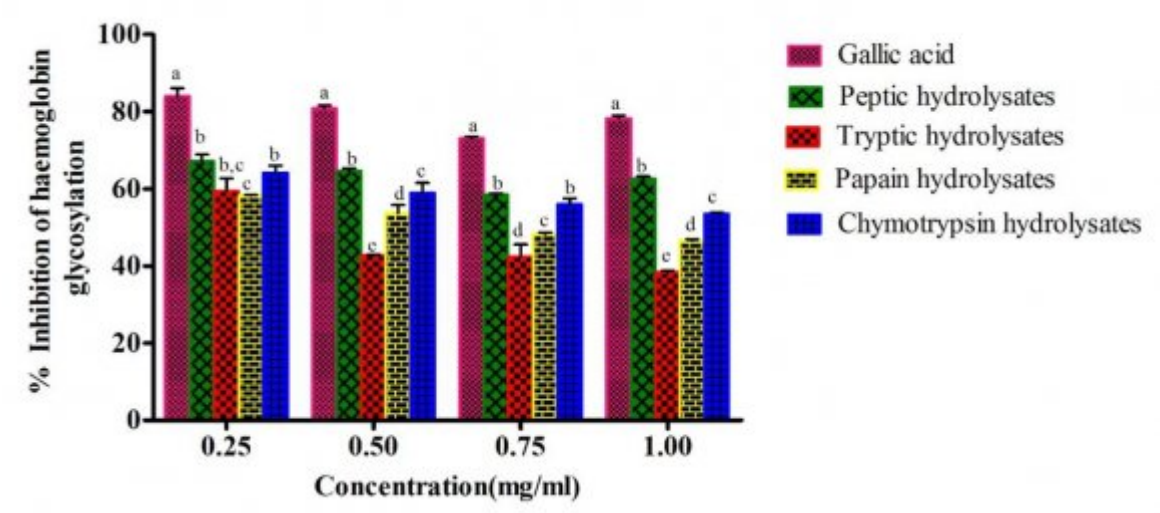
of inhibitor was determined to be 0.297mg/ml p-NPG in this study. This is slightly higher than 0.211mg/ml (0.7mM) p-NPG obtained by Awosika and Aluko (2019) and lower than 6.31mg/ml reported by Arise et al., (2019). V max, in the absence of inhibitory hydrolysates was 270.27mM/mg/ml. The Lineweaver Burk plots indicate that hydrolysates derived from peptic digestion displayed a mixed type of inhibition at 0.5mg/ml and an uncompetitive type of inhibition at 1.0mglml. This partly compares to the uncompetitive mode of inhibition obtained by Arise et al., (2019) for Luffa cylindrica seed protein hydrolysates. Papain hydrolysates on the other hand, showed mixed mode of inhibition at all concentrations. This means that the peptides in the hydrolysate preparations tend to bind and inhibit the ?-glucosidase in both its free form and p-NPG bound forms, creating dead-end complexes. Chymotrypsin and tryptic hydrolysates on the other hand exhibited a competitive type of inhibition, which was in contrast to an uncompetitive mode of inhibition for the 1kD fraction of Chymotrypsin hydrolysates derived from yellow field pea proteins as reported by Awosika and Aluko (2019). This could be because the hydrolysates used in this study were unfractionated, thus containing peptides of different lengths and molecular sizes. In addition, the presence of proline, basic and bulky aminoacyl residues in the peptide chains could confer on them, the ability to lock into the enzyme active site (Yu et al., 2011), thereby preventing substrate binding.

Maximal rate of reaction, Vmax, as well as catalytic efficiency, CE, of the enzymatic reaction were reduced by the four hydrolysates, which is usually seen with the different modes of inhibition. The enzymeinhibitor dissociation constant, k i, of 0.193mg/ml, 0.203mg/ml and 0.305mg/ml determined for chymotryptic, and peptic and tryptic hydrolysates respectively, and was lower than 10.51mg/ml and 49.83mg/ml obtained for tryptic and peptic hydrolysates Arise et al., (2019) for Luffa cylindrica seed protein hydrolysates. This indicates that hydrolysates derived from chymotrypsin and tryptic digestion showed higher binding affinity for ?-glucosidase when compared to papain hydrolysates.

V.

19 Conclusion

To summarize, the hydrolysates derived from enzymatic digestion of M. oleifera seed proteins demonstrated potential anti-diabetic activities in-vitro by inhibiting both the formation of glycosylated haemoglobin and ?-glucosidase activity. Peptic and chymotrypsin hydrolysates displayed better inhibitory effects against non-enzymatic glycosylation of haemoglobin, while chymotrypsin and tryptic hydrolysates demonstrated higher ?-glucosidase inhibitory properties. This not only justifies the use of M. oleifera seeds for alernative therapeutic purposes, but may also indicate that these proteins could be potential sources of biologically active peptides which could be optimized to formulate new and potent antidiabetic agents. Further studies such as fractionation of these hydrolysates and characterization of resulting peptides responsible for the observed biofunctional properties are suggested, and are currently underway.



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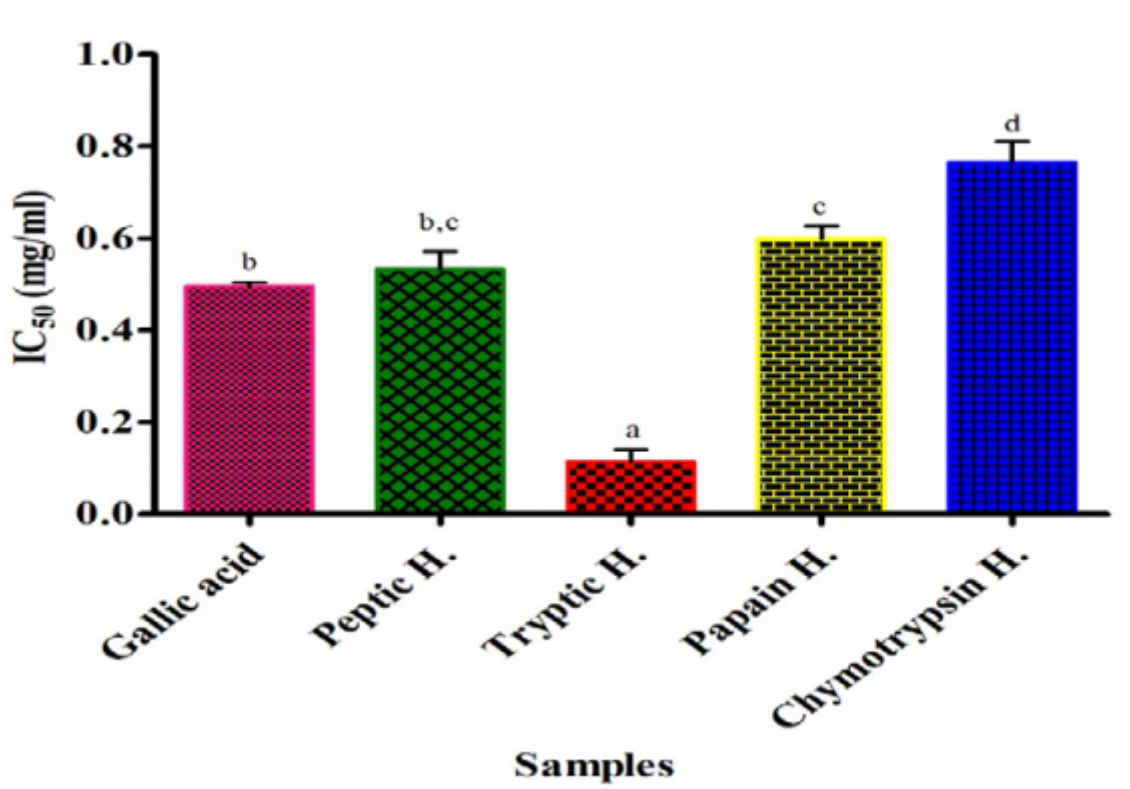

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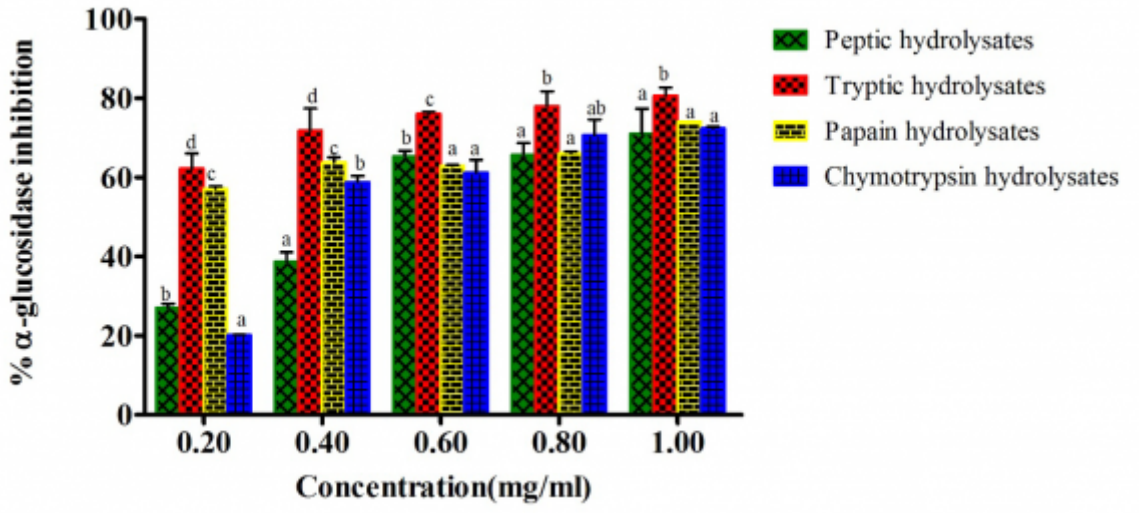

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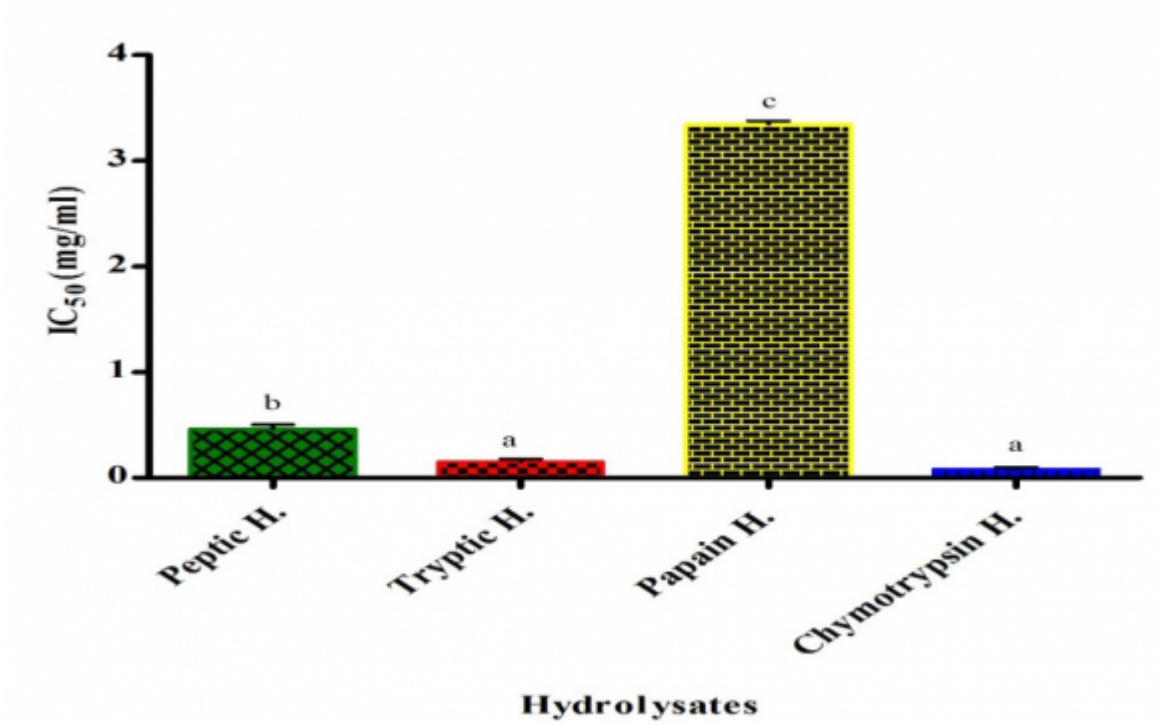

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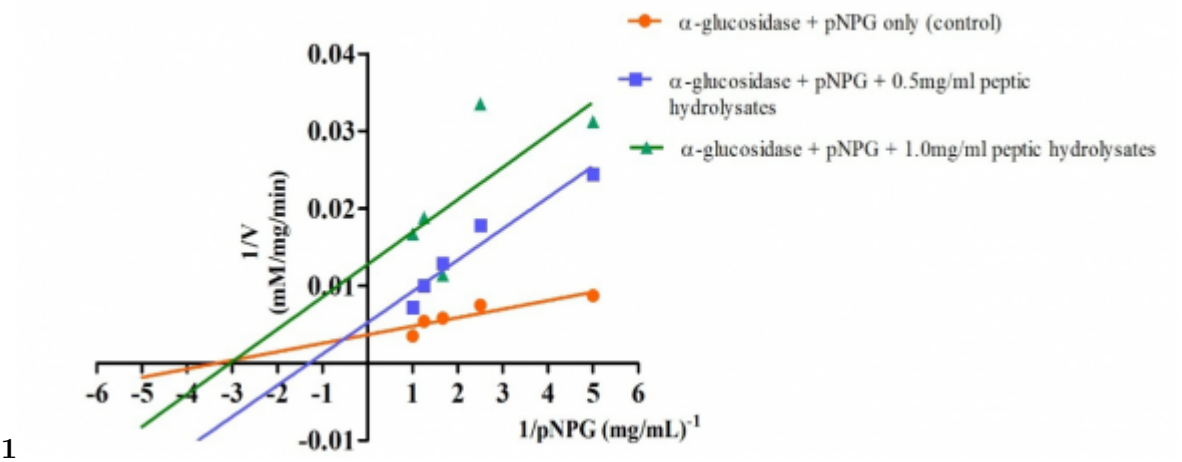
Figure 4: Figure 4 :BFigure 3 :



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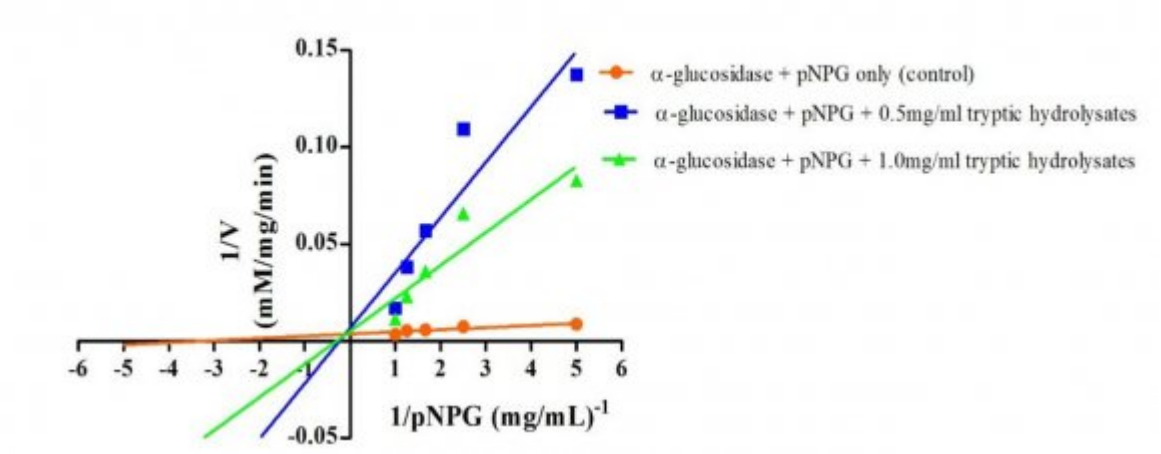

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Figure 6: Figure 5 :Figure 7 :Figure 8 :B

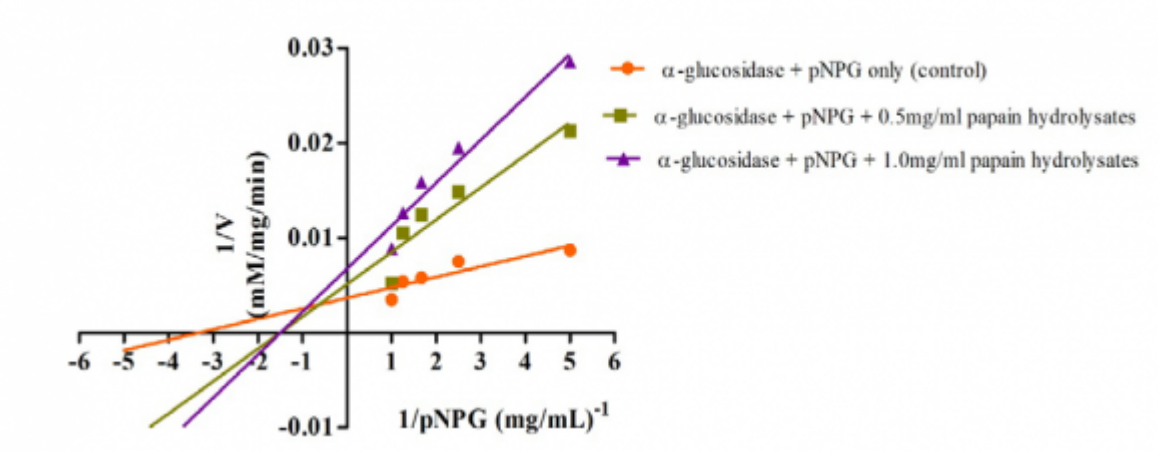

Figure 7:

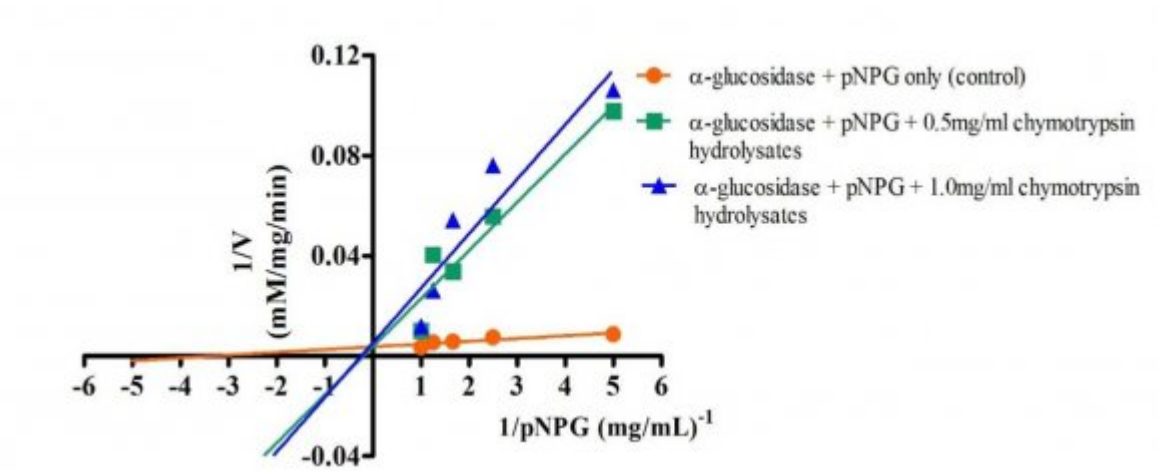

Figure 8:

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Figure 9: Table 1 :

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