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Hydrolysates from aduwa (*B.aegyptiaca* Del): A new source of inhibitory peptide

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ABSTRACT

Background: This study reports on the multi-enzyme potency of hydrolysates from Aduwa protein concentrates against α-amylase, α-glucosidase, chymotrypsin, trypsin, and pancreatic lipase inhibitory activities.

Objective: This work aims to determine the inhibitory potential of Aduwa hydrolysates derived from Aduwa protein concentrate against selected proteases and their respective enzyme–substrate activities. The work seeks to raise awareness among food processors and consumers on the importance of *Aduwa* products in modulating digestive enzymes for optimal health.

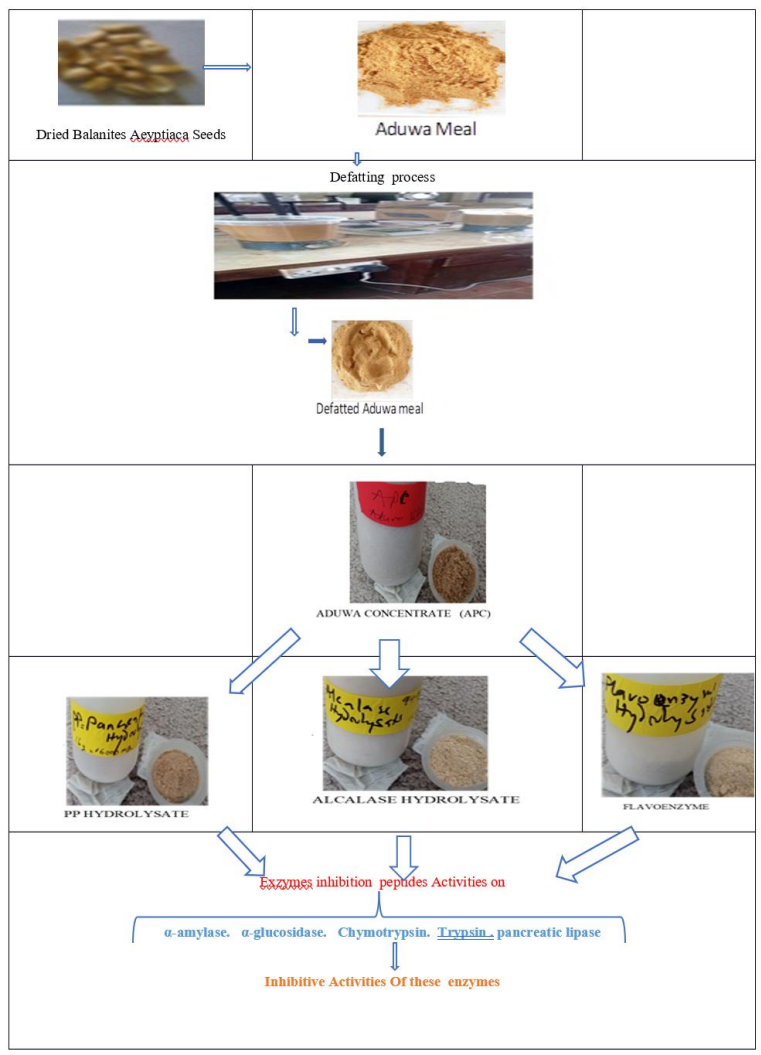
Method: Hydrolysates from *Aduwa* protein concentrate (APC) were digested using pancreatin–pepsin (PP), Alcalase (Alca), and Flavourzyme (Flav). All samples were subjected to enzyme inhibition assays using standard methods.

Results: The hydrolysate produced with Alcalase showed stronger inhibitory effects on α-amylase and trypsin activity than the PP- and Flavozyme-derived samples. The PP hydrolysates were more active against carbohydrate-, protein-, and fatty acid-related enzymatic activities than APC and Flav-derived samples. Both PP and Alca hydrolysate samples demonstrated greater proteolytic action with a high capacity to break down the protein moiety. These hydrolysates could therefore function as useful bioactive peptides or raw materials for modulating protein and fatty acid digestion, potentially reducing organ overload and promoting human health.

Novelty and Practical Implication of this study: This study corresponds to quantum and tempo theory of functional Aduwa hydrolysates as a biomarkers capable of inhibiting the degree of moieties breakdown, which should be a function of tempus or time to elicit maximum effect for optimal health, since certain threshold activities of α-amylase, α-glucosidase, chymotrypsin, trypsin, and pancreatic lipase could pose a health risk. The primary aim was to evaluate a new source of inhibitory peptide **e**nzyme inhibition peptide biomarkers that are associated with the observed functional effects of modulating food nutrients and broken-down moieties. These findings provide preliminary evidence that could support subsequent steps of functional food product development using hydrolysate from desert date seeds.

Conclusion: Findings suggest that PP and Alca hydrolysates have potential for development into functional foods or nutraceuticals for managing caloric or sugar absorption through the visceral system and into the bloodstream, with relevance to obesity, diabesity modulations, and other nutritional and health conditions.

Keywords: *Aduwa*, novel inhibitory peptides, functional hydrolysates, enzymes, inhibitions, ingredients.



Graphical abstract: Hydrolysates from aduwa (*B.aegyptiaca* Del): A new source of inhibitory peptide.

INTRODUCTION

Nut proteins are gaining global recognition due to their potential to encode active amino acid sequences [1-2]. Food-derived proteins often contain bioactive peptides that can be released through enzymatic hydrolysis either in vivo or in vitro [3]. Endogenous and exogenous proteases have been shown to release bioactive peptides with positive effects on health, including anti-diabetic properties [3-,-6]. According to [7-9], hydrolysates produced from peas, beans, and mushrooms are shown to inhibit α-amylase and α-glucosidase activities. These inhibitory effects help reduce starch breakdown and limit the release of glucose into the bloodstream. Since α-amylase and α-glucosidase are key carbohydrate-digesting enzymes. Uncontrolled activity may elevate blood sugar levels, leading to diabetes mellitus, obesity, and hypoglycemia [10]. Trypsin and chymotrypsin inhibitors have been reported as potential dietary anti-carcinogens and may play roles in preventing carcinogenesis, cardiovascular disorders, neurodegeneration, and chronic inflammation [11-15]. According to the World Health Organization, roughly 70% of the global developing nations with plant life so close to them do rely on a plant-based traditional medical system and these diverse medicinal plants are effective in the treatment of diabetes, and they have been utilized as anti-diabetic remedies; inhibitors of α-amylase from natural products may be more safer than the conventional therapy [31-33]. Physiologically, protease inhibitors are also used in food processing to prevent proteolytic degradation in animal and plant foods, functioning as texture enhancers in meat and high-quality dough [16-18]. Inhibition of trypsin, chymotrypsin, and pancreatic lipase—enzymes involved in protein and lipid digestion is essential for modulating excessive absorption of amino acids and fatty acids into the bloodstream, thereby helping reduce weight gain. High-protein and high-fat diets may stress vital organs such as the kidneys, supporting the importance of these inhibitors [19]. Synthetic digestive enzyme inhibitors in current use are often associated with negative side effects such as diarrhea and hepatotoxicity [20]. Hydrolysates from plant sources, such as *Aduwa* protein concentrate, may inhibit α-amylase, α-glucosidase, chymotrypsin, and lipase. Natural proteins and their hydrolysates are generally considered safe, inexpensive, and low risk compared to synthetic drugs such as acarbose [21]. Several plant and tree seeds have been identified as potential sources of enzyme-suppressing and anti-diabetic ingredients [5]. This study aims to determine the inhibitory potentials of *Aduwa* hydrolysates from *Aduwa* protein concentrate using selected proteases and enzyme substrate systems. There is limited information on the ability of *Aduwa* protein concentrate and its hydrolysates to inhibit proteolytic or digestive enzymes. Using active hydrolysates from *Aduwa* concentrates as functional ingredients could effectively modulate enzymatic activity in the visceral system. The objective was to produce protein concentrate hydrolysates with multifunctional inhibitory activity from *Aduwa* seed protein.

MATERIALS

Source of Raw Materials: Mature seeds of *Balanites aegyptiaca* (*Aduwa*) were purchased from Gashua Market in Yobe State, Nigeria, and transported to the Department of Biological Sciences for identification and subsequently to the Biochemistry Laboratory of the Federal University Gashua. Samples were packaged and transported to the Department of Food and Human Nutritional Sciences, University of Manitoba, Canada, for analysis. Pepsin, pancreatin, Alcalase, and Flavourzyme were purchased from Sigma-Aldrich (St. Louis, MO, USA). ACE (Sigma) and its substrate N-(3-[2-furyl] acryloyl)-phenylalanyl-glycyl-glycine (FAPGG) were also obtained from Sigma-Aldrich. All other chemicals and reagents were of analytical grade.

MATERIALS AND METHODS

Preparation of *Aduwa* (*Balanites aegyptiaca* Del.) Protein Concentrate [22]: The defatted *Aduwa* meal was mixed with double-distilled water at a 2:50 (w/v) ratio for solubilization at pH 10 using 1 M NaOH. The mixture was

stirred for 1 hour and then centrifuged at 31,000 × g for 30 minutes. The supernatant was collected, filtered through cheesecloth, adjusted to pH 4.5 with 1 M HCl, stirred for 30 minutes, and centrifuged again. The resulting precipitate was washed with running water to remove non-protein materials. After another centrifugation step, double-distilled water was added, and the pH was adjusted to 7.0. The slurry was then freeze-dried to obtain *Aduwa* protein concentrate (APC).

Preparation of *Balanites aegyptiaca* Flavoenzyme Hydrolysate: The method of [23] was adopted. Briefly, 30 g of the isolate was dispersed in water at a 1:2 (w/v) ratio to form a slurry, which was then incubated at 50 °C and adjusted to pH 6.5 with 2 M NaOH. Flavourzyme (3.04% w/w based on the protein content of APC) was added. The digestion was allowed to proceed with gentle stirring for 4 hours at a constant pH of 9, maintained with 2 M NaOH.The reaction was terminated after 4 hours by adjusting the pH to 4.5 using 2 M HCl. The mixture was then placed in a boiling water bath (95 °C) for 15 minutes to inactivate the enzyme through denaturation completely. After cooling to room temperature, the mixture was centrifuged, and the supernatant was collected and freeze-dried.

Preparation of PP Hydrolysate Using Pepsin and Pancreatin: The modified method of [24] was used. Briefly, 40 g of APC was dispersed as a 1:2 (w/v) slurry and incubated at 37 °C. The mixture was adjusted to pH 2.0, after which pepsin (4.0% w/w based on APC protein content) was added. The digestion proceeded for 2 hours at a constant pH of 2.0, maintained with 1 M HCl. For pancreatin digestion, the reaction mixture was adjusted to 40 °C and pH 7.5, then pancreatin (4% w/w) was added. The digestion proceeded for another 2 hours at constant pH 7.5, maintained using 1 M NaOH. The final digested mixture was terminated by adjusting to pH 4.5 and heating in a boiling water bath (95 °C) for 15 minutes to denature and inactivate the enzymes. After cooling, the mixture was centrifuged, and the supernatant was collected and freeze-dried.

Preparation of *Balanites aegyptiaca* Alcalase Hydrolysate: The method of [23] was used. Briefly, 40 g of APC slurry (1:2 w/v) was incubated at 50 °C and adjusted to pH 9.0 using 2 M NaOH. Alcalase (4.0% w/w based on APC protein content) was added. Digestion proceeded with gentle stirring for 4 hours at constant pH 9.0.The reaction was terminated by adjusting the pH to 4.0, followed by heating at 95 °C for 15 minutes to ensure full enzyme inactivation. After cooling, the mixture was centrifuged, and the supernatant was collected and freeze-dried.

Inhibition of α-Amylase Activity [25]: Dinitrosalicylic acid (DNS) reagent was used to measure α-amylase inhibition. One hundred microlitres (100 µL) of protein samples (0.1–0.57 mg/mL final concentrations) or standard acarbose (10 mg/mL) prepared in 0.02 M phosphate buffer (pH 6.9) containing 0.006 M NaCl were mixed with 100 µL of α-amylase (1 mg/mL in the same buffer).The mixtures were pre-incubated at 25 °C for 10 minutes, after which 200 µL of 1% starch solution (prepared in 0.02 M phosphate buffer, pH 6.9 with 0.006 M NaCl) was added. The reaction mixtures were incubated at room temperature for 10 minutes.The reactions were stopped by adding 1 mL of DNS reagent and heating the tubes in a boiling water bath for 5 minutes. After cooling to room temperature, the mixture was diluted at a 1:5 ratio with distilled water. A 200 µL aliquot was transferred to a 96-well plate, and absorbance was measured at 540 nm using a microplate reader.

The percentage inhibition was calculated as:

$$\text{Alpha–amylase inhibition} = \frac{\text{Absorbance of control} - (\text{Absorbance of sample})}{\text{Absorbance of control}} \times 100 \quad \textbf{equ (1)}$$

Inhibition of α-Glucosidase Activity [26]: Yeast α-glucosidase and p-nitrophenyl-α-D-glucopyranoside (pNPG) were used. One hundred microlitres (100 µL) of *Balanites aegyptiaca* protein samples (0.5–5 mg/mL final concentrations) or standard acarbose (10 mg/mL, dissolved in 0.1 M phosphate buffer, pH 6.9) were added

to 50 µL of α-glucosidase (1 mg/mL in 0.1 M phosphate buffer). The mixtures were pre-incubated at 37 °C for 20 minutes. After incubation, 100 µL of 5 mM pNPG (dissolved in 0.1 M phosphate buffer, pH 6.9) was added, and the mixture was incubated at 37 °C for 10 minutes. Absorbance was monitored at 405 nm every minute for 30 minutes using a microplate reader.

Alpha−glucosidase inhibition = (Final absorbance of blank− Final absorbance of sample) ×100

final absorbance of blank ----------**equ (2)**

Inhibition of Chymotrypsin Activity: Chymotrypsin-inhibitory activity was determined using the method of [27] . Briefly, 1 mg of chymotrypsin (dissolved in 0.01 M Tris–HCl buffer containing 0.02 M $CaCl_2$, pH 8.0; final concentration 20 µg/mL) was mixed with 200 µL of the samples (re-dissolved in the same buffer; final protein concentration 0.004–0.012 mg/mL). The mixture was incubated for 15 minutes at 37 °C. The reaction was initiated by adding 1 mL of 1 mM BTpNA (prepared in 0.01 M Tris–HCl buffer containing 0.02 M $CaCl_2$ and 40% (v/v) ethanol, pH 8.0). After 15 minutes at 37 °C, the reaction was stopped by adding 200 µL of 30% (v/v) acetic acid. A 200 µL aliquot was transferred to a 96-well microplate, and absorbance was measured at 410 nm. Chymotrypsin inhibition was determined based on the release of p-nitroaniline from BtpNA.

The chymotrypsin inhibitory activity (%) was calculated using the equation:

Inhibition (%) = ((Ac - As)/Ac) x 100 ----------**equ (3)**

Where Ac = absorbance of the control.

As = absorbance of the sample

Inhibition of Trypsin Activity: Trypsin-inhibitory activity was assessed using the method in a study by Girgih et al [28]. Two hundred microlitres (200 µL) of trypsin (dissolved in 20 mM Tris–HCl buffer, pH 7.5; final concentration 60 µg/mL) was pre-mixed with 200 µL of samples (final peptide concentration 0.005–0.05 mg/mL) and incubated for 5 minutes at 37 °C. The reaction was initiated by adding 500 µL of 1 mM BApNA (prepared in Tris–HCl buffer, pH 7.5, containing 1% (v/v) dimethyl sulfoxide). After 10 minutes at 37 °C, the reaction was stopped by adding 100 µL of 30% (v/v) acetic acid. A 200 µL aliquot was transferred to a 96-well microplate, and absorbance was measured at 410 nm. Trypsin inhibition was calculated based on the release of p-nitroaniline. AEBSF served as the positive control.

The trypsin inhibitory activity (%) was calculated using the equation:

Inhibition (%) = ((Ac − As)/Ac) × 100 -------**equ (4)**

Where Ac = absorbance of the control; As = absorbance of the sample

Inhibition of Pancreatic Lipase Activity: Pancreatic lipase inhibition was determined following [29] with slight modifications. Lipase activity was quantified by measuring the release of 4-methylumbelliferone (4-MU) from 4-methylumbelliferyl oleate (4-MU oleate). A 25 µL aliquot of sample (final protein concentration 0.05–0.005 mg/mL) dissolved in Tris buffer (13 mM Tris–HCl, 150 mM NaCl, 1.3 mM $CaCl_2$, pH 8.0) was mixed with 225 µL of 0.5 mM 4-MU oleate in a 96-well plate and incubated for 15 minutes at 37 °C. Then, 25 µL of pancreatic lipase (final concentration 1 mg/mL) was added to initiate the reaction, which was allowed to proceed for 1 hour at 37 °C. The release of 4-MU was measured at 400 nm using a microplate reader. Orlistat served as the positive control.

%Inhibition P = (Absorbance of blank −Absorbance of sample)/Absorbance of control x 100 ----------**equ (5)**

Ac, Absorbance of the blank

As, the absorbance of the sample.

Statistical Analysis: All assays were conducted in triplicate, and the results were expressed as mean ± standard deviation. Analysis of variance (ANOVA) was used to compare mean values, and Duncan's multiple range test was applied to determine significant differences at $P < 0.05$ were applicable. All analyses were performed using SPSS version 16.0.

RESULTS AND DISCUSSION

α-Amylase Inhibition: α-amylase is a key enzyme involved in starch digestion, releasing glucose that is subsequently absorbed into the bloodstream. Inhibiting α-amylase is an effective strategy for the management of diabetes [30,7]. The inhibitory activities of APC and its hydrolysates increased with concentration, indicating dose-dependent behaviour (Fig. 1). The high inhibitory activities for APC (20% at 0.57 mg/mL) and Alcalase (18% at 0.57 mg/mL) were much lower than the standard, acarbose (85% at 10 mg/mL).The result revealed that Alcalase, PP, Fav, and APC samples were lower than 24% against the α-amylase enzyme at 1 µg/mL, as reported in the crude extracts of *E. japonica* from different solvents [31]. This difference may be due to the APC and the hydrolysates being mixtures of peptides rather than purified bioactive compounds. Among the hydrolysates, Alcalase (19% at 0.57 mg/mL) showed the highest activity, followed by Flavourzyme. The samples, APC, Flavourzyme, and Alcalase (all at 0.57 mg/mL) showed similar and high inhibitions (19–20%) to PP, indicating that hydrolysis into PP peptides did not significantly improve α-amylase inhibition. A plausible reason may be the limited exposure of active cleavage sites, resulting in fewer released bioactive peptides. [31] observed that the inhibition of 24% observed from the leaf extract of Eriobotrya japonica using hexane is considered mild and is recommended to avoid the maximum inhibition side effects of the enzyme. The result from this study, therefore, reflected adequate inhibitory concentrations against α-amylase as well as observed with a dietary impregnated tablet on glucose metabolism in a hyperglycemic mouse model [38]. It was asserted by [31, 36-37] that compounds such as phenols, flavonoids, and tannins must be responsible for the inhibitory actions. The relatively low inhibition by PP (10% at 0.57 mg/mL) is better than Eriobotrya japonica leaf extract, and this inhibition by PP is in agreement with previous observations for pea protein hydrolysates [31-32]. Antagonistic peptides or residual toxins may have contributed to the reduced activity, as reported in hemp seed hydrolysates [6].

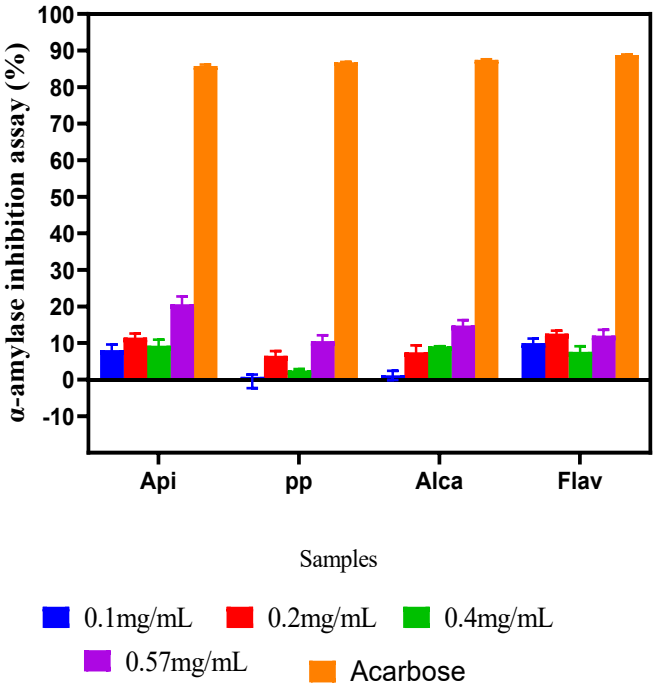


Figure 1. In vitro α-amylase inhibition activities graph Aduwa protein isolate (Aduwa isolate), pancreatin and pepsin combined hydrolysate (Panc+pepsin), Alcalase, Flavoenzyme hydrolysates, and Acarbose

α-glucosidase Inhibition: α-Glucosidase is a membrane-bound intestinal enzyme responsible for converting starch into absorbable glucose. Its inhibition helps reduce postprandial glucose levels and supports diabetes management [30,7]. The hydrolysates showed dose-dependent inhibition, except APC (Fig. 2). Inhibitory activity reached ~83% at 5 mg/mL for the hydrolysates, which was comparable to acarbose (~85% at 5 mg/mL). PP and Alcalase at 3.75 mg/mL and 5 mg/mL showed the highest activities. Lin et al. (2012) reported 56% inhibition at 4 mg/mL for brewer's spent grain hydrolysates, which is lower than the 59–60% observed in this study at 2.5–3.75 mg/mL. Alcalase hydrolysate had the highest inhibition (62–83%) among all samples. APC showed low and decreasing inhibition (~20–18%), confirming that hydrolysis significantly enhanced α-glucosidase inhibition. The strong activity of Alca, PP, and Flav hydrolysates likely results from higher peptide yield and synergistic interactions.

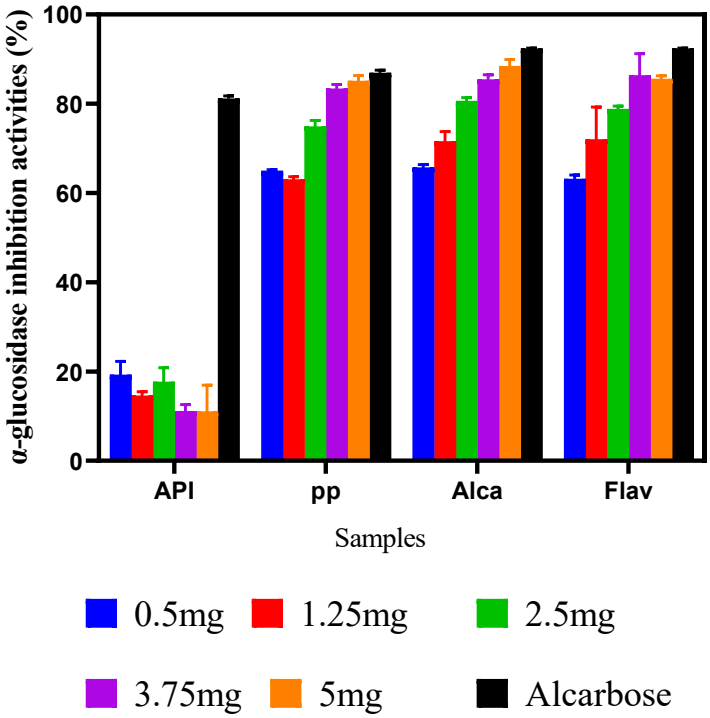


Figure 2. In vitro α-Glucosidase inhibition activities graph of Aduwa protein isolate (Aduwa isolate), pancreatin and pepsin combined hydrolysate (Panc+pepsin), Alcalase, Flavoenzyme hydrolysates, and Acarbose

Chymotrypsin Inhibition: Chymotrypsin inhibition by APC and hydrolysates was concentration-dependent (0.004–0.012 mg/mL) (Fig. 3). APC and PP showed increasing inhibition with increasing concentration, unlike Alcalase and Flavourzyme hydrolysates. The strongest inhibition (40–55%) occurred at 0.004 mg/mL and 0.008 mg/mL for all samples, whereas 0.012 mg/mL produced lower inhibition (40–41%). Among the groups, Alcalase hydrolysate exhibited the highest activity (~55%). Lower concentrations were generally more effective. It was generally observed that APC and all Aduwa hydrolysate samples under this study were below the plant Rize spp of Daphne pontica (L). (87.75%) and Mentha longifolia (L.) (84.24%) inhibition percentages [30]. They were found to have high chymotrypsin inhibitory activities above the (40-55) % hydrolysate samples, which may be due to high phenolic and flavonoid contents [37]

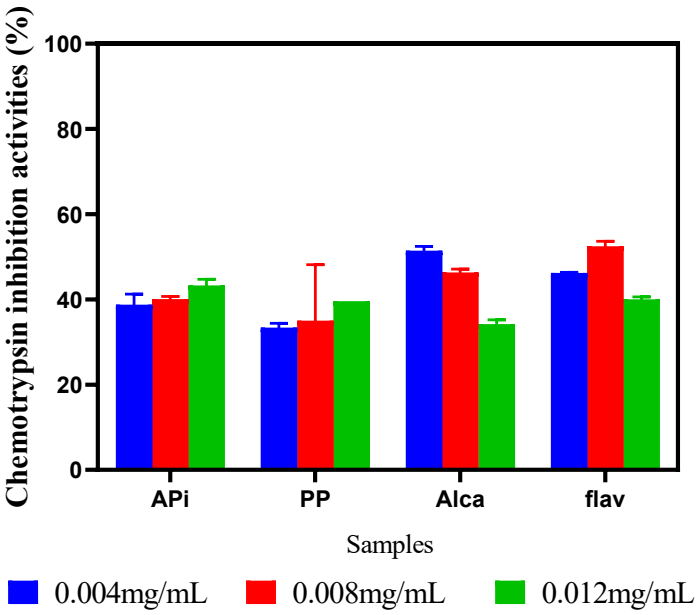


Figure 3. Chymotrypsin inhibition activities graph of Aduwa protein isolate (Aduwa isolate), pancreatin and pepsin combined hydrolysate (Panc+pepsin), Alcalase and Flavoenzyme hydrolysates.

Trypsin Inhibition: Trypsin inhibition results are presented in Fig. 4. All samples showed dose-dependent inhibition. Hydrolysates displayed higher inhibitory activity than APC. Alcalase hydrolysate exhibited the strongest and increasing trypsin inhibition, indicating its potential to delay protein digestion and reduce excessive peptide absorption. Plant Rize spp of D. pontica L and Sambucus ebulus L. flowers have been reported with the highest trypsin inhibitory activity (99.93 % and 87.47% inhibition) above all the hydrolysate samples under study [30] probably due to the nature of the sample, Rize spp plant.

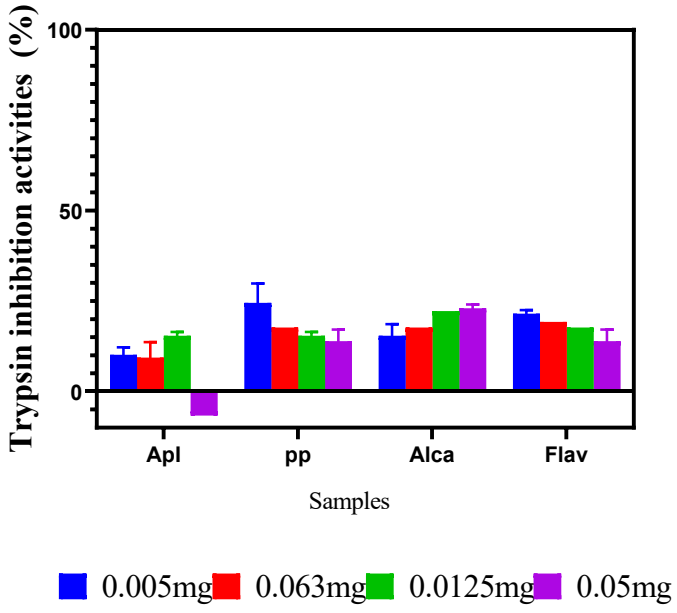


Figure 4. Trypsin inhibition activities graph of Aduwa protein isolate (Aduwa isolate), pancreatin and pepsin combined hydrolysate (Panc+pepsin), Alcalase and Flavoenzyme hydrolysates

Pancreatic Lipase Inhibition: Pancreatic lipase is essential for the digestion of dietary fat. Its inhibition reduces fat absorption and supports long-term weight management [32]. Thus, natural lipase inhibitors are potential anti-obesity agents [20]. Inhibition increased with concentration for Alcalase, Flav, and APC, although APC showed lower inhibition than Alcalase but higher than PP and Flav (Fig. 3). At 0.04 mg/mL, lipase inhibition was: APC 48%, PP 25%, Alcalase 98.9%, and Flavourzyme 30.98%, respectively. Alcalase activity was comparable to the standard inhibitor (Orlistat), indicating high potency. Differences may have resulted from the use of unfractionated peptides and synergistic interactions within the hydrolysates. Although Orlistat was more potent overall, these findings suggest that *B. aegyptiaca* proteins may serve as natural alternatives for inhibiting pancreatic lipase.

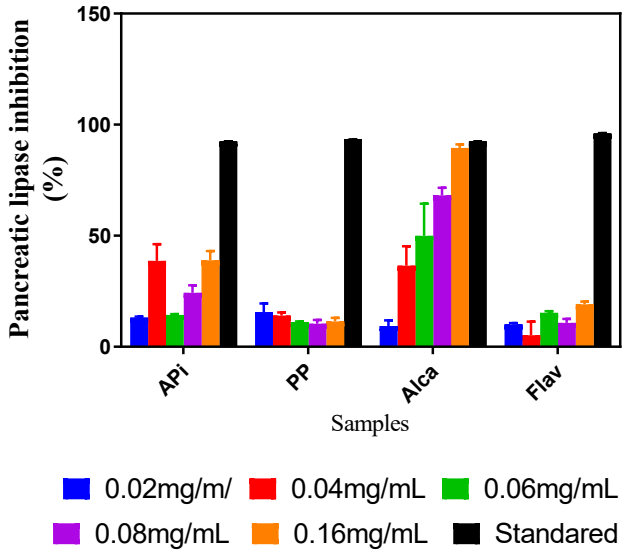


Figure 5. Pancreatic lipase inhibition activities graph of Aduwa hydrolysates; APC Aduwa protein concentrate; PP =pancreatin and pepsin combined hydrolysate; Alca = Alcalase hydrolysate; Flav Flavourzyme hydrolysates; Acarbose as standard

CONCLUSION

For the first time, this study reports the ability of *Aduwa* protein concentrate and its enzymatic protein hydrolysates to inhibit α-amylase, α-glucosidase, trypsin-, and chymotrypsin-catalysed reactions. The release and size of the peptides produced depended on the type of hydrolysing enzyme used. Alcalase generated the most effective hydrolysate and likely the smallest peptide sizes; however, peptide size did not directly correspond to complete enzyme inhibition. Overall, the hydrolysates demonstrated more potent inhibition of α-glucosidase than α-amylase, suggesting that Alcalase-derived peptides may interact more favourably with α-glucosidase enzyme. Findings from this study indicate that *the Aduwa protein and its derived peptides have potential applications in the development of functional foods and nutraceuticals to modulate* caloric or sugar absorption into the bloodstream. This may be relevant for the management of obesity, diabesity, and related metabolic health issues.

Credit authorship contribution statement: Ogori Akama F: Writing – original draft, Formal analysis, Conceptualization. Girgih, T. Abraham; Formal Abu J.

Oneh Supervision, Conceptualization. Ogori Akama F: Writing – review & editing, Software. Eke.O. Mike: Writing – review & editing. Abu J. Oneh: Writing – review and editing. Analysis, Conceptualization. Jackson Vala: editing.

Declaration of Competing Interest: The authors declare that they have no known competing interests or personal relationships that could have influenced the work reported in this paper.

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Data Availability: Data will be made available upon request.

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