

Research Article  
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## Isolation, Purification and Characterization of Proteins and Peptides from Calabash (*Crescentia cujete* L.) Fruit Flesh with Antihypertensive and Lipid-Lowering Properties

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### Abstract

Calabash (*Crescentia cujete* L.) fruit has increasingly been studied in recent years for its significant contribution to human health and well-being. The reported bioactivities are linked to the presence of phytochemicals such as polyphenols, but little is known about the role of their proteins. This represents a significant gap, as fruits are generally overlooked as sources of proteins compared to legumes and other proteinaceous products, despite their potential functional and therapeutic applications. This study aimed to isolate, purify, and characterize proteins and peptides from calabash fruits, and evaluate their potential antihypertensive and lipid-lowering activities. Proteins were extracted, purified, and characterized through a combination of biochemical techniques such as sodium phosphate buffer extraction, ammonium sulfate precipitation, gel-filtration chromatography, and *in vitro* biological assays namely pancreatic lipase inhibition, cholesterol micellar solubility inhibition, and angiotensin-converting enzyme (ACE) inhibition assays. Crude protein isolate (CPI) had a protein concentration of 688 µg/mL with observed protein bands at 12.1 kDa to 46.7 kDa. After simulated gastrointestinal digestion, CPI showed average inhibition activities of 76.5% for ACE, 22.3% for pancreatic lipase, and 84.0% for cholesterol micellar solubility, while the 2-h digest of the purified protein fraction showed 73.4%, 44.9%, and 82.2% inhibition, respectively. Proteins and peptides exhibited strong bioactivity, with inhibition responses across all assays approaching those of the assay controls under the tested conditions. This is the first study to demonstrate the bioactive potential of proteins and peptides extracted from *C. cujete* fruit, providing promising insights into their possible functional properties associated with the preventive management of obesity, cholesterol levels, and hypertension. Further peptide fractionation and sequence identification are needed to confirm the most active components.

**Keywords:** Antihypertensive, Bioactive peptides, Calabash, Cholesterol micellar, Functional food



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## Introduction

Functional foods contain bioactive compounds that provide physiological and health benefits beyond nutrition, and the emergence of these foods has led people around the world to gradually transition to a diet rich in proteins, essential oils, vitamins, polyphenols, and carotenoids (Angeles *et al.*, 2021). Among the major bioactive constituents of functional foods, proteins and peptides have attracted considerable attention because of their diverse physiological activities. Proteins play a fundamental role in providing the nutrients for human diet derived from foods. Proteins derived from functional foods may exhibit various health benefits, including regulation of blood pressure, cholesterol levels, and blood sugar.

This growing interest in functional foods is largely associated with the increasing prevalence of chronic non-communicable diseases, particularly cardiovascular diseases (CVD). Two major modifiable risk factors of CVD are hypertension and dyslipidemia. Hypertension affected around 1.3 billion adults in 2023, while dyslipidemia accounts for approximately 4 million CVD-related deaths worldwide (Zhao, 2021; World Health Organization, 2023; Abera, Worede, Hirigo, Alemayehu, & Ambachew, 2024). Increased consumption of plant proteins has been associated with reduced CVD risk. Due to this emerging need of the populace, the development of nutritionally enhanced and functional foods to address health concerns is rapidly increasing.

The physiological effects exhibited by proteins are usually present in the peptide sequences encrypted in the parent protein, which become active when released as peptide fragments or free amino acids. Bioactive peptides found in foods have been widely investigated for their potential role in the prevention of conditions such as hypertension, cancer, dyslipidemia, microbial infections, and cardiovascular diseases (Cicero, Floor & Albertoni, 2017). Protein hydrolysates and

peptides from cereals, legumes, and animal products have been widely investigated for their ACE-inhibitory and lipid-lowering activities, yet fruits are generally overlooked as sources of proteinaceous compounds due to their relatively low protein content. This gap underscores the need to explore unconventional fruit sources using targeted workflows that combine protein extraction, characterization, digestion, and *in vitro* evaluation aligned with antihypertensive and lipid-related outcomes.

Calabash (*Crescentia cujete* L.) is of particular interest because its fruit has long been used in traditional medicine and has recently gained considerable attraction in the Philippines due to anecdotal claims of treating hypertension, diabetes, dyslipidemia and cancer. Belonging to the family *Bignoniaceae*, calabash is a seasonal fruit that is widely propagated usually in home gardens in Central and South America, Africa and Asia. Calabash fruit has been incorporated into herbal mixtures in many traditional medicine systems for its anti-inflammatory, and anti-bacterial effects and its use in managing cough and colds (Shastry, Bhalodia Maulik & Aswathanarayana, 2012). Although the biological activities of calabash are commonly attributed to phytochemicals such as apigenin, quercetin, anthraquinones, carotenoids, and  $\beta$ -sitosterol, the contribution of its proteinaceous components remains poorly understood.

In this context, the present study aims to isolate, purify and characterize the proteins and peptides released after simulated gastrointestinal digestion of calabash fruit flesh, thereby providing baseline information on its protein content. This study also aims to investigate the potential effects of proteins and peptides in managing elevated blood pressure and high cholesterol levels, providing scientific validation for anecdotal claims.

## Materials and Methods

### Sample Preparation and Crude Protein Isolation

Calabash (*C. cujete*) fruits were collected from the province of Davao, Philippines. Three randomly selected trees were used as biological

sources, and three mature fruits were harvested from each tree yielding a total of nine fruits for subsequent analysis. Fruit maturity was determined based on fruit size, hard outer rind and external color, which are used for morphological identification of mature calabash fruits. The fruit flesh was lyophilized using a -85°C laboratory floor-mode freeze dryer (IlShin Biobase, Korea). Lyophilized fruit flesh from each fruit was extracted individually for generate nine independent samples, and downstream assays were performed in triplicate for each extract. Two grams of lyophilized calabash flesh were homogenized with 10 mL of 0.1 M sodium phosphate buffer pH 7.0 containing 0.05 M Tris-HCl and 0.4 M NaCl. The mixture was incubated overnight at 4°C with shaking, and then filtered through cheesecloth and centrifuged at 11,872 x *g* at 4°C for 10 min. The resulting filtrate was labeled as crude protein isolate (CPI).

#### **Purification of Proteins**

##### **Ammonium Sulfate Precipitation**

Five mL of CPI was subjected to ammonium sulfate precipitation to achieve approximately 40% saturation by slow addition of 1.21 grams of powdered ammonium sulfate under constant stirring. This saturation level was selected, based on preliminary experiments, to recover a protein-enriched fraction. The samples were centrifuged at 11,872 x *g* for 10 min at 4°C using Beckman Coulter X-30R (USA). The supernatant was discarded, and the pellet was reconstituted using 0.1 M sodium phosphate buffer pH 7.0.

##### **Dialysis**

The reconstituted proteins were dialyzed overnight at 4°C against distilled water using a cellulose dialysis tubing with molecular weight cut-off of 3.5 kDa. The distilled water was changed every 3 h.

##### **Gel Filtration Chromatography**

Dialyzed protein samples were purified using Sephadex G-100 (Sigma-Aldrich, USA)

pre-swollen for 3 days and equilibrated using extraction buffer. Three milliliters of dialyzed protein samples were loaded into the column and eluted with cold extraction buffer at a flow rate of 1.0 ml/min. Fractions of 1 mL each were collected, and absorbance of eluates was monitored at 210 nm using a UV-Vis spectrophotometer (Spectroquant Pharo 300, Merck Millipore, Germany). Pooled fractions were labelled as purified proteins (PP).

##### **Protein Quantification Using Bradford Assay**

Protein quantification was performed using Quick Start Bradford Assay (Bio-Rad, USA). A standard calibration curve was prepared using different concentrations of bovine serum albumin (BSA). In a 96-well microtiter plate, 2.5 µL of sample and 125 µL 1x Bradford reagent were thoroughly mixed and incubated for 10 min. The absorbance was monitored at 595 nm using a microplate reader (BioTek Epoch, Agilent, USA).

##### **Sodium Dodecyl Sulfate–polyacrylamide Gel Electrophoresis (SDS-PAGE)**

Twelve percent (12%) discontinuous denaturing gels were prepared and run at 110V for approximately 100 minutes using a Mini-PROTEAN Tetra Cell (Bio-Rad, USA). Gel bands were visualized using 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad, USA). A protein reference ladder (NEB, MA, USA) was used to estimate the molecular weights of the protein subunits.

##### **Enzymatic Hydrolysis of Proteins**

PP were subjected to digestion in a 12:1 protein-to-enzyme ratio. Solutions of pepsin, trypsin and chymotrypsin (1 mg/mL) were prepared in distilled water. These enzymes were chosen to simulate the main stages of human gastrointestinal protein digestion and to generate a broad spectrum of peptide fragments with different sequence specificities under physiologically relevant pH and temperature conditions. Sequential digestion was performed, wherein pepsin was added first and allowed to digest for 1 h at pH 2.0. The

remaining enzymes were then added simultaneously after adjusting the pH to 7.0 using 1.0 M NaOH. The mixture was incubated for 1, 2, 3, 4, 6 and 24 h at room temperature. The hydrolysis was terminated by immersing the samples in a boiling water bath for 5 min. Progression of hydrolysis was assessed via SDS-PAGE.

#### Normalization of Protein Activity

The normalized protein activity of CPI was obtained by dividing the percent inhibition by the mass of protein loaded per assay. For CPI, the protein mass was calculated from the Bradford-derived concentration (688 µg/mL) and the assay loading volume, and the values are expressed as apparent normalized inhibition per mg protein. Activities were therefore expressed as apparent normalized percent inhibition per mg protein loaded. Because the purified fractions fell below the Bradford detection limit, normalization of PP digest activity should be considered approximate unless verified using a more sensitive protein assay.

#### *In vitro* Assays for Antihypertensive, Anti-obesity and Hypcholesterolemic Activities

The sample preparation and laboratory analysis were all done at the Biochemistry Laboratory of the Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños. All chemicals and reagents used were of analytical grade and purchased from reputable suppliers.

#### ACE Inhibition Assay

ACE inhibition activity was determined using the method of [Cushman & Cheung \(1971\)](#) with modifications. An assay mix of 25 µL 100 mM sodium phosphate buffer pH 8.3, 25 µL 300 mM NaCl, 50 µL 5 mM hippuryl—histidyl-leucine (HHL), 25 µL of sample, and 25 µL crude ACE extract (which was added last) from pig lungs was incubated for 30 min at 37°C. After incubation, 125 µL of 1 N HCl was added to stop the reaction. A blank control was prepared by adding 25 µL of phosphate buffer

to the assay mix instead of the sample. Captopril at a concentration of 1 mg/mL was used as a positive control and added to the assay mix. Negative control was also prepared wherein the stop solution (1 N HCl) was initially added to the assay mix without ACE. Addition of 750 µL ethyl acetate into the mix was then performed to extract hippuric acid. Five hundred microliters of the ethyl acetate layer were collected and allowed to evaporate to dryness. The extracted hippuric acid was dissolved in 500 µL distilled water and the response was monitored at absorbance of 228 nm. The percent inhibition was calculated using the following equation:

$$\text{Percent inhibition} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank} - \text{Absorbance of negative control}} \times 100\% \quad (1)$$

#### Pancreatic Lipase Inhibition Assay

The pancreatic lipase inhibition activity was determined using the method of [Chheda, Kaikini, Bagle, & Seervi, \(2016\)](#). Freshly prepared 0.1 M potassium phosphate buffer pH 7.2 with 150 mM NaCl and 0.5% (v/v) Triton X-100 was used to dissolve 6 mg of lipase enzyme from porcine pancreas (≥125 units/mg protein) and diluted to 10 mL with phosphate buffer. The substrate of the assay was prepared using 8.45 µL *p*-nitrophenyl butyrate (PNPB) and diluted to 10 mL with acetonitrile. Inhibitory activity was determined by mixing 25 µL of the sample, 100 µL phosphate buffer, 50 µL lipase enzyme, and 25 µL PNPB solution. The mixture was incubated at 37°C for 30 min and the absorbance was monitored at 400 nm using a microplate reader to estimate the amount of *p*-nitrophenol released from the reaction. One mg/mL Orlistat dissolved in dimethyl sulfoxide was used as a positive control. The percent inhibition was calculated using the following equation:

$$\text{Percent inhibition} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100\% \quad (2)$$

#### Cholesterol Micellar Solubility Inhibition Assay

The cholesterol micellar solubility inhibition activity was determined using the method of [Bangoura, Wenshui, & Jiali, \(2009\)](#).

Cholesterol micellar solution was initially prepared by mixing 10 mM sodium deoxycholate, 0.4 mM cholesterol, 132 mM NaCl, 1 mM oleic acid, and 15 mM potassium phosphate buffer (pH 7.4). The mixture was sonicated before use.

A 1:1 mixture of micellar solution and sample was incubated at 37°C for 24 h and centrifuged at 11,872 x *g* for 10 min. Using a 96-well titer plate, 60 µL of supernatant was mixed with 75 µL of acetic acid followed by the slow addition of 90 µL Zak's reagent (0.1% FeCl<sub>3</sub> in concentrated H<sub>2</sub>SO<sub>4</sub>). The reaction mixture was incubated at room temperature for 30 min prior to absorbance determination at 560 nm using a microplate reader. One mg/mL cholestyramine dissolved in phosphate buffer was used as positive control. The blank sample was prepared by substituting the protein isolates with the micellar solution. The percent inhibition was calculated using the following equation:

$$\text{Percent inhibition} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100\% \quad (3)$$

#### Statistical Analysis

The data collected were expressed in mean ± standard deviation and analyzed using R Studio (Posit Software, Boston, Massachusetts). The number of samples, *n*, refers to independently prepared fruit extracts, whereas assay measurements from each extract were treated as technical replicates. The bioactivities were analyzed using Kruskal–Wallis test to determine the significant differences among the given groups. A pairwise post hoc test using Dunn's test with Benjamini-Hochberg (BH) correction was also performed to identify which specific groups differ compared to the activity of positive control.

## Results and Discussion

#### Protein Isolation and Characterization

This study extracted soluble protein-containing fractions from *C. cujete* fruit flesh to evaluate their functional applications and potential health benefits through *in vitro* bioactivity assays. The extracted protein has an

average concentration of 688 µg/mL in terms of extraction buffer or 0.344 g/100 g dry weight of fruit flesh. The median and interquartile range (IQR) values were recorded at 671 and 449 µg/mL, respectively. The quality of CPI was assessed using SDS-PAGE as shown in Fig. 1a. Five distinct bands were observed with estimated molecular weights of 12.1 kDa, 23.2 kDa, 25.7 kDa, 30.1 kDa, and 46.7 kDa. This suggests that CPI contains relatively low-molecular-weight proteins and peptides. Many classical structural and enzymatic proteins in cellular compartments have higher molecular masses (usually 40–60 kDa), but numerous functional proteins and bioactive peptides are substantially smaller. Classic examples include insulin, a 5.8 kDa hormone, and the 4-kDa hormone-like legume peptide that interacts with a 43-kDa receptor-like kinase in soybean. Plant-derived proteolytic enzymes like fruit bromelain (25–32 kDa) from pineapple, widely used for its protease activity and reported anti-inflammatory property further demonstrate that functional proteins can span a broad molecular-weight range. Reviews on plant- and food-derived bioactive peptides also report many active sequences with molecular weights below 10 kDa, supporting the view that the relatively small size of the CPI proteins and peptides observed in this study is compatible with functional and bioactive roles (Hanada & Hirano, 2004; Hikiş & Bernasinska-Slomczewska, 2021; Mayer, Zhang, & DiMarchi, 2007; Zhu *et al.*, 2023).

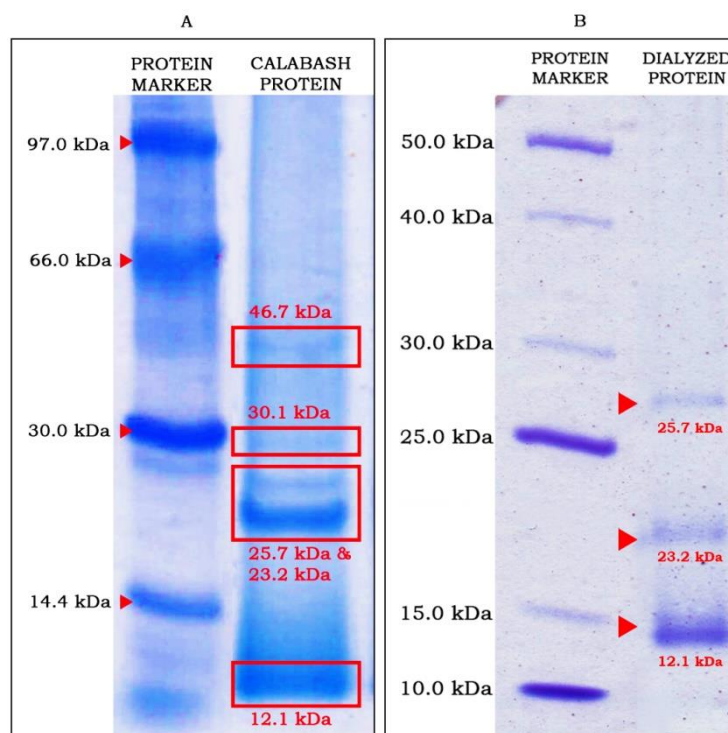
Crude proteins were isolated using sodium phosphate buffer containing Tris-HCl and NaCl. Phosphate buffers are highly effective in maintaining pH stability, thereby preventing extreme changes that could result in protein denaturation or aggregation. Maintaining a neutral pH (7.0) is crucial for preserving the native conformation of proteins, ensuring their functionality and bioactivity remain intact. Furthermore, sodium phosphate buffer is a preferred choice in protein extraction protocols due to its minimal reactivity with proteins, which reduces the likelihood of undesirable chemical interactions (Pavani, Kumar, Rani,

Venkatesu, & Lee, 2021). CPI contains less than 1% protein, consistent with the typically low protein content of most fruits and vegetables, which still offer dietary benefits (Ford *et al.*, 2023).

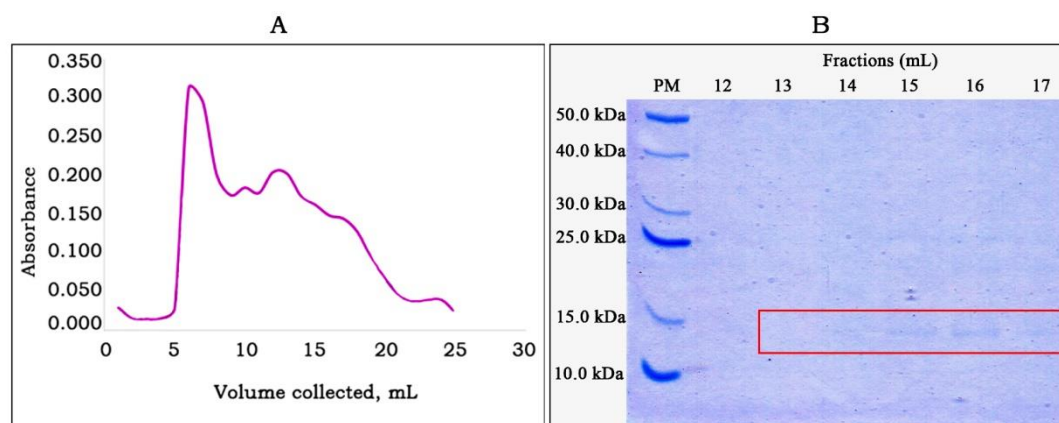
The CPI was precipitated using ammonium sulfate at 40% saturation as a partial enrichment step and subsequently dialyzed to remove excess salts. This precipitation indicates selective recovery of a subset of soluble proteins rather than confirmed precipitation of the total protein complement. Classical protein fractionation shows that proteins precipitate over a broad range of saturations (e.g., 60-80%) to recover more complete protein fractions (Tola & Missihoun, 2023; Wingfield, 1998). Future work will combine stepwise fractionation with trichloroacetic acid or total nitrogen measurements to validate total protein recovery. The partially purified proteins were evaluated using SDS-PAGE which showed three remaining distinct bands with improved resolution (Fig. 1b) which correspond to molecular weights of 12.1 kDa, 23.2 kDa, and 25.7 kDa, as estimated in CPI in Fig. 1a. Different molecular weight protein markers were used for Fig. 1a and 1b due to reagent availability during separate experimental runs; however, band size estimations were independently performed using the corresponding protein ladder for each gel. The partially purified proteins were then subjected to size-exclusion chromatography using Sephadex resin and 25 fractions of 1 mL each were collected. The elution profile showed two distinct peaks: one between 5-9 mL and another broad peak from 11-22 mL with observed peak shouldering from 15 mL to 18 mL. The presence of shouldering suggests the presence of multiple species due to close molecular weight or the existence of multiple states such as oligomer or protein aggregates. After conducting several electrophoretic runs, the

remaining visible band corresponded to a protein of approximately 12.1 kDa, matching the last broad peak observed in the elution profile as illustrated in Fig. 2. Bradford assay could not determine the remaining protein concentration in PP because of the possibility of having few low molecular weight proteins and peptides. Another possibility is that the protein concentration of PP, estimated to be less than 20 µg/mL, is already below the detection limit of the Bradford assay, thus resulting in no detectable response. Other protein quantification methods with higher sensitivity and detection limit such as Lowry and Bicinchoninic Acid (BCA) assays can be utilized in the future to verify the protein content of calabash fruit flesh.

PP was used to simulate gastrointestinal digestion. Enzymatic digestion releases peptides and amino acids that were initially inactive within the native protein structure. These peptides are known to exhibit protein-protein inhibition due to their variability in size and specificity (Daliri, Oh & Lee, 2017). After 1 h of digestion, protein bands were no longer visible in the polyacrylamide gels, indicating successful protein hydrolysis resulting in low molecular weight proteins or peptide fragments. This observation suggests the efficiency of the hydrolysis process and highlights its potential to support biochemical reactions analogous to natural digestive processes. In addition, purified protein hydrolysates were not detectable by Bradford assay, likely due to low concentration or reduced dye binding of low-molecular-weight species, hence, the extent of total protein recovery could not be fully verified in the present study.



**Fig. 1.** Electrophoretograms of (a) CPI highlighting five major protein bands; and (b) partially purified proteins from ammonium sulfate fractions



**Fig. 2.** (a) Elution profile of the dialyzed 40% ammonium sulfate-recovered protein fractions, and (b) SDS-PAGE profile of PP with visible bands with approximate molecular weight of 12.1 kDa

The apparent normalized inhibition values reported for CPI (Table 1) represent protein-normalized endpoint responses rather than classical enzyme specific activities, which are typically expressed in units per mg based on kinetic measurements. Because the purified protein fraction and its hydrolysates could not be reliably quantified by Bradford, equivalent normalization for these samples was not

performed, and their activities are presented as percent inhibition per reaction as presented in Table 2.

#### ACE Inhibition Activity

The *in vitro* antihypertensive and lipid-lowering properties of CPI and PP hydrolysates were determined spectrophotometrically. For antihypertensive activity, CPI exhibited high

ACE inhibition of 76.5%, which showed no significant difference from Captopril, a known ACE inhibitor drug, with 78.9% inhibition ( $p > 0.05$ ). CPI contributed approximately 0.0172 mg protein per reaction, corresponding to an apparent normalized inhibition of about  $4.45 \times 10^3$  % inhibition per mg protein. This value provides a protein-normalized estimate of the ACE inhibitory response of CPI and supports the observation that the crude isolates contain proteinaceous components with inhibitory potential. However, this apparent normalized inhibition should be interpreted only as an internal comparative metric because the assay was performed as an endpoint inhibition assay rather than a kinetic assay. In

addition, inhibition values of the calabash CPI approached those of captopril under the assay conditions, such comparisons should be interpreted cautiously because the tested materials differed markedly in composition, purity, and active mass. In addition, results showed that ACE inhibition of protein hydrolysates had comparable values from the 1<sup>st</sup> to 4<sup>th</sup> h of digestion but showed a significant decrease at the 6<sup>th</sup> h of digestion. However, an increase in inhibition activity was later observed for the 24-h digests. For visualization, the effect of digestion on the purified samples over time using ACE inhibitory activity is shown in Fig. 3.

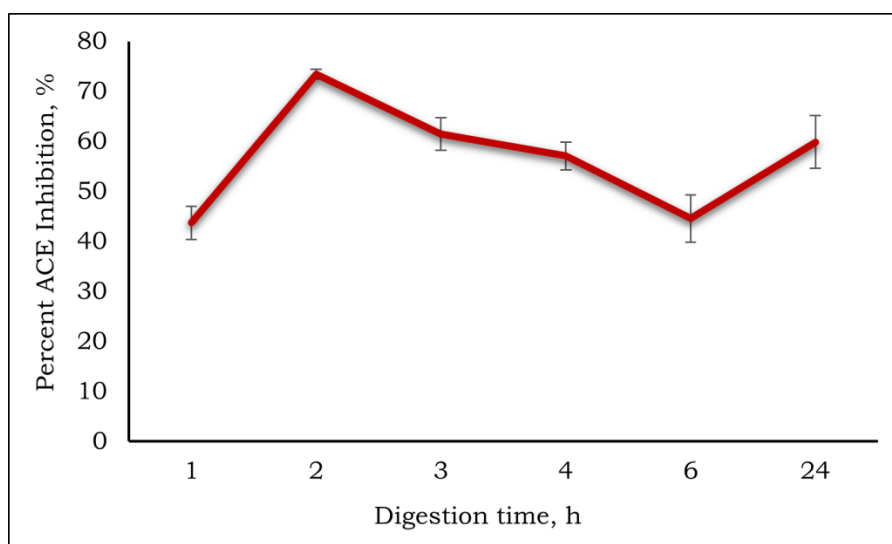


Fig. 3. Effect of digestion time on ACE inhibition of *C. cujete* fruit flesh purified protein hydrolysates

Table 1- Comparison of the apparent normalized inhibitory activities of CPI

|                             | Apparent % inhibition per milligram protein |                      |                                 |
|-----------------------------|---------------------------------------------|----------------------|---------------------------------|
|                             | ACE inhibition                              | Lipase inhibition    | Cholesterol micellar inhibition |
| Crude Protein Isolate (CPI) | $4.45 \times 10^3$                          | $1.30 \times 10^3$ % | $2.03 \times 10^3$ %            |

Table 2- Summary of inhibitory activities of CPI and two (2)-hour PP digests

|                  | ACE inhibition, %                     |                            | Lipase inhibition, %                 |                             | Cholesterol micellar inhibition, %         |                            |
|------------------|---------------------------------------|----------------------------|--------------------------------------|-----------------------------|--------------------------------------------|----------------------------|
|                  | CPI                                   | PP digest                  | CPI                                  | PP digest                   | CPI                                        | PP digest                  |
| Positive control | Captopril<br>78.9 ± 5.94 <sup>a</sup> |                            | Orlistat<br>72.6 ± 6.36 <sup>a</sup> |                             | Cholestyramine<br>91.5 ± 0.16 <sup>a</sup> |                            |
| Calabash         | 76.5 ± 3.54 <sup>a,b</sup>            | 73.4 ± 1.00 <sup>a,b</sup> | 22.3 ± 5.76 <sup>b,c</sup>           | 44.9 ± 0.680 <sup>a,c</sup> | 84.0 ± 1.20 <sup>a,b</sup>                 | 82.2 ± 8.17 <sup>a,b</sup> |

\*Values are expressed as mean ± SD (n = 3). In the same column, values followed by the same superscript letters are not significantly different at the 5% level of significance based on the Kruskal-Wallis test followed by Dunn's test. Letters before the comma indicate column-wise comparisons; letters after the comma indicate row-wise comparisons per bioassay.

For digested PP, it was observed to have high ACE inhibition after 2 h digestion time with 73.4% inhibition compared to other digestion times. The 2-h digests were selected to use in the succeeding bioassays after the ACE inhibition assay exhibited the highest bioactivity across digestion time.

Crude extracts may contain various proteins in their native conformation including the presence of other bioactive compounds such as polyphenols, which could contribute to the observed increased bioactivity. Previous studies have shown that certain phenolics and flavonoids exhibit ACE inhibitory activity (Balasuriya & Rupasinghe, 2011; Simaratanamongkol, Umehara, Noguchi, & Panichayupakaranant, 2014). The results obtained from this study were comparable to the ACE inhibitory activity of mango flesh and 'Señorita' banana cultivar flesh crude proteins with 71%-74% and 85.2% inhibition, respectively (Ferrerias, Bercansil-Clemencia, Hizon-Fradejas, Uy, & Torio, 2021; Rojas *et al.*, 2021). The highest ACE inhibition was observed in 2 h digested PP, followed by a significant decrease at the 6<sup>th</sup> h of digestion. Although the 2-hour digest exhibited highest ACE inhibition, the present study did not fractionate the hydrolysate into peptide subfractions. The initial increase in activity likely reflects the release of ACE-inhibitory peptides during early digestion time whereas the subsequent decrease may be attributed to the further hydrolysis of proteins into smaller fragments with reduced inhibitory response. ACE inhibitory peptides are typically short sequences (2–12 amino acids), because smaller peptides can more easily access the ACE active site. Their activity is commonly associated with hydrophobic or aromatic residues, especially at the C-terminal end. These features are inferred to enhance binding through hydrophobic interactions within ACE's substrate-binding pockets leading to an improved inhibitory response (Daskaya-Dikmen, Yucetepe, Karbancioglu-Guler, Daskaya, & Ozelik, 2017; Hu *et al.*, 2025). ACE inhibition activity can also be increased by using higher enzyme

concentration and longer hydrolysis time which may generate shorter peptides with potential bioactivity (Rojas *et al.*, 2021; Shalaby, Zakora, & Otte, 2006). Similar studies also reported higher ACE inhibitory activity when exposed to longer hydrolysis time (Abuine, Rathnayake & Byun, 2019). In addition, a minimal contribution can be linked to trace amounts of other compounds, such as polyphenols because PP underwent purification and dialysis utilizing a molecular weight cut-off of 3 kDa which effectively removes compounds smaller than this threshold. Any residual effect would therefore be minor compared to peptide-mediated inhibition.

#### Pancreatic Lipase Inhibition Activity

For pancreatic lipase inhibition, CPI exhibited 22.3% inhibition and showed significantly lower inhibition than Orlistat, a known lipase inhibitor, with an inhibition value of 72.6 %. The crude protein isolate corresponded to an apparent normalized inhibition of approximately  $1.30 \times 10^3$  % inhibition per mg protein. This lower normalized response relative to ACE and cholesterol micellar solubility inhibition suggests that, within CPI, lipase-inhibitory activity was weaker or less concentrated than the other measured bioactivities. On the other hand, 2-h PP digest showed higher lipase inhibition at 44.9% compared to CPI and was not statistically significant from Orlistat ( $p > 0.05$ ). The values obtained in this study were lower compared to Orlistat but higher compared to the reported lipase inhibitory activity of sea buckthorn seed protein hydrolysates with 35% inhibition (Xiang *et al.*, 2020). Studies also reported peptides isolated from millet grains, rice bran, milk, and some fish species that showed moderate to strong inhibitory activity (Jakubczyk, Szymanowska, Karaś, & Złotek, 2019; Ketprayoon *et al.*, 2021). In physiologic conditions, pancreatic lipase interacts at the lipid interface of emulsion droplets, where it hydrolyzes triglycerides to release free fatty acids. One possible mechanism for pancreatic lipase inhibition by

proteins involves the ability to reduce the interfacial tension at the lipid-water interface. This reduction allows the proteins to penetrate the monomolecular film of phospholipids and glycerides, even under high surface pressure. The disruption hampers the ability of pancreatic lipase to hydrolyze its substrate efficiently (Gargouri, Julien, Pieroni, Verger, & Sarda, 1984). Another possible mechanism includes the formation of protein aggregates or peptide complexes that limit its accessibility to lipid substrates, as demonstrated by lipase activity with flavonoids under intestinal conditions (Bustos, Hakansson, Linares-Pasten, Peñarrieta, & Nilsson, 2020). However, because of the observed low-molecular weight nature of proteins and peptide fractions, peptide-mediated inhibition is more plausible than the aggregate-related effects. Low molecular weight peptides tend to diffuse more efficiently to the lipid-water interface, adsorb at the emulsion surface, and interact directly with the substrate-binding regions of lipase, thereby interfering with substrate access that reduce enzymatic turnover (Ketprayoon *et al.*, 2021; Wang *et al.*, 2022).

#### Cholesterol Micellar Solubility Inhibition Activity

For cholesterol micellar solubility inhibition, CPI and the 2-hour PP digest showed high inhibitory responses of 84.0% and 82.2%, respectively. CPI corresponded to an apparent normalized inhibition of approximately  $2.03 \times 10^3$  % inhibition per mg protein. This suggests that CPI may contain components capable of reducing cholesterol incorporation into mixed micelles under the simplified *in vitro* conditions of the assay. Cholestyramine showed a comparable inhibition of 91.5%, with no significant difference ( $p > 0.05$ ) from CPI. Both CPI and protein hydrolysates likely bound cholesterol thereby reducing its solubility within micelles. Proteins and peptides may interact with bile components, bind free cholesterol through hydrophobic interactions or disrupt the structural conformation of micelles (Udenigwe & Rouvinen-Watt, 2015). Such interactions could potentially limit the

bioaccessibility of cholesterol during intestinal digestion. From a physiological perspective, non-solubilized cholesterol tends to segregate into an oil phase within the intestinal lumen, thereby becoming inaccessible for absorption and can be excreted through the hepatobiliary pathway or transintestinal cholesterol excretion (Estribillo *et al.*, 2022; Jesch & Carr, 2017). Similar cholesterol micellar solubility inhibition activities have been reported for peptides generated from royal jelly and fermented seabass by-product which reported suppression of cholesterol solubility greater than 80% (Chen, Lin, Huang, & Lin, 2021; Kashima *et al.*, 2014). This simplified *in vitro* assay provides an indicator of reduced cholesterol bioaccessibility potential rather than a direct measure of *in vivo* cholesterol-lowering efficacy. Overall, these findings highlight the promising potential of CPI and PP digests to contribute to the preventive management of elevated blood cholesterol level and obesity, and when combined with their ACE inhibitory activity, to support blood pressure control.

This study represents an initial screening effort on the biological activity evaluation of calabash proteins; the most active peptide fraction and individual peptide sequences were not identified. In addition, all bioactivities were evaluated using *in vitro* assays, which provide useful screening evidence but do not demonstrate *in vivo* antihypertensive or lipid-lowering efficacy. These constraints highlight the need for further work involving improved protein quantification methods, more detailed peptide fractionation and identification (e.g. LC-MS/MS), and *in vivo* validation.

#### Conclusion

Protein- and peptide-enriched fractions from *C. cujete* fruit flesh exhibited promising *in vitro* ACE inhibitory, pancreatic lipase inhibitory, and cholesterol micellar solubility inhibitory activities. The observed biological activities may be associated with the release of sufficient bioactive peptides during hydrolysis in a time-dependent manner. The 2 h hydrolysate of the

PP digest showed the most consistent *in vitro* antihypertensive potential, with high ACE inhibitory activity and notable inhibitory effects on pancreatic lipase and cholesterol micellar solubility, under the conditions used in this study. To the best of our knowledge, this is the first study to report on the isolation and characterization of bioactive peptides with ACE and lipase-inhibiting properties from *Crescentia cujete* fruit flesh. However, the present work represents an initial screening study; total protein recovery at the ammonium sulfate step was not validated as total precipitation, the most active peptide fraction was not isolated, and peptide sequences were not identified. Future work may involve validation using complementary protein precipitation techniques, peptide identification and sequencing using LC-MS/MS, and other approaches to confirm target interactions. Animal bioassays can also be conducted to further support the therapeutic potential of calabash fruit-derived proteins and peptides.

#### Authors Contributions

**JC Villanueva** conceptualized, designed, acquired funding, analyzed data, and wrote the manuscript for this study; **AC Sajise** helped in

acquiring funding, sample collection and reviewing the manuscript; **AC Laurena** assisted in reviewing and revising the manuscript; **MO Torio** provided laboratory resources and assisted in reviewing the manuscript; **VP Migo** helped in designing the study and assisted in reviewing the manuscript.

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#### Conflict of Interest

The authors declare no conflict of interest.

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مقاله پژوهشی

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## جداسازی، خالص سازی و شناسایی پروتئین ها و پپتیدهای موجود در گوشت میوه کدویی (*Crescentia cujete* L.) با ویژگی های ضد فشارخون و کاهنده چربی خون

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### چکیده

میوه کدویی (*Crescentia cujete* L.) در سال های اخیر به دلیل نقش قابل توجه آن در سلامت و تغذیه انسان، به طور فزاینده ای مورد مطالعه قرار گرفته است. فعالیت های زیستی گزارش شده برای این میوه با حضور ترکیبات فیتوشیمیایی مانند پلی فنول ها مرتبط دانسته شده اند، اما درباره نقش پروتئین های آن اطلاعات اندکی وجود دارد. این موضوع یک خلأ مهم پژوهشی محسوب می شود، زیرا میوه ها در مقایسه با حبوبات و سایر فرآورده های غنی از پروتئین، معمولاً به عنوان منابع پروتئینی نادیده گرفته می شوند؛ با وجود آنکه می توانند کاربردهای بالقوه عملکردی و درمانی داشته باشند. هدف این پژوهش، جداسازی، خالص سازی و شناسایی پروتئین ها و پپتیدهای موجود در میوه کدویی و ارزیابی توان بالقوه آن ها در کاهش فشار خون و چربی خون بود. پروتئین ها با استفاده از مجموعه ای از روش های بیوشیمیایی، از جمله استخراج با بافر فسفات سدیم، رسوب دهی با سولفات آمونیوم، کروماتوگرافی ژل فیلتراسیون و آزمون های زیستی درون کشتگاهی (*in vitro*) استخراج، خالص سازی و شناسایی شدند. آزمون های زیستی شامل مهار لیپاز پانکراس، مهار حلالیت میسلی کلسترول و مهار آنزیم مبدل آنژیوتانسین (ACE) بودند. ایزوله پروتئینی خام (CPI) دارای غلظت پروتئینی ۶۸۸ میکروگرم بر میلی لیتر بود و نوارهای پروتئینی مشاهده شده در محدوده ۱۲/۱ تا ۴۶/۷ کیلودالتون قرار داشتند. پس از شبیه سازی هضم دستگاه گوارش، CPI به ترتیب میانگین فعالیت مهار ۷۶/۵٪ برای ACE، ۲۲/۳٪ برای لیپاز پانکراس و ۸۴/۰٪ برای حلالیت میسلی کلسترول نشان داد. در حالی که هضم دوساعته فراکسیون پروتئینی خالص شده، به ترتیب ۷۳/۴٪، ۴۴/۹٪ و ۸۲/۲٪ مهار را در این آزمون ها نشان داد. پروتئین ها و پپتیدها فعالیت زیستی قابل توجهی از خود نشان دادند و میزان مهار آن ها در تمامی آزمون ها، در شرایط آزمایشی مورد بررسی، به مقادیر مشاهده شده در نمونه های کنترل آزمون نزدیک بود. این پژوهش نخستین مطالعه ای است که توان زیست فعال پروتئین ها و پپتیدهای استخراج شده از میوه *C. cujete* را نشان می دهد و دیدگاه های امیدوارکننده ای درباره ویژگی های عملکردی احتمالی آن ها در مدیریت پیشگیرانه چاقی، سطح کلسترول و فشار خون بالا ارائه می کند. با این حال، برای تأیید فعال ترین ترکیبات، انجام فراکسیون بندی بیشتر پپتیدها و شناسایی توالی آن ها ضروری است.

**واژه های کلیدی:** پپتیدهای زیست فعال، ضد فشارخون، غذای عملکردی، کدویی، میسل کلسترول

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