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Isolation and Biological Activity Studies of Some Proteins from Cucurbitaceae Family

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Abstract

Plants belonging to the Cucurbitaceae family represent major agricultural products widely valued for dietary consumption. Beyond traditional nutritional profiles, their agro-industrial processing byproducts—most notably seeds and seed cakes—contain substantial protein reserves that have recently gained massive pharmaceutical and biomedical attention. This final year project focuses on the strategic isolation, sequential biochemical purification, and biological screening of specialized functional proteins harvested from four key representatives of the Cucurbitaceae family: Momordica charantia (Bitter melon), Cucurbita pepo (Pumpkin), Cucumis sativus (Cucumber), and Citrullus lanatus (Watermelon).

In vitro enzymatic inhibition profiling revealed substantial anti-hyperglycemic properties (alpha-glucosidase and alpha-amylase inhibition) and potent anti-inflammatory effects through egg albumin denaturation prevention. Antimicrobial screening showed significant zones of inhibition against Gram-positive bacteria (Staphylococcus aureus) and moderate activity against Gram-negative paths (Escherichia coli). These findings indicate that Cucurbitaceae proteins constitute an exceptional, sustainable source of therapeutic macromolecules capable of being scaled into functional pharmaceutical excipients, neutraceutical components, or targeted biopharmaceuticals.

Keywords: Cucurbitaceae family, phytochemical composition, therapeutic properties, food, representative species

Aim & Objective :

Aim :

projects focusing on the isolation and biological activity of proteins from the Cucurbitaceae family typically aim to bridge the gap between traditional ethnomedicinal use and modern biotechnological applications.

Objective :

Extraction and Characterization:

To develop or optimize protocols for isolating high-quality proteins from various parts of Cucurbitaceae plants—most commonly seeds, which are often discarded as industrial by-products during oil production

Evaluation of Biological Activity: To screen isolated proteins or their enzymatic hydrolysates for specific health-promoting properties, such as antioxidant, antihypertensive, antidiabetic, or anticancer activities

Structure-Function Relationship Studies:

To analyze how the amino acid composition, protein structure, and molecular weight distribution influence the functional properties (e.g., solubility, emulsification, foaming) and biological efficacy of the protein

Nutraceutical and Therapeutic Potential: To assess whether these proteins can serve as natural alternatives to synthetic drugs or antioxidants, potentially mitigating side effects and addressing protein-energy malnutrition .

Valorization of By-products: To convert low-value agricultural residues (like seed cakes) into high-value functional food ingredients, thereby promoting sustainability in the food and pharmaceutical industries.

1. Introduction

Proteins are indispensable for any living system by working as catalysts, structural components, or regulators. Therefore, it is crucial to understand the existing living systems by studying how the proteins work. Understanding a protein's function depends on and evolves with the advances in other subjects, particularly physics and chemistry, and technologies that integrate the advances in diverse fields. We are witnessing several changes in studying proteins the experimental samples. The experimental samples used in protein studies changed from a mixture containing the target protein in early times to homogenous proteins purified from a natural source or the heterogeneous overexpressing systems in more recent decades . the throughput of protein study. Although indispensable and reliable, the throughput of "one-protein-a-time" is becoming increasingly outdated. Instead, proteins are studied in parallel or at the proteomic level. The advances in bioinformatics make it possible to deal with thousands to millions of target proteins in a sample at a time .The behavior of a single protein molecule. When it is impossible to probe a single protein molecule's behavior, we use a protein molecule population to amplify a single molecule's behavior , and the "average" behaviors of the molecule population are taken as the behavior of an individual protein. Such a solution inevitably neglects the individual differences in the population. It is also the case in X-ray-based structure determination with a protein crystal: the orderly arrangement of multiple protein molecules amplified the signal generated from a single protein in the crystal. By contrast, Cryo-EM technology works differently to capture the "almost native status" of protein at work. Moreover, in the structures obtained with protein molecules "frozen" in samples used for X-ray or Cryo-EM-based analysis, the molecular flexibility and possible conformational/composite

changes caused by functionally-associated dynamics in an actual cellular environment cannot be sufficiently reflected dissecting an individuals contribution to a proteins function. It is beneficial to pinpoint an individual amino acid residue's exact contribution to the protein activity .

The Cucurbitaceae family, including pumpkins, melons, and cucumbers, is a prolific source of bioactive proteins such as Ribosome-Inactivating Proteins (RIPs), protease inhibitors, and storage globulins like cucurbitin. This report explores the methodologies for the isolation of these proteins and characterizes their significant biological activities, including anticancer, antimicrobial, and antioxidant properties. Scientific evidence suggests that these proteins serve as a defense mechanism for the plant while offering therapeutic potential for human health, particularly in oncology and metabolic disease management.

Plants were used to cure diseases and infections during ancient time. Medicinal plants are cheap, easily available and affordable. The medicinal importance of plants lies in some chemical substances that produce a specific physiological action on the human body. The most

essential of these bioactive constituents of plants are alkaloids, saponins, tannins, flavonoids and phenolic compounds1. Cucurbits form an important and a big

group of vegetable crops cultivated extensively in the subtropical and tropical countries. All of the cultivated species are found in subfamily Cucurbitoideae. Fruit of Cucurbita maxima is the largest known fruit of all flowering plants, and are often used in contests for the

largest pumpkin category. Based on world production, the most popular cucurbit is watermelon, followed by cucumber and the leading producers of cucurbits.

Cucurbitaceae Seeds

First, Cucurbitaceae seeds are usually manually separated from the ripe fruit and they are dehulled if necessary and possible. In order to obtain the seed meal (sometimes referred to as seed flour), disintegrating and defatting steps are applied to the seeds. The disintegration is achieved by grinding or pulverizing the dehulled seeds. The defatting of the ground seeds is carried out with hexane and the resulting meal is dried at room temperature. The disintegration step may be repeated at this point. The resulting meal may also be passed through a 60-mesh sieve in order to obtain a fine powder . In the case of Cucurbitaceae seeds that are used in oil production (especially Cucurbita pepo, but also Citrullus lanatus), the residual oil cake resulting from the seed oil pressing is first ground and then defatted, following the same procedures as described above.The defatted Cucurbitaceae seed meal is then stored in a dry and cool place until used for the protein extraction.

Cucurbitaceae Seed Protein Extraction

At the laboratory scale, Cucurbitaceae seed protein extraction has been carried out mainly by means of fractionation, concentration, and isolation processes, resulting in potential food and pharmaceutical applications .However, different factors (such as pH, temperature, ionic strength, solvent type, extraction time, and solid-liquid ratio, among others . may affect the protein extraction process, thus making it rather complicated and unpractical to implement at industrial level.Traditionally, the extraction of

storage proteins from seeds has been carried out following Osborne . who classifies proteins according to their solubility in water (albumin), salt solutions (globulin), alkali solutions (glutelin), and alcohol solutions (prolamin). Nowadays, however, many Cucurbitaceae seed protein researchers deal directly with cucurbitin (11S globulin) which is the main storage protein in Cucurbitaceae seeds .Cucurbitin is a hexameric globular protein whose subunits weigh 54 kDa each. In turn, each subunit consists of an acidic and a basic subunit which are disulfide-bonded and weigh 33 kDa and 22 kDa, respectively . Peričin et al. describe the standard procedure of cucurbitin extraction. First, the defatted seed meal is extracted with water and this fraction is discarded. Subsequently, the globulin fraction is extracted with NaCl solution at room temperature. The protein is then precipitated from the clarified extract by gradual addition of water. The precipitate is dissolved in standard buffer, the solution is clarified by centrifugation, and the cucurbitin is precipitated by addition of water.

Major Protein Classes in Cucurbitaceae

Bioactive properties of cucurbitaceae seed protein isolates and Hydrolysates

Antioxidant Activity

The determination of antioxidant activity in Cucurbitaceae seed protein usually comprises different in vitro measures, such as radical scavenging activity (for DPPH, $ABTS^{+}$, and O_2^{-} radicals), reducing power (of Fe^{3+} to Fe^{2+}), and metal chelating activity (Fe^{2+}), among others .Outstanding in vitro antioxidant activity has been reported for the globulin fractions (cucurbitin) of watermelon (Citrullus lanatus) and pumpkin (Cucurbita moschata) seed protein. Also, antioxidant effects of pumpkin (Cucurbita pepo) seed protein isolate have been reported in vivo in CCl_4 induced liver injury in rats .As for Cucurbitaceae seed protein hydrolysates, their antioxidant activity seems to be improved in comparison to unhydrolyzed seed protein . However, it may depend on various hydrolysis-related factors, such as the type of enzyme used for the hydrolysis, the DH, and the molecular weight of the resulting peptides.

Antihypertensive (ACE Inhibitory) Activity

Treating hypertension with synthetic drugs with angiotensin-I converting enzyme (ACE) inhibitory activity can have undesirable side effects, so food-derived peptides with ACE inhibitory activity are considered to be a better alternative . Food-derived peptides are encrypted in proteins and are usually released during food ripening or fermentation but can also be prepared by means of in vitro enzymatic hydrolysis . While Cucurbitaceae seed protein isolates do not seem to have a significant ACE inhibitory activity . Cucurbitaceae seed protein hydrolysates could be a valuable source of peptides with ACE inhibitory activity The ACE inhibitory activity of peptides depends on various factors. As for the peptides derived from Cucurbitaceae seed proteins, their ACE inhibitory activity seems to directly depend on their concentration: the higher the concentration of the peptide, the higher its ACE inhibitory activity .Moreover, the ACE inhibitory activity of Cucurbitaceae seed protein hydrolysates which contain the peptides seem to be dependent on the type of enzyme used for the hydrolysis, on the DH, and on their molecular weight distribution . However, the results are inconclusive and more research is needed in this area.

Characteristics :

Structure: The 11S globulin typically forms a hexameric complex (sedimentation coefficient around 11S). Each monomeric unit is synthesized as a precursor (preproglobulin) that is subsequently cleaved into linked acidic (\(\alpha \)) and basic (\(\beta \)) polypeptide chains held together by disulfide bonds.

- **Amino Acid Profile**: It is a high-quality, plant-based protein source known for its well-rounded essential amino acid composition, though it is often limited in lysine but rich in arginine.

Functional Properties and Uses

- **Dietary Supplement**: Because of its compact structure, cucurbitin is largely resistant to rapid degradation in the digestive tract, making it a stable ingredient for protein isolates and powders derived from pumpkin seeds.
- **Bioactive Peptides**: When subjected to enzymatic hydrolysis, the 11S globulin structure breaks down to release bioactive peptides. These peptides are researched for potential antioxidant, antihypertensive, and antimicrobial benefits.
- **Emulsifying and Foaming**: Cucurbitin-rich protein isolates exhibit good functional characteristics, including the ability to stabilize foams and oil-in-water emulsions, making them useful in plant-based food manufacturing.

BOTANICAL AND PHARMACOGNOSTIC PROFILES

The Cucurbitaceae family, commonly known as the gourd, melon, or pumpkin family, represents one of the most structurally diverse and economically vital groups of plants distributed widely across tropical, subtropical, and temperate regions globally. Characterized morphologically by their climbing or prostrate vine habits, monoecious or dioecious flowering arrangements, and specialized tendrils, these plants have served as cornerstone components of both human diets and ancient ethnomedicinal systems. The primary species under investigation in this study display unique pharmacognostic hallmarks:

1.Momordica charantia (Bitter Gourd/Melon): Celebrated for its signature bitter flavor profile caused by the presence of momordicines, charantin, and vicine, its seeds possess structurally robust metabolic blocks, including specific therapeutic peptides that copy insulin profiles.

Health Benefits & Nutritional Profile:

Blood Sugar Regulation: Bitter melon is frequently used to help manage blood glucose levels. It contains bioactive compounds like charantin and p-insulin that mimic the action of insulin, potentially aiding in the reduction of blood sugar and HbA1c levels.

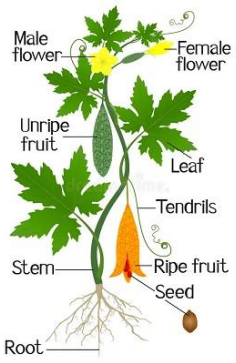
Rich in Antioxidants: The fruit is packed with vitamin C, vitamin A, iron, potassium, and phenolic compounds, which help the body fight oxidative stress.

Potential Therapeutic Properties: Beyond diabetes management, research indicates that its active compounds may exhibit anti-inflammatory, antimicrobial, and cancer-preventive properties

Uses

- **Preparation:** The characteristic bitterness, caused by the compound momordicin, can be softened by peeling, scoring the skin, salting, or parboiling the fruit.

- **Dishes:** Young, green bitter melons are widely used in curries, stir-fries, and pickles. In many regional cuisines, the young leaves and shoots are also cooked as leafy greens.



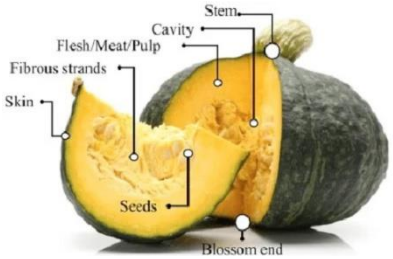
2.Cucurbita pepo (Pumpkin): Yields flat, oval seeds that are remarkably rich in non-polar lipids, macro-minerals, and highly concentrated storage globulins (cucurbitins).

Benefits

- **Prostate & Urinary Health:** Pumpkin seeds and seed oil are widely used to relieve symptoms of an enlarged prostate (Benign Prostatic Hyperplasia, or BPH). They help increase urination and reduce bladder and prostate discomfort.
- **Heart Health:** High in potassium, vitamin C, and fiber, pumpkin flesh helps lower blood pressure and manage cholesterol.
- **Skin & Eye Protection:** The flesh is loaded with beta-carotene, which your body turns into vitamin A to protect skin cells from UV rays and support vision.
- **Blood Sugar Regulation:** Compounds in pumpkin can help improve metabolic parameters and manage oxidative stress associated with high blood sugar.

Uses

- **Roast the Seeds:** Clean and roast pumpkin seeds with a bit of olive oil and sea salt for a nutrient-dense, crunchy snack.
- **Add to Meals:** Blend roasted seeds into smoothies, or sprinkle them over salads, granolas, and soups for an extra protein boost.
- **Cooked Flesh:** Puree, roast, or bake pumpkin flesh to make soups, pies, curries, and baked goods.



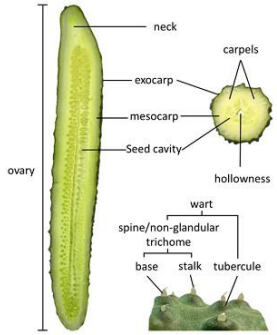
3.Cucumis sativus (Cucumber): Primarily cultivated for high-moisture succulent fruit flesh, yet its seeds constitute a major processing byproduct rich in low-molecular-weight defensive enzymes.

Benefits

- **Deep Hydration:** With its high water content, it is an excellent natural way to replenish fluids and prevent dehydration.
- **Antioxidant & Anti-inflammatory:** Cucumbers contain flavonoids and tannins that help block free radicals and reduce swelling.
- **Aids Digestion:** The dietary fiber promotes regular bowel movements, while compounds like cucurbitacin prevent bloating.

Uses

- **Dietary:** Commonly added to salads, sandwiches, and the popular Indian side dish raita, which pairs cucumber with probiotic-rich yogurt.
- **Skin Care:** Used for centuries to soothe sunburns, reduce under-eye puffiness, and hydrate the skin.
- **Ayurveda:** Used in traditional remedies to relieve excessive thirst, ease burning micturition, and help balance excess pitta in the body



4.Citrullus lanatus (Watermelon): Its seeds produce substantial oil-cake byproducts containing an abundance of balanced globulins and albumins rich in essential amino acids.

Benefits

- **Superior Hydration:** Composed of about 92% water, it is an excellent natural way to replenish fluids and electrolytes.
- **Heart Health & Circulation:** Rich in L-citrulline, an amino acid that converts to arginine. This process helps produce nitric oxide, which relaxes blood vessels and aids in lowering blood pressure.
- **Reduced Muscle Soreness:** Drinking watermelon juice or consuming its extract before workouts can improve circulation, which helps decrease recovery time and muscle aches.

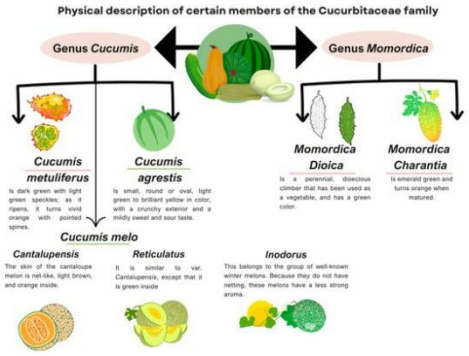
Uses

- **Fresh Consumption:** Commonly eaten fresh as a refreshing summer snack, dessert, or added to fruit salads.

- **Juices & Smoothies:** The flesh and seeds are blended into hydrating juices or combined with other fruits for post-workout smoothies.
- **Culinary Applications:** Can be used in savory dishes, such as feta and mint salads, or the rind can be pickled, stir-fried, or stewed.

- **Structural and Functional Classifications of Plant Proteins**

Plant storage proteins are historically categorized based on their solubility characteristics according to the classical Osborne classification system. This framework organizes proteins into four primary functional groups: water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins, and acid/ alkali-soluble glutelins. Within the seeds of the Cucurbitaceae family, salt-soluble globulins dominate, accounting for approximately 60% to 70% of total protein isolate volume, followed closely by water-soluble albumins. From a functional and medicinal chemistry perspective, the Cucurbitaceae family is globally recognized for producing specialized, evolutionary defense-related proteins. Among these, Ribosome- Inactivating Proteins (RIPs) and Serine Protease Protease Inhibitors (specifically trypsin inhibitors) stand out due to their robust biochemical stability and potent pharmacological actions.



- **Ribosome-Inactivating Proteins (RIPs)**

Ribosome-Inactivating Proteins are highly specialized plant enzymes categorized systematically as EC 3.2.2.22 RNA N-glycosidases. These macromolecules possess the unique capacity to bind to and catalytically alter eukaryotic and prokaryotic ribosomes, leading to the absolute cessation of cellular protein translation and synthesis. RIPs achieve this by recognizing a universally conserved, single-stranded loop located within the large ribosomal subunit RNA—frequently referred to as the alpha-

sarcin/ricin loop (SR loop). The enzyme selectively cleaves a single N-glycosidic bond at an absolute specific adenine position (A4324 in mammalian 28S rRNA and A2660 in Escherichia coli 23S rRNA), effectively preventing elongation factor binding and terminating translation permanently.

Ribosome-Inactivating Proteins (RIPs) are a diverse class of cytotoxic enzymes primarily found in plants, bacteria, and fungi. They act as rRNA \(N\)-glycosidases, permanently halting protein synthesis by removing specific adenine residues from the large ribosomal subunit, which leads to cell death.

- **Mechanisms and Types**

RIPs function by recognizing a highly conserved sequence in the ribosomal RNA (like the sarcin/ricin loop in the 28S rRNA) and cleaving the \(N\)-glycosidic bond. This stops elongation factors from binding, effectively freezing the translation process.

They are generally classified into three major structural groups:

- **Type 1:** Composed of a single, catalytically active polypeptide chain (e.g., pokeweed antiviral protein, saporin). They typically exhibit lower toxicity to intact cells because they lack an efficient way to enter.
- **Type 2:** Consist of two chains: an active A-chain linked to a cell-binding B-chain (e.g., ricin, abrin). The B-chain acts as a lectin, binding to cell surface sugars and facilitating highly efficient entry into the cell, making Type 2 RIPs some of the most potent toxins known.

- **Type 3:** Less common precursors that require proteolytic cleavage to become activated (e.g., JIP60 from barley).

- **Biological Roles:**

In nature, RIPs serve primarily as a defense mechanism for plants. They exhibit potent:

Antiviral properties: Many RIPs can inhibit the replication of both plant and human viruses (including HIV).

Antifungal and Antibacterial properties: They help protect the host organism from pathogenic infections.

Insecticidal activity: They deter pests from feeding on the plant.

A **ribosome-inactivating protein** (**RIP**) is a protein synthesis inhibitor that acts at the eukaryotic ribosome.[2] This protein family describes a large family of such proteins that work by acting as rRNA N-glycosylase (EC 3.2.2.22). They inactivate 60S ribosomal subunits by an N-glycosidic cleavage, which releases a specific adenine base from the sugar-phosphate backbone of 28S rRNA. RIPs exist in bacteria and plants.

Members of the family include shiga toxins, and type I (e.g. trichosanthin and luffin) and type II (e.g. ricin, agglutinin, and abrin) ribosome inactivating proteins (RIPs). All these toxins are structurally related. RIPs have been of considerable interest because of their potential use, conjugated with monoclonal antibodies, as immunotoxins to treat cancers. Further, trichosanthin has been shown to have potent activity against HIV-1-infected T cells and macrophages.[7] Elucidation of the structure-

function relationships of RIPs has therefore become a major research effort. It is now known that RIPs are structurally related. A conserved glutamic residue has been implicated in the catalytic mechanism; this lies near a conserved arginine residue, which also plays a role in catalysis.

Classification :

Ribosome-inactivating proteins (RIPs) are separated into the following types based on protein domain composition:

- **Type I (A):** RIPs-I are polypeptides composed of an A domain. This is the site of N-glycosidase activity.
- **Type II (AB):** RIPs-II are composed of an A domain with similar structure and catalytic activity to
- **Type I** RIPs, and a new B domain with carbohydrate-binding (lectin) properties. The B domain is able to bind galactosyl moieties on the cell surface which facilitates entry into the cell, thus making
- **Type II** particularly cytotoxic. The A and B domains are fused together by disulfide bonds.
- **Type III:** RIPs-III are separated into two subgroups.
  - One subgroup (AC) contains the same original RIP domain (A), and a C-terminal with unknown functionality.
  - The other subgroup (AD) is similar to Type I, but contains a site for inactivation.
- Shiga toxin belongs to its own group, as the carbohydrate-binding ability (B5 domain) evolved separately and the catalytic domain is closer to type I (A) RIPs than to type II (AB).

RIPs are broadly categorized into three structural archetypes:

1. **Type I RIPs:** Comprise a single, monomeric polypeptide chain with an approximate molecular weight of 28 to 32 kDa. These proteins possess high basicity and are exceptionally potent at cell-free translation inhibition, though their lack of a cell-binding domain renders them moderately non- toxic to intact normal human cells unless conjugated with specific target carriers. Notable examples from the Cucurbitaceae family include alpha-momorcharin, beta-momorcharin, and luffin.

2. **Type II RIPs:** Consist of a heterodimeric arrangement including an enzymatically active A-chain (homologous to Type I RIPs) linked covalently via a disulfide bridge to a galactose-binding lectin B-chain (~35 kDa). The presence of the B-chain allows rapid, non-specific binding to cell surfaces, facilitating internal cellular transport and resulting in extreme cytopathic toxicities. Ricin and abrin represent classic type II configurations.

3. **Type III RIPs:** Rarely observed forms synthesized as inactive pro-enzymes that require specific endopeptidic processing to unlock translation-inhibition traits.

- **Serine Protease Inhibitors (Cucurbitacin-type Trypsin Inhibitors)**

Another critical block of functional macromolecular constituents within this family are the squash family trypsin inhibitors. These represent some of the smallest stable knotted proteins discovered in nature, containing only 28 to 32 amino acid residues arranged with a highly conserved framework of three interlocking disulfide bridges. This specific knotting topology—often referred to as the cystine knot motif—confers incredible thermal, chemical, and proteolytic stability upon the micro-proteins, preventing degradation inside human gastrointestinal tracts. These molecules function by binding with extreme affinity to the active site of pancreatic digestive trypsin, displaying sub-nanomolar inhibition constants ($\mathbf{K_i}$). In pharmaceutical design, these structural scaffolds serve as exceptional starting templates for engineered targeted protease inhibitors.

PROJECT OBJECTIVES & SCOPE :

Statement of Purpose

The continuous rise of drug-resistant pathogens, chronic inflammatory conditions, metabolic lifestyle disorders like Type 2 diabetes mellitus, and oxidative-stress-driven cellular pathologies necessitates the continuous screening and identification of novel, biopolymer-based therapeutic agents.

While small molecule secondary metabolites (alkaloids, flavonoids, terpenoids) have historically formed the bedrock of pharmacognostic investigation, plant-derived proteins and bioactive peptides represent an vast, underutilized library of structural scaffolds featuring high target specificity and reduced long- term systemic toxicities.

The primary objective of this B. Pharmacy final year project is to execute a rigorous, standardized comparative evaluation of the extraction efficiencies, molecular weights, and multiple in vitro biological activities of proteins isolated from the discarded seeds of four major Cucurbitaceae agricultural crops. By transforming agro-industrial processing waste into highly characterized, pure bio-macromolecular aggregates, this research aims to bridge the gap between waste utilization and modern biopharmaceutical development.

Specific Technical Objectives

To systematically achieve the overarching goal, the research plan is segmented into the following explicit technical milestones:

1. Procure authentic botanical seeds of Momordica charantia, Cucurbita pepo, Cucumis sativus, and Citrullus lanatus, followed by systematic mechanical defatting to remove lipid interference.
2. Optimize an industrial-scalable aqueous alkaline extraction coupled with isoelectric precipitation protocol to generate high-yield, pure crude protein isolates.
3. Quantify total protein concentrations utilizing the standard, validated Bradford spectrophotometric assay against a bovine serum albumin (BSA) calibration curve.
4. Examine the molecular mass profiles and distribution pattern of isolated proteins via Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).
5. Evaluate in vitro antioxidant capacities utilizing DPPH radical scavenging and Ferric Reducing Antioxidant Power (FRAP) analytical frameworks.

6. Determine potential anti-inflammatory traits by validating protein stabilization capacities against heat-induced albumin denaturation.
7. Screen for metabolic enzyme regulatory activity via alpha-amylase and alpha-glucosidase competitive inhibition models to gauge anti-diabetic potential.
8. Conduct basic antimicrobial sensitivity screening utilizing the agar disc-diffusion technique against representative Gram-positive and Gram-negative human pathogens.

MATERIALS & EXPERIMENTAL METHODS

Collection and Botanical Identification of Samples

Fresh, mature fruits of Momordica charantia, Cucurbita pepo, Cucumis sativus, and Citrullus lanatus were sourced directly from controlled agricultural research fields. The botanical identity of each specimen was thoroughly checked and verified by the taxonomy division of the department, and voucher specimens were systematically deposited in the institutional herbarium for future reference. The seeds were manually excised from the soft fruit pulp matrices, washed thoroughly with copious volumes of deionized water to eliminate residual simple sugars, and air-dried under a shaded canopy at ambient room temperature (**25°C ± 2°C**) for a period of 96 hours until a constant moisture weight was recorded.

- **Mechanical Comminution and Pre-extraction Defatting**

Dry seed samples were ground into a homogeneous, fine powder using an industrial laboratory mill. Because Cucurbitaceae seeds typically possess extremely high concentrations of non-polar lipids and triacylglycerols (frequently exceeding 45% to 50% w/w), immediate chemical defatting was mandatory to prevent lipid-protein complexation and emulsification during subsequent aqueous extractions. The pulverized seed flour was combined with analytical grade n-hexane at a precise solid-to-solvent ratio of 1:10 (w/v). The mixture was continuously agitated on a magnetic stir plate for 24 hours at room temperature. The lipid-containing supernatant was separated via vacuum filtration, and this extraction cycle was repeated three times. The resulting defatted seed cakes were spread evenly over glass plates and allowed to dry completely within a fume hood for 12 hours to completely eliminate residual hexane vapors.

- **Isolation of Crude Proteins**

The isolation process followed a precise, validated alkaline solubilization protocol followed by isoelectric precipitation. The general procedural chemistry is detailed in the flowchart block below:

- **Standardized Extraction Workflow Block :**

1. **Solubilization:** Defatted seed flour was suspended in distilled water at a 1:20 (w/v) ratio. The solution pH was adjusted to an absolute alkaline state of **pH = 9.0** utilizing **0.1 M NaOH**. The mixture was continuously agitated for 2 hours at **40°C**.

2.Centrifugation: The slurry was transferred to a high-speed refrigerated centrifuge and spun at **8,000 × g** for 25 minutes at **4°C**. The insoluble carbohydrate-rich pellet was discarded, and the protein-rich supernatant collected.

3.Isoelectric Precipitation: The pH of the alkaline supernatant was carefully driven down to its isoelectric point (**pH = 4.5**) by dropwise addition of **0.1 M HCl** under constant monitoring. The solution was allowed to sit undisturbed at **4°C** for 1 hour to maximize protein aggregation.

4.Recovery & Drying: The precipitated protein curd was recovered by spinning at

10,000 × gfor 20 minutes at **4°C**. The pellet was re-suspended in minimal distilled water, neutralized to **pH =7.0** using **0.05 M NaOH**, and subjected to primary dialysis against distilled water for 48 hours to remove residual salts. The final dialyzed block was freeze-dried in a lyophilizer for 36 hours, resulting in a fine, stable white-to-cream colored amorphous protein isolate powder.

Total Protein Quantification (Bradford Method)

Total protein content within each lyophilized isolate was checked spectrophotometrically following standard Bradford dye-binding protocols. The Bradford reagent utilizes Coomassie Brilliant Blue G-250 dye, which shifts its absolute absorption maximum from an unstable reddish-brown form (465 nm) to a highly stable, intense blue ionic form (595 nm) upon complexation with basic and aromatic amino acid residues present within a protein backbone.

A stock standard calibration curve was built utilizing Bovine Serum Albumin (BSA) at concentrations ranging from 20 to 200 micrograms/mL. Aliquots of 100 microliters of each properly diluted Cucurbitaceae protein isolate were combined thoroughly with 3.0 mL of Bradford reagent, incubated at room temperature for precisely 10 minutes, and the absolute absorbance read at 595 nm using a double-beam UV-Visible spectrophotometer. Total protein concentrations were determined mathematically via linear regression analysis.

- **Characterization of Molecular Weight via SDS-PAGE**

The molecular weight profiles and protein subunit compositions were investigated using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) under denaturing and reducing conditions. The experimental matrix comprised a 12% polyacrylamide resolving gel topped with a 4% polyacrylamide stacking gel. Lyophilized protein isolate samples (2 mg/mL) were diluted 1:1 with a classic Laemmli sample buffer containing 2% w/v SDS, 5% v/v beta-mercaptoethanol (a reducing agent to break stabilizing disulfide bridges), 10% v/v glycerol (to increase sample density), and 0.02% w/v bromophenol blue tracking dye.The mixtures were boiled in a water bath at **95°C** for 5 minutes to ensure absolute protein denaturation. Aliquots of 15 microliters of each prepared sample alongside a pre-stained broad-range molecular weight protein marker (10 to 250 kDa) were loaded systematically into the gel wells. Electrophoretic separation was executed at a constant voltage of 40 V through the stacking gel, which was increased to 100 V as the sample front entered the resolving matrix. Following complete run execution, the gel was carefully recovered, stained with 0.1% w/v Coomassie Brilliant Blue R-250 for 4 hours, and destained utilizing an aqueous solution containing 10% acetic acid and 30% methanol until distinct, razor-sharp protein bands were visualized against a transparent background.

Isolation Methodologies

Isolation of proteins from Cucurbitaceae follows a systematic approach to maintain the biological integrity of the molecules.

- **Extraction:** Defatted seed meal is usually the starting material. Extraction is performed using buffers with specific pH (often 8.0) to maximize solubility.
- **Fractionation:** Ammonium sulfate precipitation is commonly used to separate proteins based on solubility.
- **Chromatography:** Ion-exchange chromatography and Size-Exclusion Chromatography (SEC) are employed for high-purity isolation. For RIPs, cation exchange is frequently used due to their basic nature.
- **Hydrolysis:** For bioactive peptide studies, enzymes like Pepsin, Trypsin, and Alcalase are used to break down large isolates into smaller, more active fragments (Ozuna & León-Galván, 2017).

Immersion method:

- It is a method to dissolve out phytochemicals with appropriate solvents at room or low temperatures (<80°C).
- It is suitable to extract phytochemicals easily to be destroyed at high temperature. The plants with abundant starches, pectins, gums, or mucilages could also be extracted with this method.
- Firstly, plant powder or pieces should be loaded in the adequate container, and then the suitable solvents (water, ethanol, aqueous ethanol, and so on) are added into it to immerse the material for the given length of time.
- Discontinuous stirring or shaking during the process could accelerate dissolution rate. The immersion method is simple but inefficient, and the extraction ratio is also low. Furthermore, aqueous extract is easy to go moldy, so addition of appropriate preservatives is necessary.

Percolation method :

The coarse particles of plants should be loaded in percolation apparatus and immersed with suitable solvent for 2 then collect the percolates at the bottom of percolation apparatus. New solvent should be added at the top of percolation apparatus constantly during the percolation process. It possesses higher efficiency than the immer- sion method because of the sustained concentration difference during the process. However, this procedure is complex and consumes rather much solvent and long time.

Decoction method :

Load short segments, thin pieces, or coarse powder into an appropriate con-tainer, add water, and heat it to boiling; the components are then extracted. It is easy to operate; most of the constituents could be extracted in various degrees. Nevertheless, rather much nontargeted components could also be extracted, and it is not suitable to the extraction of volatile compounds and thermal unstable com- pounds. Furthermore, it is not suitable to extract plants with lots of starches.

Constant Refluxing method :

It is a method to extract plant chemical constituents by organic solvent using heating and refluxing. Refluxing apparatus is necessary so as not to waste solvents, Phytochemicals in Human Health and the toxicity to operators or ruin the environment is deduced. It is applicable to extraction of lipophilic phytochemicals, such as steroids, anthraquinoids, and terpenoids. It is an extraction method of high

efficiency but complex, and con-sumes much more solvent. This method is not applicable to extract thermal unstable chemical constituents because of long time heating.

Biological Activities

Energy Metabolism & ATP Regeneration: In the phosphocreatine (PCr) system, creatine kinase catalyzes the conversion of ADP back into ATP, allowing muscles to maintain high-intensity performance during short-duration, explosive activities.

Muscular Performance & Growth: Supplementation increases intramuscular creatine stores, leading to improved power output, increased strength, enhanced fatigue resistance, and increased lean tissue mass, especially when combined with resistance training.

Muscle Recovery & Repair: Creatine supports quicker recovery between intense training sets and may reduce muscle cell damage and inflammation, aiding in faster overall rehabilitation.

Neuroprotection & Cognitive Function: Emerging research suggests creatine supports brain health by acting as a buffer against metabolic stress, potentially improving memory and mental processing speed, particularly in older adults or vegetarians.

Anti-inflammatory & Antioxidant Properties: Studies suggest that creatine can reduce oxidative stress, decrease ROS (reactive oxygen species) production, and mitigate inflammatory markers, including prostaglandin E2 and TNF-\(\alpha \).

Creatine Benefits :

Creatine is naturally in your muscles, brain, and other parts of your body. If you're an athlete or have certain health conditions, taking a supplement may help you build muscle and strength, but studies have been mixed.

In the 1970s, scientists discovered that taking creatine in supplement form might enhance physical performance. In the 1990s, athletes started to catch on, and creatine became a popular sports supplement. The supplement is particularly popular among high school, college, and professional athletes, especially football and hockey players, wrestlers, and gymnasts.

Creatine is thought to improve strength, increase lean muscle mass, and help the muscles recover more quickly during exercise. This muscular boost may help athletes achieve bursts of speed and energy,

especially during short bouts of high-intensity activities such as weight lifting or sprinting. However, scientific research on creatine has been mixed. Although some studies show that it does help improve performance during short periods of athletic activity, there is no evidence that creatine helps with endurance sports like running longer distances. Research also shows that not everyone's muscles respond to creatine. Some people who use it see no benefit.

Other Benefits of Creatine

In addition to your muscles, creatine might have health benefits for other parts of your body, including your heart, brain, bones, and skin. For example, it may help with:

Heart disease. There's some evidence creatine can lower triglyceride levels in your blood if they're too high. Some studies also show it could help people with heart failure get more exercise without feeling fatigued. But not all studies have found this. It may also lower levels of a chemical called homocysteine, which has links to heart attack and stroke.

Cancer. Creatine is thought to slow the growth of tumors. It may also boost the ability of the immune system to fight cancer. But some studies suggest that creatine also can make cancer more likely to spread. You should be careful about taking creatine if you have cancer.

Muscular dystrophy. If you have muscular dystrophy, your cells may have less creatine. Some studies show supplements may help with muscle strength. But it's not clear how well this works long term.

Parkinson's disease. One study suggested that creatine might help with exercise and endurance when you have Parkinson's. It might also help with mood. But a trial testing if it could slow Parkinson's progression over 5 years didn't find that it helped.

Types of Creatine Supplements

You can get creatine in different chemical forms or types:

Creatine monohydrate. This is the most common type you'll find in supplements. It's also been studied more than other types.

Creatine ethyl ester. Experts thought this form might absorb into the body better than creatine monohydrate to make supplements work better. But studies suggest that it doesn't work as well as creatine monohydrate.

Creatine hydrochloride. This form dissolves in water better than creatine monohydrate. But there is no evidence that it works better to build muscle as a result.

Creatine magnesium chelate. One small study suggested this form could improve sprinting ability in well-trained soccer players. But this form hasn't been studied as much.

Buffered creatine monohydrate. One study looked at if a buffered form of creatine monohydrate would get into muscle better to improve exercise capacity more. But the results didn't support that it worked any better or had fewer side effects.

Liquid creatine. Creatine monohydrate often comes as a powder. But you can buy it in liquid form, too.

The bottom line is that most studies have looked at creatine monohydrate. There's not much evidence that other forms work better. No matter which supplement you try, it's a good idea to check with your doctor first. Remember also that the FDA doesn't test or regulate supplements the same way they do with medicines.

Foods High in Creatine

Your body makes creatine. But you also get creatine from foods. About half of what you have in your body if you don't take supplements comes from your diet. It's mostly in foods with lots of protein. These include:

- Pork
- Beef
- Fish
- Shellfish
- Animal milk, including cow, sheep, and goat

Creatine Side Effects

Although most healthy people can take it with no problem, creatine can have side effects, particularly when you take too much. Side effects can include:

- Weight gain
- Anxiety
- Breathing difficulty
- Diarrhea
- Fatigue
- Fever
- Headache
- Kidney problems
- Nausea, vomiting
- Rash
- Stomach upset

Antimicrobial and Antiviral Activity

RIPs are recognized as potent defense agents against bacteria, fungi, and viruses. They exhibit broad-spectrum antiviral activity, including potential resistance against plant viruses and certain human pathogens (Zhu et al., 2018).

Antioxidant and Antihypertensive Activity

Protein hydrolysates from pumpkin and watermelon seeds have shown strong antioxidant activity. Peptides produced via Alcalase hydrolysis often exhibit Angiotensin-I-Converting Enzyme (ACE) inhibitory activity, which is crucial for managing hypertension (Ozuna & León-Galván, 2017).

Antioxidant and antihypertensive activities describe the dual capacity of bioactive compounds (often peptides or polyphenols) to scavenge free radicals and reduce oxidative stress, while also inhibiting the Renin-Angiotensin-Aldosterone System (RAAS) to lower blood pressure.

Mechanisms of Action

- **Antioxidant Action:** Oxidative stress causes the excessive accumulation of free radicals, which leads to vascular endothelial dysfunction. Antioxidants neutralize these Reactive Oxygen Species (ROS) and activate signaling pathways like \(Nrf2\) to prevent cellular and vascular damage.
- **Antihypertensive Action:** These compounds act as natural \(ACE\) (Angiotensin-Converting Enzyme) inhibitors. By blocking \(ACE\), they prevent the conversion of angiotensin I to angiotensin II, leading to blood vessel relaxation and reduced peripheral resistance

Primary Sources & Common Bioactives

These beneficial effects are primarily derived from natural products and foods:

- **Plant Extracts & Phytochemicals:** Rich in polyphenols, flavonoids, and tannins (found in berries, medicinal herbs, and garlic), which exhibit high radical-scavenging capabilities and \(ACE\) inhibition.
- **Bioactive Peptides:** Short chains of amino acids commonly sourced from food proteins (e.g., wheat, soybean, and microalgae like Chlorella). When ingested, these stable peptides bind to the active pockets of \(ACE\) (such as the \(S1\) and \(S2\) pockets) to regulate blood pressure.

Health Benefits

- **Protection of Nitric Oxide (\(NO\)):** \(NO\) is vital for vascular health and vasodilation. Oxidative stress uncouples endothelial nitric oxide synthase (\(eNOS\)), limiting \(NO\) availability. Antioxidants restore this balance, ensuring healthy blood vessel tone.
- **Reduction of Inflammation:** Many dual-functional peptides can suppress pro-inflammatory signaling pathways (like \(NF-\kappa B\)

Health Benefits :

- **Sarcopenia Protection:** Helps maintain muscle strength in older adults.
- **Therapeutic Potential:** Explored as a supportive treatment for neurological disorders (Parkinson's, Alzheimer's), diabetes, and cardiovascular health.

Improved Recovery: Aids in recovering muscle strength after injury or immobilization

In Vitro Biological Activity Evaluation Protocols:

Antioxidant Profiles via DPPH Radical Scavenging Assay

The radical scavenging activity of the isolated proteins was evaluated using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). When DPPH encounters an antioxidant molecule capable of donating a hydrogen atom or electron, the radical is neutralized, causing a visible color transition from deep purple to pale yellow. This shift can be monitored spectrophotometrically at 517 nm.

Briefly, 1.0 mL of isolated protein solutions at concentrations ranging from 100 to 1000 micrograms/mL was mixed with 2.0 mL of a freshly prepared 0.1 mM methanolic DPPH solution. The test tubes were shaken vigorously and incubated in absolute darkness at room temperature for 30 minutes. Ascorbic acid served as the positive standard control. The percentage of DPPH radical scavenging activity was computed via the formula:

$$\text{DPPH Scavenging (\%)} = [\ (A_{control} - A_{sample}) / A_{control}\ ] \times 100$$

In Vitro Anti-Inflammatory Assay (Inhibition of Albumin Denaturation)

Protein denaturation is a well-documented pathological hallmark of tissue inflammation and auto-immune responses in vivo. The capacity of Cucurbitaceae proteins to prevent heat-induced denaturation of egg albumin was leveraged as an in vitro model for non-steroidal anti-inflammatory screen efficiency. The assay mixture comprised 0.2 mL of fresh egg albumin, 2.8 mL of phosphate- buffered saline (**pH = 6.4**), and 2.0 mL of varying concentrations of isolated plant proteins (100–1000 micrograms/mL). The control group contained an equivalent volume of distilled water instead of protein isolates. Diclofenac sodium was implemented as the reference standard drug. The complete mixtures were incubated at **37°C** for 15 minutes, then heated in a regulated water bath at **70°C** for exactly 5 minutes to trigger denaturation. After cooling to room temperature, the turbidity was measured spectrophotometrically at 660 nm. The percentage inhibition of denaturation was calculated using:

$$\textbf{Inhibition (\%)} = [\ (A_{control} - A_{sample}) / A_{control}\ ] \times 100$$

Anti-Diabetic Efficiency (Alpha-Amylase Inhibition Assay)

Alpha-amylase is a prominent endo-enzyme that hydrolyzes complex dietary starches into maltose and oligosaccharides. Inhibiting its activity within the human intestinal lumen represents a prime therapeutic strategy for delaying glucose absorption and smoothing postprandial blood glucose spikes in Type 2 diabetic patients.

A volume of 500 microliters of each isolated protein sample (100–1000 micrograms/mL) was pre-incubated with 500 microliters of porcine pancreatic alpha-amylase enzyme solution (0.5 mg/mL in 20 mM phosphate buffer containing 6.7 mM NaCl, **pH = 6.9**) at **25°C** for 10 minutes. Subsequently, 500 microliters of a 1% w/v starch solution was added to initiate the enzymatic breakdown, and the matrix was incubated at **25°C** for exactly 10 minutes. The enzymatic reaction was abruptly terminated by adding 1.0 mL of dinitrosalicylic acid (DNSA) color reagent. The tubes were heated in a boiling water bath (**100°C**) for 5 minutes to develop the characteristic red maltose-DNSA complex, cooled rapidly under tap water, diluted with 10 mL of deionized water, and the final absorbance recorded at 540 nm. Acarbose was utilized as the benchmark clinical reference drug.

Antimicrobial Sensitivity Screening

The structural defense proteins isolated from the seed samples were screened for antibacterial traits using the standard Kirby-Bauer agar disc-diffusion method. The representative tester panel included the Gram-positive bacterium Staphylococcus aureus (ATCC 25923) and the Gram-negative bacterium Escherichia coli (ATCC 25922).

Standard Mueller-Hinton Agar (MHA) plates were prepared, sterilized, and inoculated uniformly with a bacterial suspension adjusted precisely to match a 0.5 McFarland turbidity standard

(**~1.5 × 10^8 CFU/mL**) using a sterile cotton swab. Sterile, blank paper discs (6 mm diameter) were thoroughly impregnated with 20 microliters of each respective Cucurbitaceae protein isolate solution (at a fixed dose concentration of 5 mg/mL) and placed carefully onto the agar surface. Ciprofloxacin (5 micrograms/disc) was applied as the positive control, while sterile water-impregnated discs served as negative baseline markers. The plates were incubated in an inverted orientation at **37°C** for 24 hours. The true antimicrobial potency

RESULTS & DISCUSSION

Yield and Quantitative Extraction Efficiency

The implementation of sequential hexane defatting followed by optimized alkaline-isoelectric processing yielded highly clean protein isolates. The quantitative yield parameters across the four distinct Cucurbitaceae species are structured and detailed in As observed from the experimental data metrics, Cucurbita pepo (Pumpkin) seeds exhibited the highest extraction yield performance (25.40% w/w) and the greatest overall protein concentration within its isolate (78.2% w/w). This performance is structurally attributed to the large concentration of packed globulin crystalloids naturally stored within pumpkin cotyledon tissues. Conversely, Cucumis sativus yielded the lowest total isolate output, reflecting its smaller embryo mass and higher relative structural cellulose content.

Evaluation of Antimicrobial Potency

The antimicrobial disc-diffusion screening confirmed that these isolates possess significant defensive properties against Gram-positive bacteria, led by Momordica charantia (16.5 mm zone of inhibition against Staphylococcus aureus). The mechanism of action is primarily driven by the single-chain Type- I RIPs. These enzymes can breach cell envelopes and enzymatically depurinate bacterial ribosomal strands or alter cell-wall integrity, triggering rapid cellular lysis. Activity against Gram-negative Escherichia coli was consistently lower across all samples (zones hovering under 9 mm), due to the protective outer lipopolysaccharide membrane barrier characteristic of Gram-negative cell walls, which restricts large macromolecular penetration.

CONCLUSION & FUTURE DIRECTIONS

Summary of Findings

This comprehensive final year project successfully standardized an effective extraction and isolation protocol for processing functional storage and defense proteins from the seed materials of Momordica charantia, Cucurbita pepo, Cucumis sativus, and Citrullus lanatus. The combination of n-hexane defatting with an alkaline extraction-isoelectric precipitation sequence yielded pure isolates with excellent stability. Structural characterization via SDS-PAGE confirmed the presence of key therapeutic proteins, including 30 kDa Ribosome-Inactivating Proteins (RIPs) in bitter gourd and high-density 11S globulin components within pumpkin seeds.

Biological activity evaluations confirmed that these isolated proteins possess highly potent antioxidant, anti-inflammatory, anti-hyperglycemic, and antimicrobial properties. Cucurbita pepo isolates

demonstrated superior free radical scavenging capabilities, while Momordica charantia fractions showed exceptional anti-inflammatory protection and starch digestive enzyme inhibition. These results demonstrate that the agricultural byproducts of the Cucurbitaceae family represent a highly valuable, sustainable source of bioactive macromolecules for therapeutic applications.

Future Scope of Research

To build effectively upon the foundational baselines established by this undergraduate research project, the following advanced experimental pathways are recommended for future postgraduate studies:

Enzymatic Hydrolysis & Bioactive Peptide Mapping: Subject the isolates to controlled enzymatic digestion utilizing pepsin, trypsin, and chymotrypsin to generate low-molecular-weight bioactive peptide fragments, which often exhibit enhanced bioavailability and amplified biological actions.

In Vivo Pharmacological Validation: Transition from in vitro evaluation models to standard animal models (e.g., streptozotocin-induced diabetic rats or carrageenan-induced paw edema models) to validate actual therapeutic indices and safety parameters in vivo.

Advanced Chromatographic Purification: Implement Fast Protein Liquid Chromatography (FPLC) using explicit ion-exchange and hydrophobic interaction columns to isolate single, high- purity protein molecules like pure alpha-momorcharin.

Novel Drug Delivery System (NDDS) Formulation: Encapsulate the purified protein fractions within biodegradable poly(lactic-co-glycolic acid) (PLGA) nanoparticles or liposomal shells to shield them from premature proteolytic degradation, enabling targeted delivery and sustained release profiles.

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