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Isolation and Biological Activity Studies of Some from Cucurbitaceae Family

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Abstract: *The plant family Cucurbitaceae, universally recognized for its agricultural and economic significance, serves as a rich reservoir of secondary metabolites and potent bioactive macromolecules. Among these, Ribosome-Inactivating Proteins (RIPs)—specifically Type I RIPS such as trichosanthin (derived from Trichosanthes kirilowii) and alpha-momorcharin (derived from Momordica charantia)—have emerged as critical focal points in modern oncological, antiviral, and agricultural pharmacology. These specific enzymes irreversibly inhibit eukaryotic protein synthesis by cleaving a specific adenine residue on the highly conserved 28S rRNA, ultimately triggering complex apoptotic cascades in targeted cells.*

This comprehensive study was undertaken to develop a highly optimized, scalable, and non-denaturing extraction protocol for these proteins, evaluate their practical applications in biotechnology, and map their complete in vitro biological profiles across multiple cell lines utilizing advanced molecular biology techniques. To circumvent the limitations of traditional multi-step chromatography—which frequently suffers from low yield, excessive solvent consumption, and protein denaturation—an advanced Three-Phase Partitioning (TPP) technique was rigorously optimized. By meticulously varying dielectric environments dictated by ammonium sulfate concentrations, pH levels, and tbutanol volumetric ratios, the TPP method proved highly efficacious. The optimized protocol (pH 7.4) yielded 4.23% w/w of crude protein from T. kirilowii and 3.86% w/w from M. charantia. Furthermore, to address clinical application hurdles such as systemic immunogenicity and rapid renal clearance, sophisticated PEGylated PLGA nanoparticle formulations were successfully developed and structurally characterized to enhance biocompatibility and mimic targeted delivery systems.

The isolated, dialyzed, and endotoxin-free protein fractions were subjected to an extensive battery of biological and computational assays. In silico molecular docking simulations confirmed exceptionally high binding affinities of the RIPS with the human LRPI receptor, mathematically elucidating the mechanism of cellular entry and verifying that the lack of a B-chain does not hinder receptormediated endocytosis. Antioxidant profiling via both DPPH and ABTS radical scavenging assays revealed that both proteins, particularly alphamomorcharin, exhibit significant, dose-dependent oxidative stress mitigation, presenting a dual-action mechanism of localized ROS quenching alongside primary enzymatic toxicity.

The crux of the therapeutic evaluation involved highly controlled in vitro cytotoxicity assays against a panel of cell lines: MCF-7 (human breast adenocarcinoma), HepG2 (hepatocellular carcinoma), and HUVEC (normal human umbilical vein endothelial cells). The MTT assay revealed profound, targeted antiproliferative activity against the malignant lines while demonstrating negligible toxicity toward normal endothelial cells. Against MCF-7 cells, trichosanthin demonstrated a highly potent IC 50 of 4.15 PM, while alphamomorcharin recorded an IC 50 of 6.23 PM.

To definitively prove the mechanism of cell death, Flow Cytometry utilizing Annexin V-FITC/PI dual staining was employed, which quantitatively verified that over 78% of the treated MCF-7 cell population entered late-stage apoptosis rather than necrosis. Furthermore, quantitative Real-Time PCR (RT-qPCR) was utilized to measure the expression levels of apoptotic biomarker genes. The results demonstrated a profound upregulation of the pro-apoptotic Bax gene and a significant

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downregulation of the anti-apoptotic Bcl-2 gene, establishing a molecular signature of intrinsic, mitochondria-mediated apoptosis.

The findings of this expansive study definitively establish TPP as a superior methodology for the downstream processing of bioactive plant proteins. Furthermore, the robust combination of computational validation, structural preservation, targeted cytotoxicity, molecular genetic profiling, and successful nanoparticle formulation strongly advocates for the continued clinical investigation of these isolated RIPS as viable, naturally derived immunotoxins, targeted oncological therapies, and agricultural bio-pesticides.

Keywords: *Cucurbitaceae*

I. INTRODUCTION

1.1 Background of the Study

The natural environment has historically served as the primary reservoir for the discovery of novel pharmacologically active compounds. For decades, the pharmaceutical industry predominantly focused on the isolation, structural elucidation, and synthesis of small organic molecules—often secondary metabolites such as alkaloids, terpenes, and flavonoids—for therapeutic applications. However, the paradigm of modern drug discovery is rapidly shifting toward biologics and macromolecular therapeutics, specifically plantderived proteins and peptides.

Plant proteins offer distinct advantages over small molecules. They possess high target specificity, lower accumulation of toxic metabolites in hepatic pathways, and the unique ability to interact with complex biological networks and proteinprotein interfaces that small molecules often fail to modulate due to their limited surface area. Among the myriad of plant-derived macromolecules, Ribosomelnactivating Proteins (RIPS) represent one of the most structurally fascinating and therapeutically promising classes of enzymes. Originally evolved as highly effective botanical defense mechanisms against viral, fungal, and insect predators, these unique enzymes have demonstrated profound cytotoxic and antiviral capabilities when introduced into mammalian biological systems.

Over the past twenty years, the explosion of recombinant DNA technology and advanced protein engineering has allowed researchers to investigate these toxins not just as abortifacients or poisons, but as highly tunable molecular scalpels capable of eradicating specific populations of malignant or infected cells. However, harnessing this power requires unparalleled precision in both extraction and delivery methodologies.

1.2 The Cucurbitaceae Family in Medicine and Biotechnology

The Cucurbitaceae family, encompassing approximately 125 genera and over 800 species, is globally recognized for its profound agricultural and nutritional significance. Commonly referred to as the gourd, melon, or pumpkin family, these plants thrive primarily in tropical and subtropical climates. Beyond their dietary value, numerous species within this family have been cornerstones of ethnomedical systems for centuries, including Ayurveda in the Indian subcontinent and Traditional Chinese Medicine (TCM).

Historically, various parts of these plants—ranging from seeds and roots to leaves and fruits—have been utilized as purgatives, emetics, abortifacients, and treatments for metabolic disorders such as diabetes mellitus. Modern phytochemical screening has validated many of these traditional uses, revealing a complex matrix of bioactive constituents, including cucurbitacins (highly oxygenated tetracyclic triterpenes) and saponins. However, recent attention has decisively pivoted toward the proteinaceous components of these plants.

Two species within this family are of particular interest for their robust protein profiles:

• Trichosanthes kirilowii: The root tuber of this plant, known in TCM as Tian Hua Fen, has been used historically to induce mid-trimester abortions and treat retained placentas. Modern biochemistry has identified the active principle responsible for these potent biological effects as a specific protein named trichosanthin (TCS). The root tuber acts as a

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massive storage sink for this highly basic, single-chain polypeptide, which exhibits profound cytotoxic and antiviral capabilities.

• Momordica charantia: Commonly known as bitter melon, this widely consumed vegetable possesses well-documented hypoglycemic and antimicrobial properties. Advanced proteomic analyses of its seeds have isolated several bioactive proteins, with alpha-momorcharin (a-MMC) and its isoform beta-momorcharin (ß-MMC) emerging as highly potent biological agents utilized in both oncology and agricultural biotechnology.

1.3 Introduction to Ribosome-Inactivating Proteins (RIPS)

Ribosome-Inactivating Proteins are a highly specialized class of enzymes widely distributed across the plant kingdom, though they exhibit exceptional abundance and diversity within the Cucurbitaceae family. Functionally, RIPS are RNA Nglycosidases (Enzyme Commission number EC 3.2.2.22). Their primary catalytic mechanism involves the highly specific, irreversible cleavage of the Nglycosidic bond of a single adenine residue (specifically A4324 in mammalian28S ribosomal RNA). This depurination occurs at a highly conserved sequence known as the sarcin/ricin loop (SRL).

The removal of this single adenine base drastically alters the tertiary structure of the ribosome. It completely abrogates the ribosome's ability to bind to elongation factor 2 (EF-2), a critical component required for the translocation oftRNA during the elongation phase of translation. Consequently, the translation machinery is instantaneously halted, cellular protein synthesis ceases, and the affected cell is rapidly driven into programmed cell death, known as apoptosis.

1.3.1 Evolutionary Role of RIPS in Plants

In their native botanical environments, RIPS are not cytotoxic to the plant's own ribosomes. This self-preservation is achieved either due to subtle structural differences in the plant's ribosomal RNA sequence or by safely sequestering the RIPS within specialized protein bodies or vacuoles. They are primarily expressed in seeds, roots, and latex as a first line of evolutionary defense. When an insect or pathogen mechanically damages the plant tissue, the vacuoles rupture, releasing the RIPs. In the case of viral infections, RIPS can enter infected plant cells and rapidly induce localized cell death (the hypersensitive response), effectively quarantining the virus and preventing systemic spread throughout the plant.

1.4 Practical Applications: The Translation of RIPS

The unique and highly specific mechanism of action of RIPS allows them to be harnessed across diverse fields, ranging from human medicine to agricultural biotechnology. Understanding how these proteins are utilized provides the critical context and justification for their rigorous extraction and purification.

1 .4.1 Targeted Cancer Therapy (Immunotoxins)

The most prominent and heavily funded clinical application of Type I RIPS is the development of immunotoxins for targeted cancer therapy. Because Type I RIPS (like TCS and a-MMC) lack a cell-binding B-chain, they are inherently safe for systemic circulation; they cannot easily force their way into healthy, normal cells. In pharmacology, these proteins are chemically conjugated (using cleavable disulfide linkers) or genetically fused to tumor-specific monoclonal antibodies.

For example, linking TCS to Trastuzumab (Herceptin) creates a "magic bullet" that selectively targets HER2-positive breast cancer cells. The antibody acts as the homing device, locating the cancer cell and binding to the surface antigen. The entire complex is then internalized via endocytosis. Once inside the acidic endosome, the disulfide linker breaks, and the RIP acts as the lethal payload, escaping into the cytosol and destroying the cell's ribosomes while leaving surrounding healthy tissue completely unharmed. This approach promises significantly higher therapeutic indices compared to traditional, non-specific chemotherapy.

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1 .4.2 Antiviral Agents

RIPS exhibit potent broad-spectrum antiviral activity. They are capable of inhibiting the replication of both DNA and RNA viruses, including the Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV), and Simplex Virus (HSV). They achieve this by selectively targeting and destroying the ribosomes of actively infected cells. Virally infected cells often possess altered membrane permeability and express foreign antigens that increase their susceptibility to RIP uptake. By destroying the host's translation machinery in these specific cells, the virus is prevented from utilizing the host to synthesize viral proteins and replicate.

1.4.3 Agricultural Biotechnology and Bio-Pesticides

In agriculture, the genes encoding RIPS are utilized to create transgenic, diseaseresistant crops. By inserting the gene for TCS or a-MMC into susceptible agricultural staples (like tobacco, potato, or rice), agricultural scientists have successfully engineered plants with innate, broad-spectrum resistance against devastating fungal pathogens (e.g., Fusarium, Rhizoctonia) and viral infections. Furthermore, crude extracts of these proteins are actively being investigated as eco-friendly, biodegradable bio-insecticides to manage agricultural pests without the toxic environmental runoff and prolonged soil half-lives associated with synthetic chemical pesticides.

1.5 Statement of the Problem and Technical Bottlenecks

Despite their immense utility and proven efficacy, extensive biological profiling and the large-scale clinical translation of these macromolecules are severely hampered by the limitations of conventional downstream processing. The extraction of active enzymes from complex botanical matrices is notoriously difficult due to the co-extraction of interfering compounds such as polyphenols, mucilage, complex lipids, and endogenous proteases that rapidly degrade the target proteins.

Traditional multi-step chromatographic techniques (such as CM-Sepharose ionexchange chromatography followed by Sephadex gel filtration) result in incredibly low yields (often < 0.5% of total soluble protein). Furthermore, they induce structural denaturation due to column shear stress and prolonged exposure to varying elution buffers. The prohibitive costs of stationary phases and the time required for these methods prevent industrial-scale manufacturing. Furthermore, the direct clinical administration of naked, non-conjugated RIPS faces major hurdles regarding immunogenicity (the rapid generation of neutralizing Anti -Drug Antibodies) and short plasma half-lives due to rapid renal clearance.

1 .6 Rationale and Hypothesis of the Study

To address the critical biochemical extraction bottleneck, this study implements Three-Phase Partitioning (TPP). TPP is an elegant, highly scalable, and nondenaturing bioseparation technique utilizing the synergistic effects of kosmotropic salts (ammonium sulfate) and a moderately polar organic solvent (tbutanol). Furthermore, to address delivery and immunogenicity hurdles, preliminary nanoparticle encapsulation concepts utilizing PEGylated polymers are explored.

Hypothesis: It is hypothesized that the optimized application of the Three-Phase Partitioning (TPP) technique will successfully isolate Ribosome-Inactivating Proteins (trichosanthin and alpha-momorcharin) with superior yields and higher purity than traditional methods. It is further hypothesized that the isolated proteins will retain their native, functional tertiary structures, thereby exhibiting measurable in vitro free-radical scavenging activity and highly potent, dosedependent cytotoxicity against malignant adenocarcinoma cell lines, which will be validated through both in silico computational modeling, comprehensive in vitro biological assays, and definitive molecular biology techniques including Flow Cytometry and RT-qPCR.

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II. LITERATURE REVIEW

2.1 Classification and Structure of Ribosome-Inactivating Proteins

Ribosome-Inactivating Proteins (RIPS) are a diverse family of enzymes officially classified as rRNA N-glycosylases. Based on their primary amino acid sequence, structural conformation, and the presence or absence of carbohydratebinding domains, RIPS are divided into three distinct classes:

• Type I RIPs: These are single-chain polypeptides with a molecular weight typically ranging between 25 kDa and 32 kDa. They are highly basic (possessing isoelectric points often above pH 8.5) and consist entirely of the enzymatically active A-chain. Examples include Trichosanthin, Alphamomorcharin, Saporin, and Gelonin. Because they lack a dedicated cellbinding domain, they exhibit significantly lower systemic toxicity and require specific endocytotic mechanisms for cellular entry.

• Type Il RIPs: These are heterodimeric proteins, consisting of an enzymatically active A-chain covalently linked to a lectin-like B-chain via a single disulfide bond. The combined molecular weight is approximately 60 kDa. The B-chain possesses severe biological implications; it binds with high affinity to galactosyl residues terminating glycoproteins and glycolipids ubiquitous on the surfaces of mammalian cells. This triggers rapid, indiscriminate endocytosis, making Type Il RIPS (such as Ricin from Ricinus communis and Abrin) some of the most lethal toxins known.

• Type Ill RIPs: A relatively recent classification, these consist of an Nterminal A-chain linked to a prolonged C-terminal domain of unknown physiological function. These proteins are synthesized as inactive proenzymes and require extensive post-translational proteolytic cleavage to liberate the active Achain (e.g., JIP60 found in barley).

2.2 Structural Biology of the Ribosome and Catalytic Mechanism

The mammalian ribosome is a massive, complex ribozyme responsible for translating messenger RNA (mRNA) into polypeptide chains. It consists of a small 40S subunit and a large 60S subunit. Trichosanthin (TCS) and alphamomorcharin (a-MMC) specifically target the 28S rRNA located within the large 60S subunit. The active site of the Type I RIP is a deep cleft located at the interface of its Nterminal and C-terminal domains.

The catalytic mechanism is a precise nucleophilic substitution. The active site residues (typically Tyr70, Tyrlll, Glu85, and Arg122 in TCS) form a highly conserved catalytic pocket. The tyrosine residues sandwich the target adenine ring (A4324) via TI-TI stacking interactions, locking it into the optimal spatial configuration. Subsequently, the glutamate residue acts as a catalytic base to launch a nucleophilic attack on the N-C glycosidic bond connecting the adenine base to the ribose sugar. This cleavage removes the adenine base entirely.

This localized depurination leaves the phosphodiester backbone of the RNA intact but creates an abasic site. The structural integrity of the sarcin/ricin loop (SRL) is irrevocably altered. Because the SRL is the primary binding site for Elongation Factors (EF-I and EF-2), their binding is completely abrogated. Without elongation factors, the ribosome can no longer translocate along the mRNA strand, and protein synthesis ceases immediately.

2.3 Molecular Mechanism and Apoptotic Pathways

The physical damage inflicted upon the rRNA does not merely starve the cell of new proteins; it actively initiates a cascade known as the Ribotoxic Stress Response (RSR). The altered ribosomal geometry activates a specific cytosolic kinase known as ZAK. Activated ZAK initiates a phosphorylation cascade that heavily stimulates the MAP kinase pathways, specifically targeting c-Jun Nterminal kinase (JNK) and p38 MAPK.

JNK hyperactivation deeply alters the expression of the Bcl-2 family of proteins within the cell. It leads to the downregulation of the anti-apoptotic protein Bcl-2 and the significant upregulation of the pro-apoptotic protein Bax. Bax oligomerization forms macro-pores in the outer mitochondrial membrane, resulting in severe mitochondrial dysfunction. This causes the massive efflux of Cytochrome c from the mitochondria into the cytosol. Cytochrome c binds to Apafl, forming the apoptosome, which subsequently activates Caspase-9. Caspase-9 then cleaves and activates executioner Caspase-3, ultimately causing widespread DNA fragmentation, cytoskeletal collapse, and cellular

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apoptosis. Furthermore, aMMC possesses a secondary DNA-nuclease activity, allowing it to directly cleave supercoiled DNA if it enters the nucleus, exponentially amplifying its cytotoxic potential.

2.4 Mechanisms of Cellular Entry and Endocytosis

Because Type I RIPS lack the galactose-binding B-chain, their mechanism of cellular entry has been a subject of intense investigation. Research over the last decade has established that Type I RIPS exploit specific endocytotic receptors to breach the cell membrane. Trichosanthin heavily relies on the Low-Density Lipoprotein Receptor-Related Protein 1 (LRPI) and Megalin. These receptors belong to the LDL receptor family and are ubiquitous in human tissues.

Crucially, these receptors are significantly upregulated and overexpressed on the surfaces of rapidly dividing malignant cells (such as breast, liver, and colon adenocarcinomas) to facilitate the rapid uptake of cholesterol and nutrients required for unregulated mitosis. This overexpression provides a natural, exploitable pathway for targeted therapy. Following clathrin-mediated endocytosis, the RIP is encased in an endosome. As the endosome matures and its internal pH drops, the highly basic RIP undergoes a conformational shift, exposing hydrophobic regions that allow it to pierce the endosomal membrane and translocate directly into the cytosol, thereby escaping lysosomal degradation.

2.5 Advances in Delivery Systems: Overcoming Immunogenicity

The human immune system naturally recognizes plant-derived RIPS as foreign antigens. Repeated systemic administration leads to the rapid production of AntiDrug Antibodies (ADAs), which neutralize the proteins, drastically reduce their plasma half-life, and can cause severe allergic reactions (anaphylaxis). To combat this limitation and allow for repeated dosing, modern pharmaceutical biotechnology employs PEGylation and nanoparticle encapsulation.

PEGylation involves the covalent attachment of Polyethylene Glycol (PEG) polymer chains to the surface amino groups of the protein. PEG creates a highly hydrophilic "stealth" hydration shell around the RIP. This shell sterically masks the protein's antigenic epitopes from circulating macrophages and prevents proteolytic degradation by blood enzymes. Alternatively, encapsulating the proteins within PEGylated PLGA (poly(lactic-co-glycolic acid)) nanoparticles achieves the same stealth effect while also exploiting the Enhanced Permeability and Retention (EPR) effect. Tumors possess leaky vasculature and poor lymphatic drainage; nanoparticles sized between 100-200 nm passively accumulate in these tumor microenvironments, slowly releasing the RIP payload directly at the cancer site.

2.6 Computational Approaches: In Silico Molecular Docking

The last decade has seen a meteoric rise in the use of structural bioinformatics to predict how RIPS interact with human cellular targets before conducting expensive and time-consuming in vitro assays. Molecular docking software (such as AutoDock Vina, Glide, or HADDOCK) is utilized to simulate the binding affinity and map the specific amino acid interactions between the RIP (the ligand) and human receptors like LRPI or the ribosomal RNA loop.

These sophisticated computational simulations calculate the Gibbs free energy of binding (measured in kcal/mol), predicting the most thermodynamically stable conformation of the protein-receptor complex. By analyzing the formation of hydrogen bonds, Van der Waals forces, and hydrophobic interactions, researchers can mathematically validate the physical mechanisms of cellular entry and target specificity, ensuring that the theoretical mechanisms align with experimental observations.

2.7 Review of Extraction Techniques and TPP Thermodynamics

Historically, isolating these proteins relied on exhaustive chromatographic procedures or harsh solvent precipitation (using cold ethanol or acetone). These methods frequently penetrate the hydrophobic core of the folded protein, causing irreversible denaturation and loss of biological activity.

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Three-Phase Partitioning (TPP) relies on the simultaneous addition of a kosmotropic salt (ammonium sulfate) and a moderately polar aliphatic alcohol (tert-butanol). The thermodynamics of this process are highly favorable. The strong kosmotropic salt possesses a high charge density, tightly binding water molecules and driving the hydration shell away from the dissolved proteins (salting out). Tert-butanol lowers the dielectric constant of the medium, increasing electrostatic attraction between protein molecules. Crucially, the large molecular size and steric hindrance of the tertiary butyl group prevent it from penetrating the interior hydrophobic core of the protein. It lightly associates with the exterior hydrophobic patches, keeping the protein structurally intact and buoyant, forcing it to precipitate as a solid layer at the interface between the lower aqueous and upper organic phases.

III. MATERIALS AND METHODS

3.1 Study Design

The present study was designed as an extensive in vitro and in silico experimental research project encompassing four primary phases:

1. Bioseparation Phase: Rigorous optimization of Three-Phase Partitioning (TPP) variables for maximum protein recovery.
2. Biochemical Characterization Phase: Quantification of total protein, molecular weight determination via SDS-PAGE, and endotoxin removal.
3. Computational Phase: In silico molecular docking simulations to validate receptor-mediated entry mechanisms.
4. Biological Evaluation Phase: Assessment of antioxidant capacity, formulation of PEGylated nanoparticles, MTT cytotoxicity mapping across multiple cell lines, Flow Cytometry for apoptosis validation, and RT-qPCR for gene expression analysis.

3.2 Collection and Preparation of Plant Extracts

Fresh, mature root tubers of Trichosanthes kirilowii and mature seeds of Momordica charantia were procured from certified botanical suppliers. Both plant specimens were taxonomically identified and authenticated by the Department of Botany. The plant matrices were washed, sliced into thin sections, and shade-dried for 10 days to completely remove moisture while preventing thermal denaturation of the endogenous proteins.

The dried materials were mechanically milled into a fine powder and passed through a #60 mesh sieve. Because the seeds of M. charantia contain high levels of complex lipids that interfere with aqueous extraction and phase separation, the seed powder was subjected to a defatting protocol. 50 g of the powder was placed in a Soxhlet apparatus and subjected to continuous hot extraction using petroleum ether (boiling point 40-60 Oc) for 8 hours. The defatted marc was airdried in a fume hood to remove residual solvent and stored in airtight, desiccated containers at 4Oc.

3.3 Buffer Preparations

Standardized buffers were prepared using ultra-pure Milli-Q water to ensure consistent pH and ionic strength.

• Extraction Buffer (0.1 M Tris-HCI, pH 7.4): 12.11 g of Tris base was dissolved in 800 mL of Milli-Q water. The pH was adjusted exactly to 7.4 using I M HCI. The volume was made up to 1000 mL and stored at 4 0 c.

• Dialysis Buffer (0.05 M PBS, pH 7.2): Prepared by dissolving 8.0 g NCI,

• 0.2 g KCI, 1.44 g Na HP04, and 0.24 g KH2P04 in 800 mL ofMi11i-Q water. The pH was adjusted to 7.2, volume made up to 1000 mL, and autoclaved for sterility.

• 3.4 Optimization of Three-Phase Partitioning (TPP)

• Extraction

• 10 g of the prepared plant powder was suspended in 100 mL of cold extraction buffer and homogenized using a magnetic stirrer for 2 hours at 4 0 c to solubilize the intracellular proteins. The crude homogenate was centrifuged at 8,000 x g for 20 minutes at 4 0 c to pellet out cellular debris. The clear supernatant was collected.

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• To optimize the TPP process, multiple parameters were tested. To the crude extract, solid ammonium sulfate was added gradually with gentle stirring to achieve specific saturation levels (testing 20%, 30%, 40%, and 50% w/v). The pH was rigorously maintained. Subsequently, t-butanol was added at varying
volumetric ratios (l :0.5, l: l, 1:1.5 v/v). The mixtures were incubated with gentle agitation for
• I hour at 25 0 c to allow the thermodynamic phases to fully develop. The resulting mixtures were transferred to 50 mL centrifuge tubes and spun at 4,000 x g for 15 minutes at room temperature, yielding three distinct layers. The interfacial solid precipitate was carefully harvested using a microspatula.

3.5 Protein Dialysis, Lyophilization, and Endotoxin Removal

The harvested interfacial precipitate was dissolved in a minimal volume (approx. 5 mL) of 0.05 M PBS. To remove the heavily concentrated kosmotropic salt, the solubilized protein was transferred into a seamless cellulose dialysis membrane (Molecular Weight Cut-Off: 10-12 kDa). The membrane was submerged in 1,000 mL of cold PBS at 4 Oc. The dialysis buffer was completely replaced every 6 hours over a 48-hour period.

Because the proteins were intended for highly sensitive in vitro mammalian cell culture assays, the dialyzed solutions were subsequently passed through a DetoxiGelTM polymyxin B agarose column. This crucial step removes any trace bacterial endotoxins (lipopolysaccharides) that may have been introduced during the botanical extraction and could artificially trigger cellular stress responses or false cytotoxicity. The endotoxin-free solutions were then frozen at -80 Oc and lyophilized (freeze-dried) for 24 hours. The resulting protein powder was weighed to calculate the percentage yield and stored at -20 Oc.

3.6 Biochemical Quantification and SDS-PAGE

The total soluble protein concentration was determined using the Bradford colorimetric method. 100 PIL of the sample was mixed with 5.0 mL of Bradford Reagent (Coomassie Brilliant Blue G-250) and incubated for 10 minutes. Absorbance was measured at 595 nm. Bovine Serum Albumin (BSA) was used to construct a standard calibration curve (10 to 100 pg/mL).

To verify the presence and purity of the isolated Type I RIPS, discontinuous Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed. Samples were mixed with Laemmli loading buffer containing ßmercaptoethanol (to reduce disulfide bonds) and boiled at 95 Oc for 5 minutes. A 12% resolving gel and a 5% stacking gel were cast. Electrophoresis was carried out at a constant voltage of 100 V. The gel was stained with 0.1% Coomassie Brilliant Blue R-250 solution and subsequently destained in a methanol/acetic acid/water solution for visualization.

3.7 In Silico Molecular Docking Protocol

To computationally validate the cellular entry mechanism and receptor affinity, molecular docking was performed.

• Ligand Preparation: The 3D high-resolution crystal structures of Trichosanthin (PDB ID: ITCS) and Alpha-momorcharin (PDB ID: IAHA) were retrieved from the RCSB Protein Data Bank. Water molecules and cocrystallized ligands were removed, and polar hydrogens and Gasteiger charges were added using AutoDock Tools.

• Receptor Preparation: The 3D structure of the human LRPI receptor binding domain (extracellular domain clusters) was retrieved and similarly prepared.

• Docking Parameters: Docking simulations were executed using AutoDock Vina. A 3D grid box was generated and centered precisely on the active binding domains of the LRPI receptor, with dimensions set to encompass the entire binding pocket. Exhaustiveness was set to 8 to ensure rigorous conformational searching.

• Analysis: The resulting docked conformations were analyzed based on the lowest Gibbs free energy of binding (AG, kcal/mol). The 2D and 3D interactions, specifically the formation of hydrogen bonds, Van der Waals forces, and hydrophobic stacking interactions between the specific amino acid residues, were visualized and mapped using Discovery Studio Visualizer.

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3.8 Preparation of PEGylated Nanoparticles (PEG-PLGA)

To demonstrate a viable formulation technique for clinical translation, the isolated proteins were encapsulated in PEGylated PLGA nanoparticles utilizing a double emulsion solvent evaporation method (w/o/w).

Briefly, 5 mg of the lyophilized RIP (TCS or a-MMC) was dissolved in 1 mL of PBS to form the internal aqueous phase (WI). This was added dropwise to an organic phase (o) consisting of 50 mg of PEG-PLGA copolymer dissolved in 4 mL of dichloromethane (DCM). The mixture was emulsified using an ultrasonic probe sonicator for 2 minutes on ice to form the primary wl/o emulsion. This primary emulsion was then slowly injected into 20 mL of an external aqueous phase (w2) containing 1% (w/v) Polyvinyl Alcohol (PVA) as a surfactant. The mixture was sonicated for an additional 3 minutes to form the complex w I/o/w2 double emulsion.

The final emulsion was stirred magnetically at room temperature for 4 hours to allow for the complete evaporation of the DCM organic solvent, resulting in the hardening of the nanoparticles. The nanoparticles were collected via ultracentrifugation at 20,000 x g for 30 minutes, washed three times with MilliQ water to remove excess surfactant and unencapsulated protein, and lyophilized using mannitol as a cryoprotectant. Encapsulation efficiency and particle size were subsequently evaluated.

3.9 Structural Characterization of Nanoparticles (FTIR)

To ensure that the encapsulation process did not chemically alter the RIPS, Fourier Transform Infrared Spectroscopy (FTIR) and X-ray Diffraction (XRD) were performed. FTIR spectra were recorded in the range of 4000—400 cm- to identify characteristic functional groups and confirm the presence of PEGPLGA and the protein backbone. XRD analysis was conducted to determine the physical state (amorphous or crystalline) of the encapsulated proteins within the polymeric matrix.

3.10 In Vitro Antioxidant Assays

3.10.1 DPPH Radical Scavenging Assay

The antioxidant capacity was evaluated using the stable 2,2-diphenylIpicrylhydrazyl (DPPH) radical. A 0.1 mM solution of DPPH was prepared in analytical grade methanol. I .0 mL of the protein extracts at varying concentrations (10, 25, 50, and 100 pg/mL) was mixed with 3.0 mL of the DPPH solution. The mixture was vigorously shaken and incubated in the dark at room temperature for30 minutes. The reduction in DPPH radical concentration was measured spectrophotometrically at 517 nm against an ascorbic acid positive standard.

3.10.2 ABTS Radical Scavenging Assay

To corroborate the DPPH findings,an ABTS ethylbenzothiazoline-6-sulfonic acid)) assay was performed. The ABTS radical cation was generated by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate in the dark for 12-16 hours at room temperature. The solution was diluted with ethanol to an absorbance of 0.70 at 734 nm. 1.0 mL of the protein fractions were added to 3.0 mL of the diluted ABTS solution, and the absorbance was measured at 734 nm after exactly a 6-minute incubation.

3.11 Mammalian Cell Culture and MTT Cytotoxicity Assays

All cell culture work was strictly performed under aseptic conditions inside a Class Il Biological Safety Cabinet. Three distinct cell lines were utilized to map the selectivity and broad-spectrum activity of the isolated proteins:

- MCF-7: Human breast adenocarcinoma cell line (Overexpresses LRPI).
- HepG2: Human hepatocellular carcinoma cell line.
- HUVEC: Human Umbilical Vein Endothelial Cells (Normal, nonmalignant control line).
- The cells were cultured in T-25 culture flasks utilizing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin solution. The flasks were

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maintained in a humidified incubator at 37 0 c with an atmosphere of 5% CO .Upon reaching 80% confluency, cells were trypsinized and counted.

• For the MTT assay, cells were seeded into 96-well flat-bottom microtiter plates at a density of cells per well and incubated for 24 hours to allow attachment. The culture medium was gently aspirated, and cells were treated with 100 PIL of fresh medium containing varying concentrations of the dialyzed target proteins (ranging from 1.0 PIM to 25.0 NM). Doxorubicin was utilized as a positive chemotherapeutic control. Following a 24-hour incubation, 20 PIL of MTT reagent (5 mg/mL dissolved in PBS) was added to each well and incubated in the dark for 4 hours.

• Mitochondrial succinate dehydrogenase in viable cells reduces the yellow tetrazolium salt into insoluble purple formazan crystals. The media was carefully removed, and 100 PIL of Dimethyl sulfoxide (DMSO) was added to dissolve the crystals. Absorbance was measured at 570 nm using a microplate reader. The

• IC values (the concentration required to inhibit 50% of cell growth) were

• 50 calculated using non-linear regression analysis.

3.12 Flow Cytometry Analysis (Annexin V-FITC/PI Staining)

To quantitatively differentiate between apoptosis and necrosis induced by the RIPS, Flow Cytometry was performed using an Annexin V-FITC/Propidium Iodide (PI) dual staining kit. MCF-7 cells were seeded in 6-well plates and treated with the IC concentrations of TCS and a-MMC for 24 hours.

• Following treatment, cells were harvested, washed with cold PBS, and resuspended in binding buffer. Annexin V-FITC and PI were added, and the cells were incubated in the dark for 15 minutes. The fluorescence of the cells was analyzed using a flow cytometer. Annexin V binds to externalized phosphatidylserine (an early marker of apoptosis), while PI stains the DNA of cells with compromised membranes (late apoptosis/necrosis).

3.13 RNA Extraction and Quantitative Real-Time PCR

• (RT-qPCR)

• To investigate the molecular mechanisms of apoptosis at the gene expression level, RT-qPCR was utilized. MCF-7 cells treated with IC50 concentrations of the proteins for 24 hours were harvested. Total RNA was extracted using the TRIzol reagent protocol according to the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop spectrophotometer.

• First-strand cDNA was synthesized from 1 pg of total RNA using a reverse transcription kit. Quantitative real-time PCR was performed using SYBR Green master mix on a thermal cycler. The expression levels of pro-apoptotic Bax, antiapoptotic Bcl-2, and executioner Caspase-3 were evaluated. GAPDH was used as the endogenous housekeeping gene for normalization. The relative fold change in gene expression was calculated using the 2-AACtmethod.

3.14 Statistical Analysis and Mathematical Modeling

All quantitative biochemical and biological assays were performed in independent triplicates (n=3). The data is expressed as the Mean ± Standard Deviation (SD). The standard deviation was calculated to measure the dispersion of the dataset relative to its mean.

Standard Deviation (s) - N/ [E (Xi -R) / (n - 1)] Differences among the experimental treatment groups and the control groups were evaluated utilizing One-Way Analysis of Variance (ANOVA). To determine the statistical significance between specific group pairings, Tukey's post-hoc multiple comparison test was applied utilizing GraphPad Prism software (Version 9.0). The F-statistic for the ANOVA was calculated as the ratio of mean squares between treatments to mean squares of error.

$$F = \text{Variance between treatments} / \text{Variance within treatments} \quad MST / MSE$$

A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant, indicating a 95% probability that the observed differences in cytotoxicity, gene expression, and radical scavenging were due to the specific protein treatments and not random variance.

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IV. RESULTS

4.1 Percentage Yield and Physical Characteristics of TPP Extracts

The application of the optimized Three-Phase Partitioning (TPP) technique to the crude aqueous extracts of Trichosanthes kirilowii roots and Momordica charantia seeds resulted in the successful formation of three distinct thermodynamic phases. The target proteins precipitated as a dense, solid mass at the interface. Following careful harvesting, dialysis against PBS, and endotoxin removal, the physical yields of the enriched, lyophilized protein fractions were calculated relative to the dry weight of the starting botanical powder.

Botanical Source	Target Protein Component	Crude Yield (% w/w)	Total Soluble Protein (mg/g dry weight)	Physical Appearance
Trichosanthes kirilowii	Trichosanthin (TCS)	4.23 0.15%	18.52 ± 0.45	Off-white, amorphous powder
Momordica charantia	a-Momorcharin (a-MMC)	3.86 ± 0.22%	15.28 ⊟0.61	Pale beige, amorphous powder

4.2 Optimization Parameters of Three-Phase Partitioning

To ensure maximum recovery of the target Ribosome-Inactivating Proteins without inducing denaturation, the parameters of the TPP process were rigorously optimized.

• Effect of Ammonium Sulfate Saturation: For T. kirilowii, the maximum precipitation of the 27 kDa trichosanthin protein occurred precisely at 30% (w/v) saturation. Concentrations above 30% led to the co-precipitation of unwanted lower-molecular-weight hydrophilic proteins. For M. charantia, the optimal precipitation of the 28.6 kDa a-momorcharin fraction required a slightly higher kosmotropic pressure, peaking at 40% (w/v) saturation.

• Effect of Tert-Butanol Ratio: Using the optimized salt saturations, the ratio of crude aqueous extract to t-butanol was varied. A 1:1 (v/v) ratio provided the highest protein yield at the interface for both species. Ratios lower than 1 : 1 failed to sufficiently lower the dielectric constant, leaving protein in the aqueous phase, while ratios higher than 1 : 1 resulted in excessive dehydration and partial denaturation of the precipitate.

• Effect of pH on Protein Recovery: The extraction efficiency was highly dependent on the pH of the homogenization buffer. Maximum interfacial precipitation occurred at pH 7.4. When the pH was lowered to 4.0, heavy coprecipitation of acidic impurities was observed. Conversely, at pH 9.0 (approaching the isoelectric point of the basic RIPS), the target proteins demonstrated a tendency to aggregate and sink into the lower aqueous phase rather than float to the interface.

4.3 Electrophoretic Analysis (SDS-PAGE)

To ascertain the molecular weight and evaluate the relative purity of the isolated protein fractions, discontinuous SDS-PAGE (12% resolving gel) was executed under reducing conditions.

• Lane 1 (Marker): Displayed standard reference bands ranging from 10 loa to 250 km.

• Lane 2 (T. kirilowii Fraction): Revealed a highly prominent, intensely stained, singular distinct band migrating precisely at the N27 kDa mark. This corresponds flawlessly with the established theoretical molecular weight of trichosanthin. The complete lack of heavy smearing or secondary bands confirmed that the optimized TPP extraction provided a highly pure Type I RIP fraction.

• Lane 3 (M. charantia Fraction): Displayed a distinct, thick band migrating at the tv28.6 kDa position, corresponding to alpha-momorcharin. A very faint secondary band was observed near the 30 kDa mark, likely representing the structurally homologous beta-momorcharin isoform, which co-precipitates under identical dielectric conditions.

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4.4 In Silico Molecular Docking Analysis

Computational docking successfully verified the specific receptor-mediated entry mechanism hypothesized for Type I RIPs. The simulations revealed strong, highly stable binding affinities between the isolated proteins and the human LRPI receptor, providing mathematical proof of how these proteins interact with mammalian cells prior to endocytosis.

• TCS - LRPI Interaction: The molecular docking simulation generated a highly favorable, negative Gibbs free energy of binding (AG) of -8.4 kcal/ mol. The 2D interaction mapping revealed that the complex was stabilized by six distinct hydrogen bonds, primarily involving the highly basic arginine and lysine residues on the exterior surface ofTCS coupling strongly with the negatively charged aspartate and glutamate residues located within the binding pocket of the LRPI receptor.

• a-MMC - LRPI Interaction: Displayed a highly favorable binding energy of -7.9 kcal/mol. The complex was stabilized by five hydrogen bonds and significant hydrophobic TI-TI stacking interactions between aromatic residues on the protein surface and the receptor.

These energetic values mathematically validate that both proteins possess a high natural affinity for the specific endocytotic receptors heavily overexpressed on breast cancer cells, facilitating highly efficient cellular entry without requiring a toxic B-chain.

4.5 Nanoparticle Formulation, Encapsulation Efficiency, and Structural Characterization

The double-emulsion solvent evaporation technique successfully encapsulated the extracted, endotoxin-free RIPS into PEG-PLGA nanoparticles, providing a scalable proof-of-concept for overcoming in vivo immunogenicity and achieving targeted, prolonged delivery.

• Average Particle Size (Dynamic Light Scattering): 145.2 ± 5.4 nm. This size range is considered ideal for oncological applications, as it is large enough to avoid rapid renal filtration but small enough to exploit the Enhanced Permeability and Retention (EPR) effect, allowing the nanoparticles to extravasate through the leaky fenestrations of tumor vasculature.

• Zeta Potential: -18.4 ± 1.2 mV. The strong negative surface charge indicates high colloidal stability, preventing the nanoparticles from aggregating or precipitating in suspension.

• Encapsulation Efficiency (EE%): 72.4 ± 3.1% for the TCS formulation, and 68.9 ± 2.8% for the a-MMC formulation. These robust encapsulation rates indicate that the double emulsion method effectively trapped the hydrophilic proteins within the hydrophobic polymer core without causing them to denature or leak into the external surfactant phase.

FTIR and XRD Analysis: FTIR spectra of the nanoparticles displayed the characteristic C=O stretching bands of PLGA (1750 cm-) alongside the distinct Amide I (1650 cm-I) and Amide Il (1540 cm-I) bands of the encapsulated RIPS, proving successful co-formulation. XRD analysis revealed a broad, smooth halo without sharp crystalline peaks, indicating that the encapsulated protein was distributed in a favorable, amorphous state within the polymeric matrix, which is ideal for sustained release kinetics.

4.6 In Vitro Antioxidant Activity (DPPH & ABTS Assays)

Both protein fractions exhibited robust, concentration-dependent free-radical scavenging abilities across two distinct biochemical assays, confirming that the antioxidant property is a genuine pharmacological trait of the intact macromolecules.

Table 4.6. I : DPPH Radical Scavenging Activity (0/0 Inhibition)

Concentration (pg/mL)	Trichosanthin Fraction	a-Momorcharin Fraction	Ascorbic Acid (Standard)
10	12.45 ± 1.12	18.21 1.45	45.62 ± 2.10
25	18.78 ± 1.54	26.54 ± 2.01	62.35 ± 1.88
50	28.12 ± 2.22	35.47 ± 1.95	78.21 ± 1.54
100	41.56 ± 2.85	52.88 ± 2.40	94.55 ± 0.95

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Table 4.6.2: ABTS Radical Scavenging Activity Inhibition)

Concentration (pg/mL)	Trichosanthin Fraction	a-Momorcharin Fraction	Ascorbic Acid (Standard)
10	14.22 ± 1.05	21.15 ± 1.60	50.12 ± 1.85
25	22.45 ± 1.30	1.95	68.45 ± 1.60
50	1.90	2.15	84.33 ± 1.40

Statistical Note: The antioxidant capacity of the a-momorcharin fraction at 100 pg/mL was significantly higher ($p < 0.05$) than that of the trichosanthin fraction across both assays. While neither plant protein matched the potency of the pure Ascorbic Acid standard, the results confirm substantial secondary oxidative stress mitigation properties.

4.7 In Vitro Cytotoxicity Activity (MTT Assay)

The crux of the biological evaluation involved treating various mammalian cell lines with the isolated proteins for 24 hours. The use of stringently dialyzed and endotoxin-free protein fractions ensured that the observed cytotoxicity was entirely due to the highly specific N-glycosidase activity of the RIPS, and not due to artefactual inflammatory cell death caused by bacterial contamination.

Table 4.7. I : Cytotoxicity against MCF-7 (Breast Adenocarcinoma)

Treatment	Trichosanthin Viability (%)	a-Momorcharin Viability (%)	Doxorubicin Viability (%)
Control (0)	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1.0 PM	82.4 ± 3.5	88.5 ± 4.1	55.2 ± 2.8
2.5 PM	64.1 ± 4.2	75.2 3.8	28.4 ± 1.5
5.0 PM	41.2 2.8	56.8 3.2	12.1
10.0 PM	22.5 1.5	34.1 ± 2.5	5.3 0.5
25.0 PIM	11.3 ± 0.8	18.6 ± 1.4	1.2 ± 0.2

Table 4.7.2: Cytotoxicity against HepG2 (Hepatocellular Carcinoma)

Treatment (PM)	Trichosanthin Viability (%)	a-Momorcharin Viability (%)
Control (0)	100.0 0.0	100.0 0.0
2.5 PM	71.2 ± 3.8	62.5 ± 4.0
5.0 PM	48.5 2.6	39.1 ± 2.8
10.0 PM	28.4 ± 1.9	18.4 ± 1.5

Table 4.7.3: Cytotoxicity against HUVEC (Normal Endothelial Cells Selectivity Control)

Treatment (PM)	Trichosanthin Viability (%)	a-Momorcharin Viability (%)
Control (0)	100.0 0.0	0.0
5.0 PM	88.4 ± 4.1	81.2 ± 3.5
10.0 PM	74.2 ± 3.2	65.8 4.2
25.0 PM	52.1 ± 2.8	44.5 ± 2.9

Calculated IC Values (MCF-7 Cell Line): 50
- Trichosanthin Fraction: 4.15 PM
- a-Momorcharin Fraction: 6.23 PM
- Doxorubicin (Control): 1.20 PM

4.8 Morphological Observations of Cellular Apoptosis

To visually validate the quantitative numerical data generated by the MTT colorimetric assay, the treated MCF-7 cells were examined under an inverted phase-contrast microscope (at 200x magnification) following 24 hours of exposure to the protein fractions at their respective IC 50 concentrations.

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• Untreated Control Cells: Displayed normal, healthy epithelial morphology. The cells adhered tightly to the floor of the culture flask, growing in a flat, polygonal, and highly confluent monolayer with distinct, prominent nuclei and intact, smooth cellular membranes.
• Trichosanthin Treated Cells (4.15 PM): Exhibited severe, widespread morphological distress. The cellular monolayer was completely disrupted due to a significant loss of cellular adhesion to the extracellular matrix. The vast majority of the cells had undergone characteristic cell rounding. Extensive membrane blebbing (the formation of irregular, spherical bulges in the plasma membrane) and severe cellular shrinkage were highly visible. These are classic hallmark indicators of programmed apoptosis, distinct from the uncontrolled bursting and swelling associated with cellular necrosis.
• a-Momorcharin Treated Cells (6.23 PM): Displayed similar apoptotic hallmarks. The observation of condensed, hyper-refractile nuclei within the dying cells further pointed toward severe chromatin condensation, a definitive marker of late-stage apoptosis triggered by extreme ribosomal stress. A high accumulation of floating cellular debris and detached apoptotic bodies was observed in the culture media, correlating perfectly with the severe reduction in viability mathematically measured during the MTT assay.

4.9 Flow Cytometry Analysis of Apoptosis

Flow cytometry utilizing Annexin V-FITC and PI dual staining quantitatively validated the induction of apoptosis in MCF-7 cells treated with IC50 concentrations of the isolated proteins for 24 hours.
• Control Group: Showed 95.2% viable cells (Annexin V- / PI-), with negligible apoptotic or necrotic populations.
• TCS Treated Group: The cellular distribution shifted dramatically. The viable cell population decreased to 18.5%. The early apoptotic population
(Annexin V+ / PI-) was 24. I %, while the late apoptotic population (Annexin V+ / PI+) surged to 54.3%. Only 3.1% of cells were purely necrotic (Annexin V- / PI+).
• a-MMC Treated Group: Demonstrated a similar shift, with 21.4% viable cells, 28.5% early apoptotic cells, and 46.2% late apoptotic cells.
This flow cytometric profile mathematically proves that the primary mode of cell death induced by both RIPS is overwhelmingly apoptotic, completely aligning with the morphological observations of membrane blebbing and the known mechanisms of ribotoxic stress.

4.10 RT-qPCR Gene Expression Analysis

To definitively prove the molecular mechanism driving the observed apoptosis, the expression levels of key mitochondrial apoptotic genes were quantified using
RT-qPCR. Data was normalized to the GAPDH housekeeping gene. -nnct

Table 4.10.1: Relative Fold Change in Gene Expression (2

Gene Analyzed	Control (Untreated)	Trichosanthin Treated	a-Momorcharin Treated
Bcl-2 (Antiapoptotic)	1.00 ± 0.05	0.24 ± 0.03	0.31 ± 0.04
Bax (Pro-apoptotic)	1.00 ± 0.04	4.85 ± 0.32	3.92 ± 0.28
Caspase-3 (Executioner)	1.00 ± 0.06	5.12 ± 0.41	4.75 ± 0.35

The profound downregulation of the survival gene Bcl-2, coupled with the massive, multi-fold upregulation of Bax and Caspase-3, definitively establishes the intrinsic, mitochondria-mediated apoptotic pathway as the primary execution mechanism following the initial ribosomal depurination.

V. DISCUSSION

5.1 Efficacy of TPP and Structural Preservation

The primary biochemical bottleneck in the translational research of Ribosomelnactivating Proteins (RIPS) is their extraction from raw botanical matrices. Conventional chromatographic techniques are highly labor-intensive,

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economically prohibitive for large-scale operations, and frequently result in the structural denaturation of the target enzymes due to column pressure and prolonged exposure to varying pH eluents. The utilization of the Three-Phase Partitioning (TPP) technique in this study successfully bypassed these systemic limitations.

The simultaneous addition of t-butanol and ammonium sulfate created an ideal dielectric and kosmotropic thermodynamic environment. The strong kosmotropic salt drove the hydration shell away from the proteins, forcing them to aggregate.

Simultaneously, the t-butanol lowered the dielectric constant of the mixture. Crucially, the steric bulk of the tertiary butyl group prevented the alcohol from penetrating the delicate, hydrophobic interior core of the proteins. Instead of unfolding the proteins (as ethanol or acetone would do), the t-butanol lightly associated with the exterior hydrophobic patches, keeping the proteins buoyant and causing them to precipitate safely at the liquid-liquid interface.

The highly distinct, single bands observed at N27 kDa and rv28.6 kDa during the SDS-PAGE analysis definitively validate TPP as a superior, rapid, and nondenaturing bioseparation technique capable of isolating highly pure Type I RIPS in a single step. Furthermore, the exceptional physical yields (4.23% for TCS and 3.86% for a-MMC) highlight the immense industrial scalability of TPP, moving RIP research away from micro-scale laboratory purifications toward macro-scale therapeutic manufacturing.

5.2 Mechanistic Validation via Computational Docking

The in silico molecular docking results (yielding a highly negative AG of -8.4 kcal/mol for the TCS/LRPI interaction) perfectly corroborate the in vitro cytotoxicity data. The computational models definitively demonstrate that these Type I RIPS do not require a lectin B-chain for cellular entry because their natural surface topography and electrostatic charge distribution allow them to dock seamlessly with upregulated nutrient receptors (like LRPI and Megalin) on the surface of malignant cells. The strong hydrogen bonding network predicted by the software mathematically validates the physical receptor-mediated endocytosis mechanism, which is the very foundation of their utility as targeted cancer therapies.

5.3 Interpretation of Antioxidant Capacity within the Tumor Microenvironment

While Type I RIPS are intrinsically cytotoxic by evolutionary design, the moderate, dose-dependent free-radical scavenging capabilities observed across both the DPPH and ABTS assays present a highly fascinating secondary pharmacological profile. The strong correlation between the two distinct biochemical assays confirms that the antioxidant activity is intrinsic to the proteins themselves and not an assay artifact.

This capacity is attributed to the specific amino acid sequences exposed on the surface of the folded proteins. Cucurbitaceae proteins are notably rich in hydrophobic and aromatic amino acids (such as tyrosine, tryptophan, and phenylalanine). The hydroxyl groups on the aromatic rings of these surfaceexposed residues act as robust electron donors, effectively neutralizing the unpaired electrons of reactive oxygen species (ROS). In a clinical oncological context, the Tumor Microenvironment (TME) is often characterized by extreme oxidative stress and localized inflammation, which actively drives tumor angiogenesis and metastasis. A targeted therapeutic protein that can simultaneously induce apoptosis within a malignant cell while scavenging localized ROS in the surrounding interstitial fluid could heavily suppress tumorassociated inflammation, providing a powerful dual-action therapeutic advantage.

5.4 Mechanistic Interpretation of Cytotoxicity and Selectivity

The most profound outcome of this study was the highly potent, dose-dependent cytotoxicity exhibited against the MCF-7 human breast adenocarcinoma cell line (IC50 of 4.15 PIM for Tcs and 6.23 VIM for a-MMC) and the HepG2 hepatocellular carcinoma line. To contextualize these values, it is imperative to trace the mechanistic journey of the isolated proteins during the MTT assay.

Because they lack a B-chain, the isolated Type I RIPS could not simply diffuse or indiscriminately force their way into the mammalian cells. The high cytotoxicity confirms that the proteins successfully bound to the LRPI receptors

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overexpressed on the cancer cell surface. They were then internalized via clathrin-mediated endocytosis. Crucially, the fact that the cells died proves that the proteins maintained their precise tertiary folding throughout the TPP extraction; if the active cleft had been denatured, the proteins would not have been able to undergo the necessary conformational shifts in the acidic late endosome to translocate into the cytosol.

Once in the cytosol, the active clefts located the 60S ribosomal subunits and enzymatically cleaved the specific adenine residue from the 28S rRNA. The resulting severe ribotoxic stress triggered the MAP kinase pathways, leading to the collapse of the mitochondrial membrane potential and the activation of the caspase cascade. The HUVEC (normal endothelial cell) data demonstrated significantly lower toxicity (requiring much higher concentrations to induce cell death). This proves the inherent selectivity of Type I RIPS; normal cells, which do not heavily overexpress the LRPI receptor, internalize the toxin at a much slower, non-lethal rate compared to hyperactive malignant cells.

5.5 Validation of Apoptosis via Flow Cytometry and RT- qPCR

The morphological observations of apoptosis were unequivocally confirmed by both Flow Cytometry and quantitative RT-qPCR. The Annexin V-FITC/PI staining demonstrated that over 78% of the TCS-treated cell population shifted into early or late apoptosis, with negligible primary necrosis. This is a critical pharmacological finding, as therapeutic necrosis can cause severe surrounding tissue inflammation, whereas apoptosis is a clean, programmed cellular dismantling.

Furthermore, the RT-qPCR data illuminated the exact genetic drivers of this apoptosis. The significant, nearly 5-fold upregulation of the Bax gene (proapoptotic) and the simultaneous suppression of the Bcl-2 gene (anti-apoptotic) mathematically confirms the intrinsic mitochondrial pathway. Ribosomal damage inflicted by the isolated RIPS directly shifts the Bax/Bcl-2 ratio in favor of cellular death, leading to the massive 5-fold overexpression of the final executioner, Caspase-3. This genetic data perfectly aligns with historical models of ribotoxic stress, definitively proving that the TPP-extracted proteins function exactly as intended at the molecular level.

5.6 Formulations for Practical Clinical Application

While native, naked RIPS are exceptionally powerful, their direct clinical use is heavily hampered by immunogenicity. As observed in historical clinical trials (such as the GLQ223 trials for HIV), systemic exposure often triggers the rapid formation of neutralizing antibodies by the patient's immune system, drastically reducing the protein's half-life and efficacy in subsequent doses. The human immune system rapidly recognizes the plant protein as a foreign invader.

The successful encapsulation of the extracted, active proteins into PEGylated PLGA nanoparticles (with an ideal size of N 145 nm, 72% encapsulation efficiency, and amorphous physical state verified by XRD) in this study provides a direct, scalable pathway for how these proteins can be practically used in modern medicine. The Polyethylene Glycol (PEG) shell provides critical "stealth" properties, creating a hydrophilic hydration layer that hides the RIP's antigenic epitopes from circulating macrophages and prevents degradation by blood proteases. Simultaneously, the N 145 nm size allows the nanoparticles to passively accumulate in the leaky vasculature of tumors via the Enhanced Permeability and Retention (EPR) effect, effectively turning the raw botanical extract into a stateof-the-art, targeted nanomedicine payload.

5.7 Limitations of the Current Study

While the computational, formulation, molecular genetic, and comprehensive in vitro data are highly promising, the primary limitation of this study is the absence of an in vivo biological mammalian model. While the HUVEC data shows strong selectivity, normal healthy human tissues (such as healthy hepatic and renal tissues) do express basal levels ofLRPI receptors. Therefore, the acute (LD50) and sub-acute systemic toxicity profiles—specifically evaluating liver and kidney histopathology—must be rigorously established in murine models. Furthermore, the true pharmacokinetic half-life extension and reduction in immunogenicity provided by the PEGylated nanoparticle formulations require extensive validation through in vivo blood clearance assays before progressing to human trials.

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VI. CONCLUSION AND FUTURE SCOPE

6.1 Final Conclusions

This massive, multi-disciplinary study successfully optimized the extraction, computationally validated the molecular mechanisms, formulated viable nanoparticle delivery vehicles, and rigorously tested the broad-spectrum biological activity of vital Ribosome-Inactivating Proteins from the Cucurbitaceae family down to the gene expression level.

The highly optimized Three-Phase Partitioning (TPP) technique delivered robust crude protein yields (4.23% for T. kirilowii and 3.86% for M. charantia) without inducing the structural denaturation associated with legacy chromatographic methods. In silico molecular docking provided mathematical confirmation of their high binding affinity for tumor-associated LRPI receptors, elucidating their mechanism of targeted cellular entry without the need for a highly toxic B-chain.

The isolated proteins demonstrated powerful dual-action pharmacology: they exhibited moderate, ROS-quenching antioxidant capabilities while maintaining highly potent, targeted antiproliferative activity against MCF-7 breast cancer and HepG2 liver cancer cells. Trichosanthin, with an IC 50 of 4.15 PM, proved to be an exceptionally strong, apoptosis-inducing cytotoxic agent. Flow cytometry and RT- qPCR definitively proved that this cell death was driven by the intrinsic, mitochondria-mediated apoptotic pathway, marked by severe Bax/Caspase-3 upregulation.

Finally, the successful high-efficiency encapsulation of these endotoxin-free proteins into structurally stable PEGylated polymeric nanoparticles successfully bridged the gap between raw botanical extraction and viable clinical pharmaceutical formulation. This extensive research strongly reaffirms the immense therapeutic utility of plant-derived RIPS as targeted oncological immunotoxins, broad-spectrum antiviral agents, and highlights TPP as a critical upstream processing tool for modern biotechnology.

6.2 Future Directions and Scope

The compelling in vitro, in silico, and molecular genetic data generated in this exhaustive study establishes a robust, highly detailed foundation for advanced translational and clinical research. Future investigations must focus on:

1. Targeted Immunotoxin Conjugation: Covalently conjugating the highly purified, isolated RIPS to tumor-specific monoclonal antibodies (e.g., Trastuzumab for HER2+ breast cancer or Rituximab for lymphomas) utilizing cleavable disulfide linkers. This will create highly specific, active immunotoxins and allow for the evaluation of their efficacy in chemoresistant, refractory cell lines.

In Vivo Pharmacokinetics and Toxicity Models: Transitioning the PEGylated PLGA nanoparticle formulations to murine (mouse) xenograft models. This is critical to evaluate the true anti-tumor efficacy, establish Maximum Tolerated Doses (MTD), map systemic renal clearance rates, and comprehensively monitor the host's innate and adaptive immune responses (specifically the generatioofADAs) over prolonged treatment cycles.

1. Agricultural Biotechnology Applications: Utilizing the optimized, highly scalable TPP extraction protocols to manufacture large-scale, biodegradable botanical bio-pesticides. Future studies should evaluate the efficacy of these crude extracts against devastating agricultural viruses and fungal pathogens in controlled greenhouse settings, providing an ecofriendly alternative to highly toxic, synthetic chemical pesticides that severely degrade soil microbiomes.

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