Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Article
Volume 15, Issue 6, 2025, 81
https://doi.org/10.33263/BRIAC156.081

In Silico Characterization of *c-Sarcin* Ribotoxin from *Aspergillus clavatus* as a Potential Anticancer Agent

Dyah Wulandari [1,2,*], Diva Zahra Rahmatika [1], Alberta Rika Pratiwi [1], Lu-Sheng Hsieh [3,*], Anto Budiharjo [2,4], R. Haryo Bimo Setiarto [5], Waheni Rizki Aprilia [6], Yosa Prawiratama [2]

1 Food Technology Department, Faculty of Agricultural Technology, Soegijapranata Catholic University, Jl. Rm. Hadisoebeno Sosro Wardoyo, Semarang 50219, Indonesia; dyahwulandari@unika.ac.id; (D.W.); pratiwi@unika.ac.id (A.R.P.); divazahrarahmatika@gmail.com (D.Z.R.);
2 Molecular and Applied Microbiology Laboratory, Central Laboratory of Research and Service, Diponegoro University, Jl. Prof. Sudharto SH, Semarang 50275, Indonesia; anto.budiharjo@live.undip.ac.id ; https://orcid.org/0000-0002-4815-5138 (A.B.);
3 Department of Food Science, Tunghai University, No. 1727, Sec. 4, Taiwan Boulevard, Xitun District, Taichung 407224, Taiwan; lshsieh@thu.edu.tw (L.S.H.);
4 Biotechnology Study Program, Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Semarang, Central Java, Indonesia, 50275; anto.budiharjo@live.undip.ac.id (A.B.);
5 Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Jl. Raya Jakarta-Bogor KM. 46, KST Soekarno, Cibinong, Bogor, Indonesia,16911; rhar002@brin.go.id (R.H.B.S);
6 School of Biotechnology, Faculty of Agricultural Technology, Suranaree University of Technology, Thailand 30000; wahenira@gmail.com (W.R.A);
* Correspondence: dyahwulandari@unika.ac.id (D.W.); lshsieh@thu.edu.tw (L-S. H.);

Received: 5.05.2025; Accepted: 25.07.2025; Published: 14.11.2025

Abstract: Cancer remains the leading cause of premature death in Indonesia, with approximately 147,500 deaths annually, underscoring the urgent need for more effective therapies. Ribotoxin c-sarcin from *Aspergillus clavatus* has demonstrated potential as a therapeutic agent by irreversibly inhibiting protein synthesis and inducing apoptosis in cancer cells. In this study, recombinant c-sarcin was successfully expressed in *Komagataella phaffii* using a methanol-induction system at 30°C, achieving a peak protein yield of 48,018 mg/L on day 6 of cultivation. Molecular docking analysis using α-sarcin as a structural model revealed a strong interaction with the sarcin/ricin loop of mouse 28S rRNA, with a binding energy of -1.531 kJ/mol. Key hydrogen bonds were formed by residues Lys29, Phe94, and Pro99. Phylogenetic analysis revealed 100% sequence identity to c-sarcin from Aspergillus clavatus, indicating its functional similarity. These quantitative findings support the therapeutic relevance of c-sarcin and underscore the role of bioinformatics in accelerating the discovery of novel anticancer agents.

Keywords: Cancer; ribotoxin c-sarcin; *Aspergillus clavatus*; bioinformatics; anticancer therapy

© 2025 by the authors. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. The authors retain copyright of their work, and no permission is required from the authors or the publisher to reuse or distribute this article, as long as proper attribution is given to the original source.

1. Introduction

Cancer is among the major contributors to deaths in Indonesia, accounting for a considerable number of untimely deaths. Specifically, about one in every eleven deaths occurring before the age of 75 is due to cancer, which leads to a total of approximately 147,500 untimely deaths each year [1]. The most commonly diagnosed cancers are breast, lung, and cervical cancer. Mortality can be improved through timely diagnosis and positive action [2].

However, traditional therapies such as chemotherapy can weaken the immune system by destroying immune cells and their functions, as well as disrupting communication pathways. For this reason, improving the efficiency of cancer treatments is crucial for developing novel treatment strategies and new therapeutic options [3].

Bioinformatics has become a crucial player in the quest for new cancer drugs by utilising computer methods that accelerate drug development. By applying biological network theory, researchers can gain a deeper understanding of the intricate ways in which proteins, drug targets, and the relationships between genes and diseases interact [4]. This discipline deepens our understanding of complex biological systems while facilitating the discovery of bioactive compounds, particularly those from natural sources, including fungal organisms that serve as potential sources for identifying and discovering bioactive compounds suitable for therapeutic development [5].

The widespread occurrence of *Aspergillus clavatus* in Indonesian food products highlights a significant concern regarding mycotoxin contamination and its public health implications. This species produces several toxic metabolites, including patulin, cytochalasins, tryptoquivalines, and tryptoquivalones, well known for their roles in food spoilage and animal mycotoxicoses [6]. Importantly, its therapeutic relevance lies not in these toxins, but in ribotoxins such as α-sarcin, which are ribosome-inactivating proteins with potential applications in cancer immunotherapy. Beyond toxin production, A. clavatus is notable as a source of antibacterial compounds (ascladiol, clavatol, xanthocillin X dimethylether), as a model organism for studies on alkalitolerance, phototropism, and secondary metabolism, and as an environmentally versatile fungus found in soil, dung, cereals, and malting systems. Its impact extends further to human and animal health, being linked to extrinsic allergic alveolitis (“malt worker’s lung”) and veterinary toxicoses, underscoring its dual significance as both a health risk and a source of biotechnologically valuable metabolites [7].

As a member of the Ascomycota phylum, *A. clavatus* is most well-known for producing ribotoxin, including c-sarcin, which shows promise as a therapeutic agent in cancer, as it can inhibit protein synthesis and induce apoptosis in cancer cells. Among these, c-sarcin, a well-characterized ribotoxin produced by *A. clavatus*, has attracted interest due to its ability to inactivate the eukaryotic ribosome irreversibly has attracted interest due to its ability to irreversibly inactivate the eukaryotic ribosome by specifically cleaving a single phosphodiester bond within the sarcin-ricin loop (SRL) of the large ribosomal RNA [8].

The first members of this family (α-sarcin, restrictocin, and mitogillin) were discovered in the early 1960s as secreted proteins of *Aspergillus giganteus* and *A. restrictus* with anti-tumour activity" [(829)]. α-sarcin is a ribosome-inactivating protein (RIP) that cleaves a specific bond in the 28S rRNA, halting protein synthesis. It can translocate across membranes and selectively target ribosomes, which is essential for its cytotoxic (including antitumor) action [9]. Its cytotoxic effect has been evaluated on cultured cells (e.g., reticulocyte lysates, tumour cell lines) to demonstrate its functional impact.

Ribonuclease clavin (c-sarcin) has the same substrate specificity as alpha-sarcin. It is specific for purines in both single- and double-stranded RNA. Its toxic action on eukaryotic cells is the result of cleavage of a single phosphodiester bond in the 60S subunit of ribosomes. Furthermore, the recombinant and purified c-sarcin protein was shown to effectively inactivate ribosomes from rabbit reticulocyte lysates. This was evidenced by the generation of a characteristic RNA fragment (α-fragment), similar to that produced by α-sarcin and other ribotoxins known to inhibit protein synthesis [10]. Both the monomeric and dimeric forms of

c-sarcin exhibited strong ribonuclease activity on RNA-impregnated gels, indicating their potential for cytotoxicity through RNA degradation. C-sarcin is structurally and functionally homologous to α-sarcin and restrictocin, both of which have been previously evaluated for antiviral and anticancer applications [11,12]. These agents can be incorporated as an immunotoxin for targeted cancer therapy. The combination of ribotoxin and immunotoxin offers targeted cancer treatment with potential activity against several cancer types and may also confer resistance to traditional therapies [13,14].

Recent advances in bioinformatics tools have enabled the in silico prediction and functional annotation of protein sequences, providing a cost-effective and time-efficient approach to screening potential anticancer agents. This study utilises various bioinformatics platforms to analyse the structural and functional characteristics of c-sarcin ribotoxin from *A. clavatus*, aiming to assess its viability as a candidate for anticancer therapy. By integrating sequence analysis, structural modeling, and molecular docking simulations, we aim to predict the anticancer potential of c-sarcin and identify potential molecular interactions that underlie its therapeutic effect. The findings from this study may contribute to the foundation for future experimental validation and development of ribotoxin-based anticancer treatments. The originality of this research lies in its application of computational models to elucidate the mechanisms of c-sarcin, providing insights that can inform the development of targeted cancer therapies. This study aimed to predict the effects of c-sarcin on cancer cells and explore its potential as a component of immunotoxins for targeted cancer treatment.

2. Materials and Methods

2.1. Sources of reagents and materials.

The following reagents were obtained from Bio-Rad (Hercules, CA, USA): protein dye-binding reagents, DNA size ladders, molecular mass protein standards, and reagents for SDS-PAGE. SeaKem® LE Agarose was sourced from Lonza (Morristown, NJ, USA). Restriction enzymes EcoRI, NotI, and SacI were acquired from Takara Bio (San Jose, CA, USA). Geneticin™ Selective Antibiotic, Pierce™ biotin, agar powder, dextrose, peptone, yeast extract, and yeast nitrogen base were purchased from Cyrusbioscience (New Taipei, Taiwan).

2.2. Cloning and expression vector construction.

The coding sequence for ribotoxin c-sarcin from *A. clavatus* (GenBank accession No. U48731.1) was inserted into the pPIC9K plasmid between the *EcoRI* and *NotI* restriction sites, generating the pPIC9K-cSAR expression vector. The c-sarcin gene was amplified by PCR using gene-specific primers containing EcoRI and NotI restriction sites. The forward primer was designed as 5´- GACTGGTTCCAATTGACAAGC-3´, and the reverse primer was designed as 5´-GACTGGTTCCAATTGACAAGC-3´. The validity of the construct was confirmed by EcoRI and NotI digestion, agarose gel electrophoresis, and sequencing [15,16]. *E. coli* DH5α was employed for propagating plasmids, while *Komagataella phaffii* X-33 functioned as the host for expressing and producing the c-sarcin protein.

2.3. Electroporation and selection of K. phaffii transformants.

Electrocompetent *K. phaffii* cells were generated following the manufacturer's instructions from the EasySelect™ Pichia Expression Kit (Invitrogen, USA). pPIC9K-cSAR

vector was linearised with SacI, and approximately 10 µL of the linearised plasmid was introduced into 80 µL of the cells. The transformed cells were spread onto YPD plates (1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar) containing 1.0, 2.0, and 3.0 mg/mL of geneticin. Successfully transformed colonies were then transferred to minimal dextrose (MD) or minimal methanol (MM) plates for further screening and incubated at 30°C for 48 hours.

2.4. Protein expression and production.

K. phaffii transformants were cultivated on MM plates and inoculated into 50 mL of buffered glycerol-complex (BMGY) medium (1% yeast extract, 2% peptone, 1.34% YNB, 4× 10–5% biotin, 1% glycerol, 100 mM potassium phosphate buffer, pH 6.0) at 30°C for 16 h. After centrifugation at 8000×g for 5 min, the cells were resuspended in 250 mL of buffered methanol-complex (BMMY) medium (1% yeast extract, 2% peptone, 1.34% YNB, 4× 10–5% biotin, 0.5% methanol, 100 mM potassium phosphate buffer, pH 6.0) and cultured at 20°C or 30°C for 5 days, with methanol (1%, v/v) added daily for protein expression induction. Protein samples were analysed using SDS-PAGE, and concentrations were determined by measuring absorbance at 260/280 nm with a NanoDrop 2000 spectrophotometer. Graphs of the mean absorbance against known concentrations were plotted.

2.5. Sequence confirmation and phylogenetic analysis.

DNA sequencing was conducted at Tri-Biotech in New Taipei, Taiwan. The sequence was analyzed using BLAST to determine the base pair match percentage with the reference isolate from the gene bank. Multiple sequence alignments of the FASTA sequences were performed using MEGA X software, followed by phylogenetic analysis. Protein isolate sequences were compared with other proteins using a neighbour-joining tree and the bootstrap method.

2.6. Anticancer activity analysis.

Molecular docking was conducted using the automated docking tool, ClusPro 2.0 web server. The optimal protein complex was selected based on the lowest energy score among all the generated clusters or poses. Utilising the PDBsum tool facilitated the identification of amino acid residue interactions within the protein chain [17].

3. Results and Discussion

3.1. Amino acid sequence analysis of ribotoxin c-sarcin.

Computational analyses revealed key molecular characteristics of c-sarcin, a 374-amino-acid ribotoxin that has been extensively characterized at the protein and physicochemical levels. The protein exhibits a theoretical isoelectric point (pI) of 6.74, indicating that it remains nearly charge-neutral under physiological pH conditions. Its amino acid composition shows a relative enrichment of negatively charged residues (Asp + Glu) compared to positively charged residues (Arg + Lys), with alanine (10.2%), leucine (8.8%), and lysine (6.4%) as the most abundant contributors to overall composition, collectively accounting for its near-neutral pI. A high aliphatic index (78.34) implies strong thermostability, which is further corroborated by an instability index (II) of 27.22, indicating that the protein is stable. In addition, its GRAVY score of –0.295 reflects hydrophilic properties consistent with

reported aqueous solubility. Together, these physicochemical features underscore c-sarcin's structural stability and functional robustness as a ribotoxin, supporting further investigation into its mechanistic and therapeutic applications. From a biophysical perspective, the pI plays a critical role in modulating intermolecular interactions. At pH values near the isoelectric point, the protein assumes a neutral net charge, thereby minimizing electrostatic repulsion and potentially facilitating closer intermolecular contacts. In eukaryotic systems, interaction patterns are strongly influenced by pI distribution: acidic proteins typically exhibit higher interaction kinetics, whereas basic proteins display comparatively reduced interaction frequencies across diverse biological contexts. These findings emphasize the importance of pI in defining protein–protein interaction dynamics and provide a framework for understanding c-sarcin's molecular behavior in complex cellular environments [18,19].

The amino acid sequence analysis of c-sarcin, a well-characterised ribotoxin produced by *A. clavatus*, provides important insights into its catalytic mechanism, structural integrity, and evolutionary context [3]. Ribotoxins are a subclass of ribonucleases that specifically cleave a phosphodiester bond within the sarcin/ricin loop (SRL) of the 28S rRNA, irreversibly inhibiting protein synthesis and inducing apoptosis in eukaryotic cells [14]. Our sequence analysis confirms the presence of highly conserved catalytic residues, notably histidine and glutamic acid residues, critical for enzymatic activity. In particular, His50 and Glu96 (numbering based on the c-sarcin mature peptide) are preserved across the ribotoxin family and are predicted to participate in general acid-base catalysis during RNA cleavage. These findings are consistent with previous mutagenesis studies, which demonstrate that alteration of these residues leads to a complete loss of activity. Their conservation underscores the evolutionary pressure to maintain an efficient catalytic core essential for ribotoxin function [13,14].

Sequence analysis also highlights the presence of three intramolecular disulfide bridges, a hallmark of ribotoxins that contributes to their remarkable thermal and proteolytic stability. These disulfide bonds not only stabilise the tertiary structure but also likely play a role in the precise positioning of catalytic residues [13,20]. The conserved cysteine residues involved in disulfide bridge formation suggest a rigid and well-maintained structural scaffold critical for biological activity. The amino acid composition of c-sarcin exhibits a predominance of basic residues, resulting in a highly cationic surface. This property facilitates interaction with the negatively charged phosphodiester backbone of rRNA and may also enhance affinity for the ribosomal surface. This electrostatic complementarity is essential for the ribotoxin's selective binding to the SRL, a feature that distinguishes it from non-specific RNases [14,21].

Phylogenetic comparisons reveal high sequence homology between c-sarcin and other fungal ribotoxins, particularly restrictocin and α-sarcin [13]. The evolutionary conservation of functional domains, coupled with variability in peripheral regions, supports the hypothesis that ribotoxins have diverged to accommodate different ecological niches or host interactions while retaining a conserved mechanism of action. Given its stability and specificity, c-sarcin represents a promising scaffold for developing targeted cytotoxic agents, such as immunotoxins [14]. However, its intrinsic cytotoxicity necessitates engineered modifications to enhance selectivity and reduce systemic toxicity. Targeted delivery strategies, including fusion with tumour-specific antibodies or ligand peptides, are being explored to harness the ribotoxin's potency in a controlled therapeutic context [21].

3.2. Characterisation of the pPIC9K-cSAR expression construct.

The ppic9k-CSR construct, which was deliberately designed for this study, is positioned between the EcoRI and NotI restriction sites (Figure 1). The expression gene weighs 454 kilobases (kb) in molecular weight. In contrast, the rest of the plasmid is approximately 9.270 kb in length, resulting in a total size of 9.724 kb for the pPIC9K-cSAR construct. Verifying ribotoxin c-sarcin expression vector for the *P. pastoris* system involves key steps. First, the ribotoxin c-sarcin gene must be cloned into a suitable vector, such as pPIC9k, which includes the AOX1 promoter, the secretion signal factor-α, and selection markers for identifying transformed cells. These components are fundamental to achieving successful protein expression and secretion in *P. pastoris*. A thorough verification process ensures the accurate integration of these elements, optimising c-sarcin expression [22]. Once the gene sequence has been incorporated, it is essential to verify its accuracy and identify any accidental variants. Furthermore, it is crucial to verify the presence of key components within the vector, as these elements significantly influence protein production and gene expression [23].

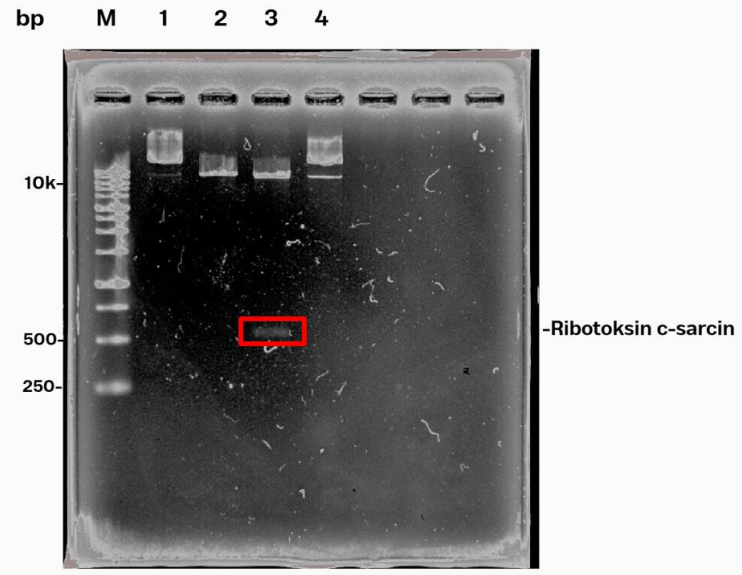


Figure 1. Agarose gel electrophoresis of restriction digests confirmed the pPIC9K-c-sarcin construct. Lanes show undigested vector (1), EcoRI digest (2), EcoRI/NotI digest (3), and SacI digest (4). M: 1 kb marker.

3.3. Protein concentration.

The production pattern of c-sarcin was examined extensively during 7-day cultivation at 30°C with methanol (1%, v/v) as an inducer (Figure 2). Time series analysis revealed a fluctuating pattern of production, starting with a level of 33,401 mg/L on day 0, then decreasing to 17,810 mg/L on day 2. This was followed by a significant spike, reaching a peak level of 48,018 mg/L on day 6, before gradually decreasing to 45,773 mg/L on day 7. This pattern suggests the potential onset of the stationary phase or a reduction in protein production capacity over time. Thorough knowledge of the c-sarcin production profile is critical to its biosynthesis at both industrial and laboratory levels. The protein concentration of the ribotoxin may vary depending on the culture conditions and purification methods used. Li and Xia [24] reported the effective development and refinement of a protocol for expressing the heterologous fungal ribotoxin hirsutellin A (HtA) in the yeast strain *P. pastoris*. Their study involved a detailed characterisation of the resulting ribotoxin.

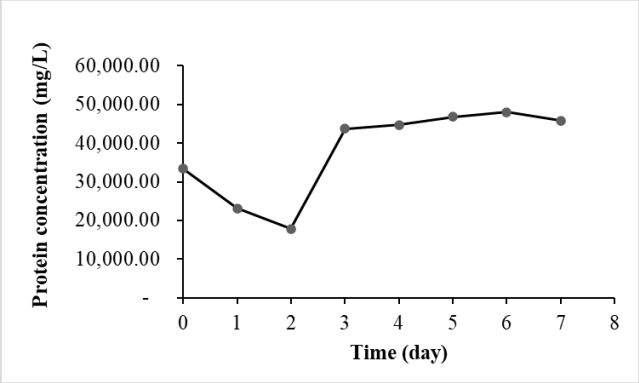


Figure 2. Crude extracts from cultures incubated at 30°C with 1% methanol were used to measure ribotoxin c-sarcin concentration over a period of seven days.

The authors produced the protein HtA in *P. pastoris* using a high-cell-density fermentation system, optimising the expression system to achieve high yields of the ribotoxin. Eventually, the optimised system allowed the expression of HtA up to a concentration of 80 mg/L [24]. The therapeutic efficacy of ribotoxin across various species and its potential medical applications are highly dependent on its concentration. When ribotoxin is conjugated with specific antibodies, such as RIP type 2 nigrin b combined with a CD105 antibody linked to an anti-CD105 antibody, their immunotoxin performance is significantly enhanced. These conjugates enable precise, targeted cytotoxicity at considerably lower concentrations than ribotoxin alone. This delivery system increases their therapeutic potency and the quantity needed for clinical use. These advancements are among the most significant developments in medical technology, significantly enhancing the precision and efficacy of ribotoxin-based cancer therapeutics [25].

3.4. SDS-PAGE analysis of c-sarcin expression.

The SDS-PAGE analysis successfully demonstrated the expression of the c-sarcin protein in the chosen expression system. SDS-PAGE tests revealed protein bands between 15–18 kDa in all samples from day 0 to day 7 (Figure 3). Notably, the intensity of the c-sarcin band remained consistent throughout the incubation period, demonstrating its stability under the experimental conditions. The tests also identified several higher molecular weight bands associated with host proteins. Methanol induction and incubation time in *P. pastoris* have a significant influence on ribotoxin expression levels. As a eukaryotic expression system, *P. pastoris* can make key post-translational changes, which help to boost recombinant protein yields. The AOX1 gene promoter enables controlled expression through methanol induction, thereby regulating the metabolic processes that produce ribotoxin. To cut toxicity and increase yields, scientists must fine-tune methanol levels and incubation time [26]. Studies show that *P. pastoris* produces the most heterologous protein at 2.5% methanol concentration and when induced for 48 hours [27]. These results highlight the importance of maintaining well-controlled environmental conditions for maximising recombinant protein production yields.

Additionally, the SDS-PAGE profile revealed other protein bands, representing host cell proteins, indicating that the expressed c-sarcin was present in a complex protein mixture [22]. If a purification step (such as Ni-NTA affinity chromatography for His-tagged c-sarcin) were performed, a cleaner band corresponding to c-sarcin would further confirm its identity and purity [23].

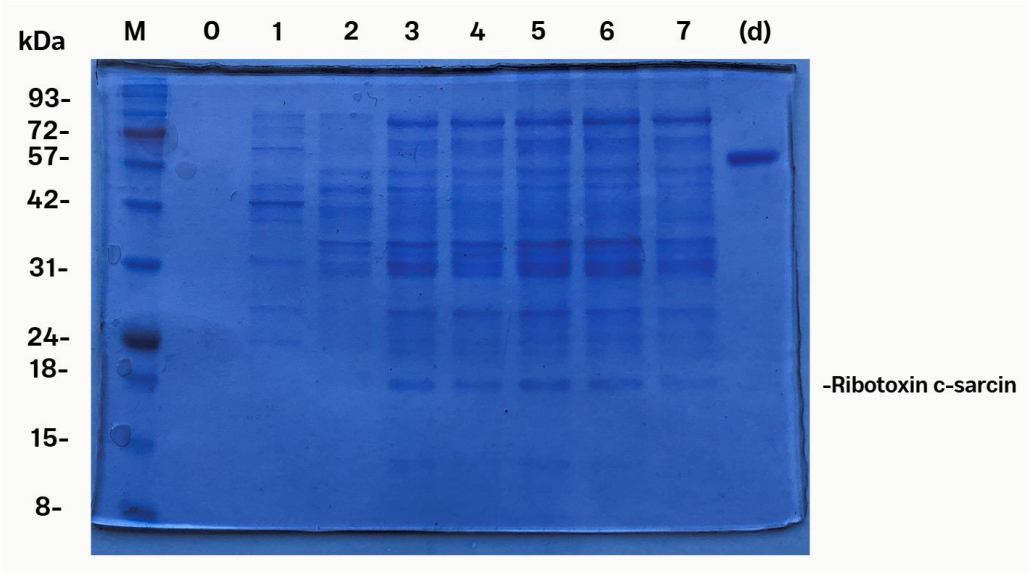


Figure 3. SDS-PAGE analysis of c-sarcin (17 kDa) expression and stability during a 7-day incubation with methanol.

In this case, if the protein band appeared at the expected size in the eluted fractions, it would demonstrate successful purification [24]. It is also important to note the solubility of the expressed protein. If the c-sarcin band was primarily present in the insoluble fraction, it may suggest the formation of inclusion bodies, which would require additional steps such as solubilization and refolding [25]. Conversely, strong bands in the soluble fraction would be favourable for downstream applications and functional assays. The SDS-PAGE analysis indicates successful expression of c-sarcin, with the presence of a prominent protein band at the expected molecular weight in the induced sample. Further confirmation through Western blotting or mass spectrometry could verify the identity of the expressed protein. Optimising expression conditions, such as temperature, inducer concentration, and host strain, could further enhance yield and solubility for future studies [26].

3.5. Phylogenetic tree.

The new isolate, *A. clavatus*, is also most evolutionarily related to the type strain, with a bootstrap value of 100%, confirming its taxonomic status (Figure 4). It clusters with the other Aspergillus species (*A. acanthosporus*, *A. giganteus*, *A. rhizopodus*), showing a common evolutionary origin of ribotoxin c-sarcin. Certain Penicillium species, such as *P. spinulosum* and *P. digitatum*, are also closely related, suggesting evolutionary relationships with Aspergillus ribotoxin. Phylogenetic analysis reveals rich diversification in Aspergillus, where well-developed branches, such as *A. felis* and *A. fumigatus*, have been identified. Phylogenetic analysis validates the phylogenetic relationships between these species and the potential conservation of ribotoxin function. Protein sequence alignment of the new isolate with the protein sequence of α-sarcin of *A. giganteus* (CAA43180.1) by NCBI BLAST revealed high similarity, with 87-93% identity and without any gaps of significance. This highlights their structural and functional homologies, suggesting equivalent functions and likely shared cytotoxic activities.

The evolutionary history was inferred using the Neighbor-Joining method [28]. The bootstrap consensus tree inferred from 1000 replicates [29] is used to represent the evolutionary history of the analysed taxa [29].

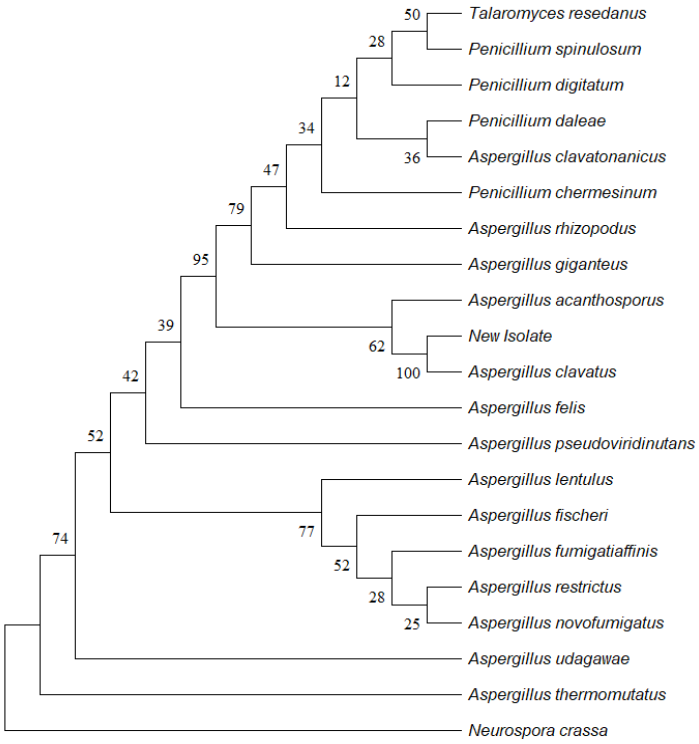


Figure 4. A phylogenetic tree using Neurospora crassa as an outgroup demonstrates a close evolutionary relationship between c-sarcin from *A. clavatus* and other Aspergillus and Penicillium ribotoxins. High bootstrap values support the robustness of the tree.

Branches corresponding to partitions that are reproduced in fewer than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [29]. The evolutionary distances were computed using the Poisson correction method [30] and are expressed in units of amino acid substitutions per site. This analysis involved 21 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The final dataset comprised a total of 224 positions. Evolutionary analyses were conducted in MEGA11 [31].

Ribotoxin c-sarcin from *A. clavatus* demonstrates a substantial similarity to α-sarcin from *A. giganteus* with a particular emphasis on their conserved amino acid sequences. Although the coding sequences are identical, the intron sequences differ, with an overall similarity of 34% observed. The removal of polyadenine from c-sarcin intron enhances the similarity to 83%. This implies that despite the differences in non-coding sequences, ribotoxin is a highly conserved functional element. This analogy highlights that ribotoxin remains the primary cytotoxic agent, and the illness will be addressed through its antigens [32].

Ribotoxin α-sarcin and c-sarcin, part of the fungal ribotoxin family, are known for their ability to precisely focus on the sarcin-ricin loop within the large ribosomal subunit. These ribotoxins act as highly specific endoribonucleases that catalyse the hydrolytic cleavage of a conserved phosphodiester bond within the sarcin–ricin loop (SRL) of 28S ribosomal RNA. This modification prevents the proper binding and function of elongation factors EF-1 and EF-2, thereby irreversibly arresting protein translation. The resulting translational blockade activates stress-response signalling cascades and ultimately triggers apoptosis through both intrinsic and extrinsic pathways. Notably, α-sarcin and c-sarcin have emerged as promising therapeutic candidates due to their capacity to inactivate ribosomes and induce programmed cell death selectively. Moreover, their ability to recognise and bind to overexpressed cell-

surface receptors on malignant cells suggests a mechanism for tumour-specific targeting, potentially enabling the selective eradication of cancerous tissues while sparing normal cells. This tumour tropism significantly strengthens their potential as molecular tools for targeted cancer therapy [33].

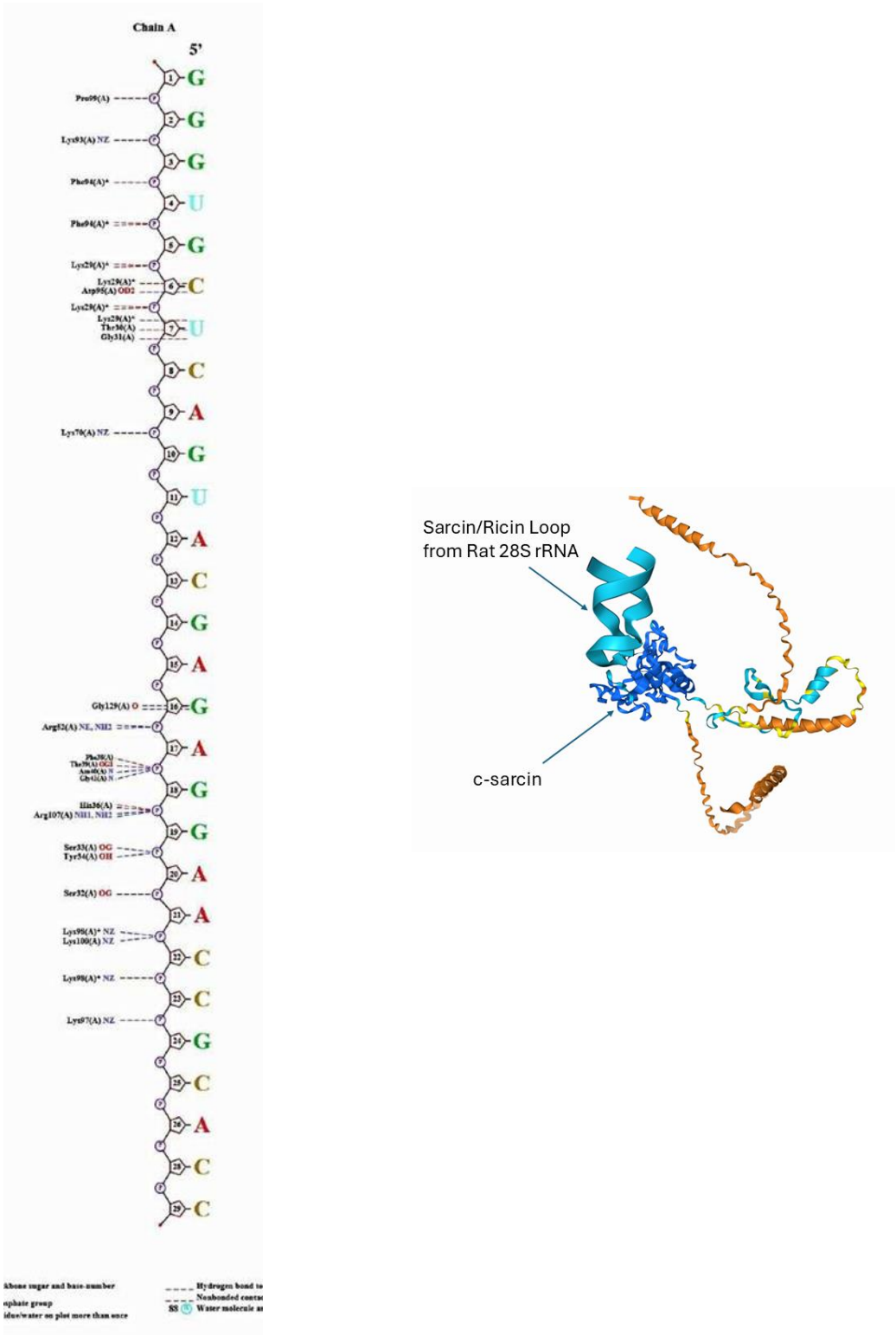
3.6. Molecular docking reveals key interactions between C-sarcin and 28S rRNA.

Ribosome-inactivating proteins (RIPs) are toxins that inhibit protein synthesis by catalytically modifying ribosomal RNA (rRNA). The fungal RIP C-sarcin is a prototypical example, cleaving a single phosphodiester bond within the conserved sarcin–ricin loop (SRL) of 28S rRNA, thereby blocking elongation factor binding and stalling translational elongation [32]. Its high specificity and catalytic efficiency have motivated detailed investigations of the molecular determinants governing SRL recognition. Molecular docking provides a computational framework for modeling these interactions and predicting the binding orientation of C-sarcin on the SRL [33]. Functionally, the SRL is indispensable for elongation factor–dependent GTP hydrolysis, and its structural integrity is critical for ribosomal activity. C-sarcin cleaves a specific bond between G4325 and A4326 (human numbering) in the 28S rRNA, effectively halting protein synthesis. Unlike ricin, which depurinates the SRL, C-sarcin acts as a site-specific endoribonuclease. Given the essential and conserved nature of the SRL, understanding how C-sarcin interacts with this region is key to grasping its high specificity and potential toxicity [34,35].

In this study, we conducted a molecular docking analysis of the ribotoxin, focusing on its interaction with the sarcin/ricin loop of mouse 28S ribosomal RNA (rRNA) (Figure 5). Due to the lack of a Protein Data Bank (PDB) file for c-sarcin, we used α-sarcin from *A. giganteus* as a substitute, based on the structural similarity noted by Huang *et al.* in 1997 [32]. Our analysis revealed specific interactions between α-sarcin and the rRNA, including contacts with the backbone phosphates and nucleotide bases, which contribute to its recognition and catalytic activity. The binding energy was calculated to be -1.531 kJ/mol, indicating a strong interaction. Key residues, such as Lys29, Phe94, and Pro99, played a crucial role in forming hydrogen bonds, with Lys29 engaging in four interactions with the RNA phosphate backbone.

Several amino acid residues, such as Lys29, Phe94, and Pro99, were identified as key components in hydrogen bond formation, with Lys29 appearing four times, indicating its crucial role in electrostatic interactions with the RNA phosphate backbone. Additionally, the analysis revealed the presence of 88 water molecules acting as mediators in these interactions. Non-bonded contacts detected at distances less than 3.35 Å suggest strong potential interactions between α-sarcin and rRNA, providing further insights into the specific binding mechanism and the resulting toxic effects of α-sarcin.

Recent studies have shown that several lysine residues, particularly in loop 3, play a crucial role in forming stable interactions between α-sarcin and rRNA. Lysine residues 111, 112, and 114 have been identified as key contributors to this specific recognition process, enabling α-sarcin to interfere with protein synthesis [34] effectively. Additionally, the NH_2-terminal β-hairpin region of α-sarcin contains several basic residues, primarily lysines, which are important for determining the overall charge and structural stability of the protein. The presence of these basic residues not only contributes to the electrostatic properties of the protein but also influences its interactions with other molecules, including ribosomal proteins and cellular membranes.



(a) **(b)**

Figure 5. (a) The interaction between the rat 28S rRNA sarcin/ricin loop with ribotoxin α-sarcin; **(b)**Three-dimensional structure binding between the rat 28S rRNA sarcin/ricin loop with c-sarcin.

The structural stability of the resulting β-hairpin enables α-sarcin to maintain the conformation necessary for its binding process and biological activity. This distribution enhances the protein's ability to interact specifically with ribosomal proteins that play critical roles in protein synthesis. Moreover, the β-hairpin region also interacts with the cell membrane, influencing various cellular processes, including membrane binding and transport. The structural determinants of α-sarcin mediate highly specific interactions with the sarcin–ricin loop of 28S rRNA, emphasizing its critical role in modulating ribosomal conformational

dynamics and rRNA tertiary structure. Such interactions not only perturb local folding and stability of the SRL but may also alter long-range allosteric communication within the ribosome, ultimately compromising ribosomal integrity and translational fidelity [35].

Eighty-eight water molecules were identified as integral mediators stabilizing α-sarcin–rRNA interactions. α-Sarcin exerts toxicity by specifically cleaving the sarcin/ricin loop (SRL) of 28S rRNA, thereby abolishing elongation factor binding and halting translation. Stable complex formation is driven by electrostatic interactions involving loop 3 lysines (Lys111, Lys112, Lys114), which anchor the toxin to the SRL backbone [34]. The NH_2-terminal β-hairpin contributes additional lysine-mediated charge stabilization, enhancing affinity for rRNA and mediating contacts with ribosomal proteins and membranes [22]. Structural modeling with AlphaFold3 demonstrated precise docking of c-sarcin at the SRL, with catalytic residues oriented toward the G4325–A4326 phosphodiester bond, enabling nucleophilic attack and site-specific cleavage [35].

Critical residues on C-sarcin, including histidine and aspartate residues, form hydrogen bonds with the phosphate backbone and bases near the cleavage site, stabilising the enzyme-substrate complex [32,33]. Aromatic residues in C-sarcin participate in π-stacking interactions with the purine bases of rRNA, while positively charged residues such as lysine or arginine enhance binding through electrostatic attraction to the negatively charged RNA backbone [34]. The docking simulations indicate that induced fit may occur, with slight conformational adjustments in both the SRL and C-sarcin to optimise binding interactions and catalytic alignment [35]. Sequence and structural conservation of the SRL across eukaryotes suggests a broad potential efficacy of C-sarcin, supporting its role as a natural defence mechanism in fungi [34,35].

Understanding the molecular docking of C-sarcin to 28S rRNA not only enhances our knowledge of ribosome inactivation mechanisms but also opens avenues for therapeutic design. For instance, modified versions of C-sarcin could be engineered for use in targeted cancer therapies, exploiting tumour-specific delivery systems [32]. Additionally, insights from this interaction can inform the development of small molecules that mimic C-sarcin binding, potentially serving as novel antibiotics or antitumor agents [33,35]. Molecular docking provides a powerful tool for examining the interaction between C-sarcin and the 28S rRNA. The precise alignment, hydrogen bonding, and electrostatic interactions revealed through docking simulations underscore the specificity and efficiency of this ribotoxin. These findings not only enrich our understanding of ribosome-targeting toxins but also suggest new frontiers in drug discovery and therapeutic engineering. As computational models become increasingly accurate, the integration of docking with molecular dynamics and experimental validation will continue to advance our knowledge of RNA-protein interactions at the molecular level [34,35].

4. Conclusions

Genetic engineering techniques were employed to facilitate the expression of ribotoxin c-sarcin from *A. clavatus* in a methanol-induced system, grown at 30°C. Optimization was done to achieve a maximum protein concentration of 48,018 mg/L. Phylogenetic tree construction revealed that α-sarcin shares close evolutionary kinship with other Aspergillus species, specifically *A. acanthosporus*, *A. giganteus*, and *A. rhizopodus*, indicating a shared evolutionary lineage. Additionally, certain species of Penicillium, specifically *P. spinulosum* and *P. digitatum*, exhibited genetic relationships with the Aspergillus group. These findings

highlight the therapeutic applications of ribotoxin and our understanding of the evolutionary processes and mechanisms of ribotoxin.

Author Contributions

Conceptualization, D.W. and L.-S.H.; methodology, D.W., A.R.P., L.-S.H., and A.B.; data curation, D.Z.R., W.R.A., and Y.P.; formal analysis, D.W., A.B., R.H.B.S., W.R.A., and Y.P.; writing—original draft preparation, D.W., A.B., R.H.B.S., W.R.A., and Y.P.; writing—review and editing, D.W., A.B., R.H.B.S., W.R.A., and Y.P. All authors contributed equally to this work and have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

Funding

This research was funded by (i) PPS-PTM BIMA 2025: Pengaruh Ribotoksin c-sarcin dari *Aspergillus clavatus* terhadap Viabilitas Sel Kanker Hati HepG2, Grant Number: 127/C3/DT.05.00/PL/2025, (ii) RPI: Diversity of Entomopathogenic Fungi and Their Biotechnological Potential Application in Agriculture, Grant Number 185-80/UN7.6.1/PP/2022. (iii) Diponegoro University for the World Class University Program, Indonesia Endowment Fund for Education 2024 61/UN7.A/HK/XII/2024.

Acknowledgments

The authors gratefully acknowledge Tunghai University for providing access to its laboratory facilities and essential resources, which supported the completion of this research.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. OECD. Tackling the Impact of Cancer on Health, the Economy and Society, OECD Health Policy Studies. OECD Publishing: Paris, **2024**; https://doi.org/10.1787/85e7c3ba-en.
2. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *C CA Cancer J. Clin.* **2021**, *71*, 209–249, https://doi.org/10.3322/caac.21660.
3. Sharma, A.; Jasrotia, S. Kumar, A. Effects of Chemotherapy on the Immune System: Implications for Cancer Treatment and Patient Outcomes. *Naunyn Schmiedebergs Arch. Pharmacol.* **2024**, *397*, 2551-2566, https://doi.org/10.1007/s00210-023-02781-2.

4. Li, K.; Du, Y.; Li, L.; Wei, D.-Q. Bioinformatics Approaches for Anti-cancer Drug Discovery. *Curr. Drug Targets* **2020**, *21*, 3–17, https://doi.org/10.2174/1389450120666190923162203.
5. Olombrada, M.; Medina, P.; Budia, F.; Gavilanes, J.G.; Martínez-del-Pozo, Á.; García-Ortega, L. Characterization of a new toxin from the entomopathogenic fungus Metarhizium anisopliae: the ribotoxin anisoplin. *Biol. Chem.* **2017**, *398*, 135–142, https://doi.org/10.1515/hsz-2016-0119.
6. Pitt, J.I.; Hocking, A.D. Front Matter. In Fungi and Food Spoilage, 3rd Edition; Springer: New York, US, **2009**; pp. i-xv.
7. Varga, J.; Due, M.; Frisvad, J. C.; Samson, R. Taxonomic revision of Aspergillus section Clavati based on molecular, morphological and physiological data. *Stud. Mycol.* **2007**, *59*, 89-106, https://doi.org/10.3114/sim.2007.59.11.
8. Olson, B.H.; Goerner, G.L. Alpha-sarcin, a new antitumor agent, I. Isolation, puri®cation, chemical composition, and the identity of a new amino acid. *Appl. Microbiol.* **1965**, *13*, 314-321, https://doi.org/10.1128/am.13.3.314-321.1965.
9. Martínez-Ruiz, A.; Kao, R.; Davies, J.; del Pozo, Á. M. Ribotoxins are a more widespread group of proteins within the filamentous fungi than previously believed. *Toxicon* **1999**, *37*, 1549-1563, https://doi.org/10.1016/s0041-0101(99)00103-8.
10. Huang, K. C.; Hwang, Y. Y.; Hwu, L.; Lin, A. Characterization of a new ribotoxin gene (c-sar) from Aspergillus clavatus. *Toxicon* **1997**, *35*, 383-392, https://doi.org/10.1016/s0041-0101(96)00170-5.
11. Almela, M. J.; Gonzale, M. E; and Carrasco, L. Inhibitors of poliovirus uncoating efficiently block the early membrane permeabilization induced by virus particles. *J. Virol.* **1991**, *65*, 2572-2577, https://doi.org/10.1128/jvi.65.5.2572-2577.1991.
12. Wawrzynczak, E.J.; Henry, R.V.; Cumber, A.J.; Parnell, G.D. Biochemical, cytotoxic and pharmacokinetic properties of an immunotoxin composed of a mouse monclonal antibody Fib 75 and the ribosome-inactivating protein alpha-sarcin from *Aspergillus giganteus*. *Eur. J. Biochem* **1991**, *196*, 203-209, https://doi.org/10.1111/j.1432-1033.1991.tb15805.x.
13. Citores, L.; Ragucci, S.; Ferreras, J.M. Di Maro, A.; Iglesias, R. Ageritin, a Ribotoxin from Poplar Mushroom (*Agrocybe aegerita*) with Defensive and Antiproliferative Activities. *ACS Chem. Biol.* **2019**, *14*, 1319–1327. https://doi.org/10.1021/acschembio.9b00291.
14. Varga, J.; Samson, R.A. Ribotoxin genes in isolates of Aspergillus section Clavati. *Antonie van Leeuwenhoek* **2008**, *94*, 481–485, https://doi.org/10.1007/s10482-008-9266-7.
15. Lee, P.Y.; Costumbrado, J.; Hsu, C-Y.; Kim, Y.H. Agarose gel electrophoresis for the separation of DNA fragments. *J. Vis. Exp.* **2012**, *62*, e3923, https://doi.org/10.3791/3923.
16. Hsieh, C.-Y.; Hsieh, L.-S. Cloning of Three Cytokinin Oxidase/Dehydrogenase Genes in Bambusa oldhamii. *Curr. Issues Mol. Biol.* **2023**, *45*, 1902–1913, https://doi.org/10.3390/cimb45030123.
17. Laskowski, R.A. PDBsum1: A standalone program for generating PDBsum analyses. *Protein Sci.* **2022**, *31*, e4473, https://doi.org/10.1002/pro.4473.
18. Dumetz, A.C.; Chockla, A.M.; Kaler, E.W.; Lenhoff, A.M. Effects of pH on protein–protein interactions and implications for protein phase behavior. *Biochim. Biophys. Acta, Proteins Proteom.* **2008**, *1784*, 600–610, https://doi.org/10.1016/j.bbapap.2007.12.016.
19. Chasapis, C.T.; Konstantinoudis, G. Protein isoelectric point distribution in the interactomes across the domains of life. *Biophys. Chem.* **2020**, *256*, 106269, https://doi.org/10.1016/j.bpc.2019.106269.
20. Yueh, C.; Hall, D.R.; Xia, B.; Padhorny, D.; Kozakov, D.; Vajda, S. ClusPro-DC: Dimer Classification by the Cluspro Server for Protein–Protein Docking. *J. Mol. Biol.* **2017**, *429*, 372–381, https://doi.org/10.1016/j.jmb.2016.10.019.
21. Jones, G.; Jindal, A.; Ghani, U.; Kotelnikov, S.; Egbert, M.; Hashemi, N.; Vajda, S.; Padhorny, D.; Kozakov, D. Elucidation of protein function using computational docking and hotspot analysis by ClusPro and FTMap. *Struct. Biol.* **2022**, *78*, 690–697, https://doi.org/10.1107/S2059798322002741.
22. Kukk, K. Assembly of pAOXHygR vector for protein expression in the yeast Pichia pastoris. **2024**, https://dx.doi.org/10.17504/protocols.io.kxygx9pdzg8j/v1.
23. Logez, C.; Alkhalfioui, F.; Byrne, B.; Wagner, R. Preparation of Pichia pastoris Expression Plasmids. In Recombinant Protein Production in Yeast: Methods in Molecular Biology; Bill, R., Ed.; Humana Press: Totowa, US, **2012**; Volume 866, pp. 25–40, https://doi.org/10.1007/978-1-61779-770-5_3.
24. Li, H.; Xia, Y. High cell density fed-batch production of insecticidal recombinant ribotoxin hirsutellin A from *Pichia pastoris*. *Microb. Cell Factories* **2018**, *17*, 145, https://doi.org/10.1186/s12934-018-0992-x.
25. Muñoz, R.; Arias, Y.; Ferreras, J.M.; Rojo, M.A.; Gayoso, M.J.; Nocito, M.; Benitez, J.; Jiménez, P.;

Bernabéu, C.; Girbés, T. Targeting a marker of the tumor neovasculature using a novel anti-human CD105-immunotoxin containing the non-toxic type 2 ribosome-inactivating protein nigrin b. *Cancer Lett.* **2007**, *256*, 73–80, https://doi.org/10.1016/j.canlet.2007.05.012.
26. Türkanoğlu Özçelik, A; Yılmaz, S.; Inan, M. *Pichia pastoris* Promoters. In Recombinant Protein Production in Yeast: Methods in Molecular Biology. Gasser, B., Mattanovich, D., Eds.; Humana Press: New York, US, **2019**; Volume 1923, pp. 97–112, https://doi.org/10.1007/978-1-4939-9024-5_3.
27. Santoso, A.; Herawati, N.; Rubiana, Y. Effect of Methanol Induction and Incubation Time on Expression of Human Erythropoietin in Methylotropic Yeast Pichia Pastoris. *Makara J. Technol.* **2012**, *16*, 29-34, https://doi.org/10.7454/mst.v16i1.1041.
28. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406-425, https://doi.org/10.1093/oxfordjournals.molbev.a040454.
29. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783-791, https://doi.org/10.1111/j.1558-5646.1985.tb00420.x.
30. Zuckerkandl, E.; Pauling, L. Evolutionary Divergence and Convergence in Proteins. In Evolving Genes and Proteins, Bryson, V., Vogel, H.J., Eds.; Academic Press: **1965**; pp. 97-166 https://doi.org/10.1016/B978-1-4832-2734-4.50017-6.
31. Tamura, K.; Stecher, G.; Kumar, S. MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022-3027, https://doi.org/10.1093/molbev/msab120.
32. Lin, A.; Huang, K-C.; Hwu, L.; Tzeab, S.S. Production of type II ribotoxins by Aspergillus species and related fungi in Taiwan. *Toxicon* **1995**, *33*, 105-110, https://doi.org/10.1016/0041-0101(94)00140-4.
33. Masip, M.; Lacadena, J.; Mancheño, J.M.; Oñaderra, M.; Martínez-Ruiz, A.; Martínez del Pozo, Á.; Gavilanes, J.G. Arginine 121 is a crucial residue for the specific cytotoxic activity of the ribotoxin α-sarcin. *Eur. J. Biochem.* **2001**, *268*, 6190–6196, https://doi.org/10.1046/j.0014-2956.2001.02566.x.
34. Castaño-Rodríguez, C.; Olombrada, M.; Partida-Hanon, A.; Lacadena, J.; Oñaderra, M.; Gavilanes, J.G.; García-Ortega, L.; Martínez-del-Pozo, Á. Involvement of loops 2 and 3 of α-sarcin on its ribotoxic activity. *Toxicon* **2015**, *96*, 1–9, https://doi.org/10.1016/j.toxicon.2015.01.007.
35. Álvarez-García, E.; Martínez-del-Pozo, Á.; Gavilanes, J.G. Role of the basic character of α-sarcin's NH_2-terminal β-hairpin in ribosome recognition and phospholipid interaction. *Arch. Biochem. Biophys.* **2009**, *481*, 37–44, https://doi.org/10.1016/j.abb.2008.10.012.

Publisher's Note & Disclaimer

The statements, opinions, and data presented in this publication are solely those of the individual author(s) and contributor(s) and do not necessarily reflect the views of the publisher and/or the editor(s). The publisher and/or the editor(s) disclaim any responsibility for the accuracy, completeness, or reliability of the content. Neither the publisher nor the editor(s) assume any legal liability for any errors, omissions, or consequences arising from the use of the information presented in this publication. Furthermore, the publisher and/or the editor(s) disclaim any liability for any injury, damage, or loss to persons or property that may result from the use of any ideas, methods, instructions, or products mentioned in the content. Readers are encouraged to independently verify any information before relying on it, and the publisher assumes no responsibility for any consequences arising from the use of materials contained in this publication.