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In-silico Identification and Experimental Validation of Rainbow Trout-Derived Anticancer Peptides

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Abstract: Anticancer peptides have gained increasing attention as therapeutic agents, with marine species recognized as potential sources of bioactive peptides for pharmacological uses. This study aimed to evaluate the anticancer activity of *in silico*–predicted peptides derived from rainbow trout proteins. Peptides were synthesized using the solid-phase Rink amide/Fmoc method and tested for cytotoxicity via the sulforhodamine B assay across seven cancer cell lines. The results showed that, at concentrations from 0.01 µg/mL to 1 mg/mL, the five synthesized peptides generally did not exhibit significant toxicity against the cell lines tested, except for peptide CG, which resulted in 60.8% cell viability in the MCF-7 breast cancer line. These findings reveal the limitations of *in silico*–predicted peptides and emphasize the importance of refining predictive methods by combining multiple computational tools and physicochemical criteria to improve the chances of discovering anticancer peptides suitable for developing new cancer treatments.

Keywords: solid phase; cytotoxicity; cancer.

1. Introduction

Cancer is a group of disorders characterized by the invasion and growth of abnormal cells [1]. Globally, it ranks among the leading causes of death, with 20 million new cases and 9.7 million deaths in 2022 [2]. Standard treatments such as surgery, radiation, and chemotherapy have significant drawbacks, including damage to normal cells and the development of tumor cell resistance [3]. Faced with this problem, more effective advanced therapeutic approaches have been suggested, such as gene therapy, stem cell use, ablation, and

targeted pharmacological treatments [4]. Likewise, bioactive peptides, both natural and synthetic, have been regarded as promising therapeutic options [2]. Specifically, marine peptides derived from proteins in edible products or by-products show potential for developing drugs with anticancer activity and treating conditions related to oxidative stress [3, 5]. These peptides are typically short, highly selective, exhibit effective tumor penetration, and are less immunogenic [6]. Additionally, because of their short length, their synthesis is simpler and less expensive.

In general, several mechanisms by which marine peptides induce apoptosis have been described, including microtubule or mitochondrial disruption, vascular inhibition, activation of death receptors, and direct cytotoxicity [7]. Some marine peptides approved by the US FDA include ziconotide, Adcetris®, eribulin, and trabectedin, which are derived from marine mollusks, sponges, and tunicates [8]. Several studies have characterized peptides obtained from collagen and from rainbow trout byproducts, revealing diverse bioactivities, including anticancer potential [9-11]. However, most of these studies do not report the peptide sequences.

A valuable approach to predicting peptide sequences with anticancer activity from proteins is *in silico* analysis [12]. In this context, the BIOPEP database enables the identification of potential bioactive sequences in food proteins using integrated tools [13]. This database is recognized and used in research for its broad coverage of real bioactive peptides. It also allows simulating hydrolysis conditions to predict peptides and their potential bioactivities with an accuracy of up to 54% [14]. The main limitation affecting the agreement between theoretical and experimental results is that not all laboratory conditions can be simulated.

These peptides can be produced through enzymatic hydrolysis, fermentation, or chemical synthesis. Chemical peptide synthesis relies on the condensation reaction between the carboxyl group of one amino acid and the amino group of another. In solid-phase synthesis, the peptide is attached to a resin, and amino acids protected by their amino groups and side chains are added sequentially, with their carboxyl groups coupling to the growing chain. Once the desired peptide is formed, it is separated from the resin and purified [5, 15]. This method enables the mass production of bioactive peptides for pharmaceutical applications [15].

Although the potential of *in silico* analysis and chemical synthesis for identifying and producing anticancer peptides has been recognized, evidence confirming their biological activity remains limited. Based on this gap, we hypothesized that peptides predicted *in silico* from rainbow trout proteins exhibit measurable anticancer activity upon chemical synthesis. Therefore, this study aimed to identify promising sequences through *in silico* screening, synthesize the selected peptides by solid-phase methods, and validate their biological potential by assessing *in vitro* cytotoxicity.

2. Materials and Methods

2.1. In-silico search for peptides in the BIOPEP database.

The protein sequences of *Oncorhynchus mykiss* were obtained from the UniProt database (https://www.uniprot.org), selecting only reviewed and complete proteins associated with this species (taxid: 8022). The sequences were downloaded in FASTA format and checked for redundancies or incomplete fragments. Then, each sequence was analyzed in the BIOPEP-UWM database (https://biochemia.uwm.edu.pl/biopep/start_biopep.php) using the Profile of Bioactive Peptides tool to identify peptides previously reported with anticancer activity. To do this, the sequences were entered individually into the search engine, and all subsequences

matching database entries that had the "anticancer" activity annotation or synonyms accepted by BIOPEP were recorded. In addition, database filtering criteria were applied, prioritizing peptides with higher frequency across multiple proteins, documented experimental evidence, and activity annotations supported by peer-reviewed literature. Only peptides with complete sequence information and confidence indicators reported in BIOPEP were retained for further analysis.

For each match, the peptide identifier in BIOPEP, the amino acid sequence, the protein of origin, and the associated bibliographic reference were documented. The position of the peptide sequence within the original protein and the type of experimental evidence reported by BIOPEP were also recorded. This process was repeated for all selected proteins, ensuring the traceability of the results by preserving the consultation dates, the database version used, and the search history. Based on these criteria, the final five peptides were selected as the most promising candidates for experimental validation. Finally, a summary table was created with the list of identified anticancer peptides, previously reported in *O. mykiss*.

2.2. Solid-phase chemical synthesis of peptides.

Peptide synthesis was performed in reactors with Rink amide resin 0.74 meq/g. The resin was washed and deprotected.

The amino acid was then added according to the peptide sequence, along with the corresponding coupling activator, the Oxime additive (Iris Biotech GmbH), and N, N-diisopropylethylamine (Iris Biotech GmbH). The activators used in the synthesis included: TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) (Iris Biotech GmbH); HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (Iris Biotech GmbH); and HCTU (O-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (Merck).

Once all the amino acids in the sequence were added, the peptide was cleaved. For peptide CG, a solution of TFA, TIS, ultrapure water, and 2,2'-(ethylenedioxy) diethanol (DOTA) at 92.5:2.5:2.5:2.5 was used. After cleavage, the peptides were precipitated with cold ether and centrifuged for 10 minutes at 3500 rpm. Finally, the solvent was evaporated, and the peptides were lyophilized until further use. The mass of the synthesized peptide was confirmed by electrospray ionization mass spectrometry (ESI-MS), and its purity was analyzed through reversed-phase high-performance liquid chromatography (RP-HPLC).

The peptides were purified on Sephadex-G10 to be used in functional analysis.

2.3. Sulforhodamine B cytotoxicity assay.

The cytotoxic effect of synthetic peptides was tested on seven cell lines (PC-3, AGS, MBA-MB-231, MCF-7, HT29, HELA, and GES-1). Each cell line was plated in 96-well plates at a density of 5 x 10^3 cells per well in 100 µL of medium and incubated for 24 hours before treatment. Treatments included a control with no intervention (C), a solvent control (CS) with added Milli-Q water, and the synthesized peptides at six different concentrations from 1 mg/mL to 0.01 µg/mL, these values were chosen from published data that relate concentrations below 125 µg/mL as extracts with high potential as therapeutic agents against cancer, while concentrations above 5 mg/mL have low potential [16]. The treatments were incubated for 72 hours. After incubation, cells were fixed with 50% w/v trichloroacetic acid for one hour at 4°C. They were washed with distilled water and stained with 50 µL of 0.1% w/v sulforhodamine B

in 1% v/v acetic acid for 30 minutes. To remove excess dye, samples were washed with 150 µL of 1% acetic acid, and the dye was solubilized with 100 µL of 10 mM Tris Base. Absorbance was measured at 540 nm using a microplate reader. The percentage of viable cells was calculated using the viability formula:

$$\%\ viability = \left(\frac{Abs_{sample}}{Abs_{control}}\right) x\ 100 \qquad (1)$$

Untreated cells were used as the reference control and considered as 100% viability. The results of six replicates were expressed as the mean ± standard deviation. Based on these data, the mean effective concentration (EC_{50}) for each extract and each cell line was determined using the SigmaPlot 11.0 software.

3. Results and Discussion

3.1. Peptide identification.

In silico analysis identified five sequences with potential anticancer activity derived from rainbow trout proteins. The features of these small peptides are shown in Table 1, including net charge, isoelectric point, and hydrophobicity analysis [17]. These peptides consist of combinations of two or three amino acids, including arginine, proline, lysine, valine, cysteine, glycine, leucine, and tyrosine. It has been reported that the presence of tyrosine, glycine, cysteine, and proline in ultrashort peptide sequences can significantly enhance their bioactivity [6]. Additionally, peptides containing basic residues such as arginine are common in tumor-homing peptides, which bind to cancer cells [18].

Table 1. *In silico* prediction of anticancer peptides from O. mykiss proteins using the BIOPEP database.

Sequence	Peptide ID	Name of peptide	Protein source (O. mykiss)	Net charge (pH 7) + Theoretical pI	Brief analysis
RPK	5472	Anticancer peptide	Beta-2 adrenergic receptor	+2; pI ≈ 11.8	Dominated by Arg and Lys, giving a strongly basic profile; high pI suggests strong cationic behavior typical of membrane-active anticancer peptides.
VVV	8318	Dvl protein binding	β2-adrenergic receptor; Na/K ATPase α-2; Gonadoliberolin III precursor	0; pI ≈ 6.0	Neutral and hydrophobic, with a pI close to physiological pH; lacks ionizable side chains, contributing to a balanced but highly hydrophobic character.
CG	10468	Antioxidative peptide	Glucocorticoid receptor; Na/K ATPase α-2; Triosephosphate isomerase	0; pI ≈ 5.6	Contains Cys (pKa 8.3) but overall behaves as a neutral peptide at pH 7; slightly acidic pI due to terminal charges.
LKK	5456	Anticancer peptide	Na/K ATPase α-2; Gonadoliberolin III precursor; Desmin	+2; pI ≈ 11.1	Highly basic due to two Lys residues; elevated pI suggests strong positive charge density, favoring interaction with negatively charged membranes.
YK	4017	Anticancer peptide	Desmin; Triosephosphate isomerase	+1; pI ≈ 9.8	Lys contributes to a basic pI; Tyr adds moderate hydrophobicity and potential for π–interactions, balancing charge and aromaticity.

One of the identified peptides, made up entirely of three valine residues, can be considered hydrophobic. In this context, hydrophobicity has been shown to affect the anticancer and hemolytic activity of peptides, as well as their selectivity [19]. Peptide selectivity refers to the ability to target cancer cells without harming healthy tissues [20].

Additionally, ultrashort peptides are deemed suitable for biomedical uses and the development of new drugs.

The inclusion of additional predictive tools such as PeptideRanker, ToxinPred, and other complementary algorithmic platforms could further enhance the accuracy and robustness of peptide selection. While BIOPEP provides valuable information on previously reported bioactivities, tools like PeptideRanker offer probabilistic scoring based on machine-learning models that estimate the likelihood of a peptide exhibiting biological activity, enabling more quantitative prioritization of candidates. Likewise, ToxinPred enables the assessment of potential toxicity, an essential parameter for early filtering when selecting anticancer peptides, by helping differentiate active peptides from those with undesirable toxic profiles. Integrating multiple predictive approaches would therefore broaden the evaluation criteria, reduce false positives, and increase confidence in the final peptide shortlist by combining empirical evidence, activity likelihood estimates, and safety predictions into a unified selection strategy [21].

However, although several peptides with promising biological activities have been identified as potential drugs, their bioactivity is not always confirmed due to costs or difficulties in obtaining them [3]. In this context, the development of solid-phase peptide synthesis enables the large-scale chemical production of peptides [22]. This method was used to produce the five peptides listed in Table 1, which, after synthesis and cleavage, were analyzed by ESI-MS and RP-HPLC. Figure 1 displays the mass spectra together with their corresponding chromatograms, where each spectrum exhibits a dominant peak matching the expected molecular mass, and chromatographic profiles confirm the high purity of the synthesized peptides. Table 2 summarizes the analytical characterization of five synthesized peptides, including retention time (rt), purity percentage, and both theoretical and experimentally observed molecular masses. The retention times ranged from 1.31 to 1.42 minutes, indicating consistent chromatographic behavior typical of low–molecular–weight peptides. Purity levels exceeded 90% for most peptides, with YK achieving 100% purity, confirming the efficiency of both the synthesis and purification processes. The observed molecular masses, determined by ESI-MS, showed close agreement with the theoretical values. This strong correspondence validates the chemical identity of the synthesized peptides and supports the reliability of the synthetic procedure. Overall, the results confirm that the peptides obtained meet the analytical standards required for subsequent biological activity assays or functional studies.

Therefore, the sequences obtained are considered to have potential as anticancer agents. In the search for therapeutic alternatives for cancer, *in silico* prediction tools, combined with chemical synthesis and *in vitro* validation, offer a strategy to reduce time, costs, and effort.

Table 2. Analytical characterization of the synthesized peptides.

Peptide	rt*(min)	% purity	Molecular mass (Da)	
			Theoretical	Observed
RPK	1.38	90.2	398.4	399.3
VVV	1.38	90.7	314.4	315.3
YK	1.42	100	308.3	309.2
CG	1.31	91.7	177.2	352.5
LKK	1.38	96.5	386.5	387.4

* retention time

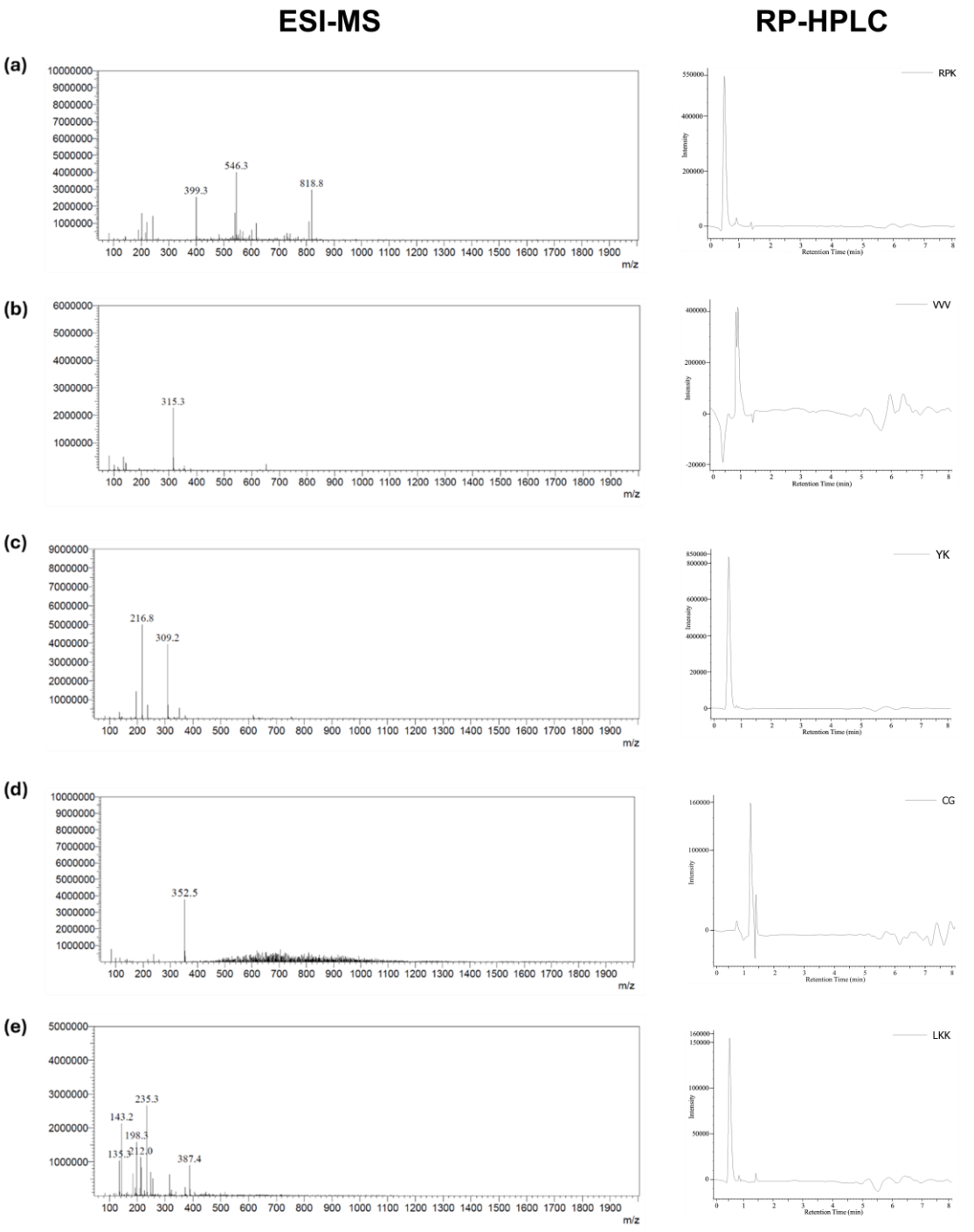
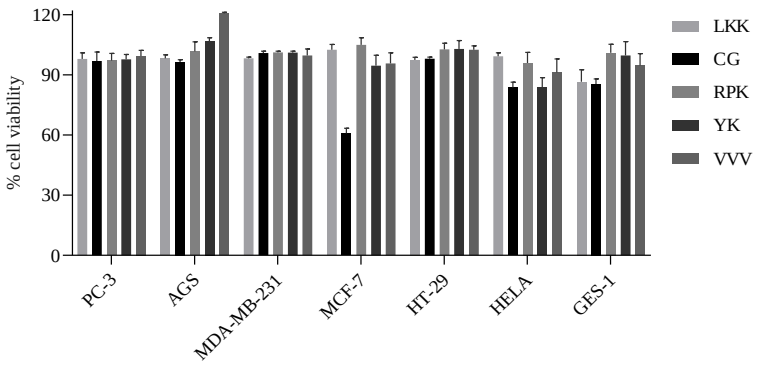


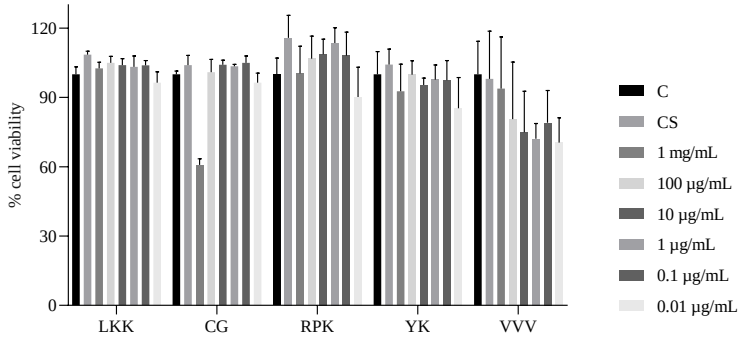
Figure 1. ESI-MS spectrum and chromatograms of the synthesized peptides obtained *in silico*. **(a)** RPK peptide; **(b)** VVV peptide; **(c)** YK peptide; **(d)** CG peptide; **(e)** LKK peptide. Improved resolution of chromatogram axes.

3.2. In-vitro cytotoxicity.

The cytotoxicity of six concentrations of the synthesized peptides, ranging from 0.01 µg/mL to 1 mg/mL, was evaluated against seven tumor cell lines and one non-tumor cell line. Overall, the peptides did not show toxicity against the cells at these concentrations (data available in the supplementary material), as they presented viability percentages greater than 80%, with the exception of the CG peptide, which al 1 mg/mL showed a viability of 60.80 ± 2.62% in the MCF-7 breast cancer cell line, compared to 85.46 ± 2.52% in the non-tumorous line GES-1 (Figure 2). Due to its concentration and the effect observed in the GES-1 line, the peptide was considered to have mild cytotoxicity. The IC_{50} of all peptides is>1 mg/mL.



(a)



(b)

Figure 2. Percentage of cell viability in cell lines exposed to synthesized peptides. **(a)** cell lines exposed to 1 mg/mL of the synthesized peptides; **(b)** cell viability in breast cancer cell line MCF-7 exposed to different concentrations (0.01 µg/mL – 1 mg/mL) of the synthesized peptides. Data expressed as the mean of 6 replicates ± standard deviation. PC-3: Human prostate cancer, AGS: Human gastric adenocarcinoma, MDA-MB-231: Human breast cancer, MCF-7: Human breast cancer, HT-29: Human colorectal adenocarcinoma, HELA: Cervical cancer, and GES-1: Human gastric epithelial cell.

These results indicate that the in silico strategy for predicting peptides with potential anticancer activity was not sufficiently accurate, as significant activity was not confirmed in *in vitro* tests on the selected cell lines.

The results may be related to the characteristics of the peptides. The VVV peptide, being short and highly hydrophobic, is prone to forming insoluble aggregates due to the tendency of its side chains to enclose themselves within the structure, thereby decreasing their interaction with the cell membrane [23, 24]. Conversely, the LKK and RPK peptides are not very hydrophobic, which may hinder their interaction with membranes [24, 25]. In the specific case of peptide YK, its composition of an aromatic and a polar amino acid allows it to behave as a minimal amphipathic motif, a characteristic that may favor its interaction with membranes. However, its short length may limit its cytotoxic effect [24, 26]. With peptide CG, we observed a slight toxic effect, which could be related to cysteine´s ability to form disulfide bonds and alter the peptide´s structure, helping it to remain stable [27].

These results suggest the need for more selective and precise methodologies are needed for the identification of bioactive peptides. Specific refinements could further strengthen peptide selection. Computationally, molecular docking and short molecular-dynamics simulations would help assess binding affinity and structural stability beyond sequence-based predictions. QSAR models trained on anticancer peptide datasets could also improve discrimination of active motifs. Biochemically, testing simple stability-enhancing

modifications, such as N-terminal acetylation, C-terminal amidation, or limited D-amino acid substitutions, could increase peptide resistance to degradation while preserving activity [28].

Previous studies have identified *in silico* peptides with potential anticancer activity. For example, Wu *et al*. [13] used a bioinformatics strategy combining tools such as BIOPEP-UWM, PeptideRanker, ToxinPred, and ProtParam to analyze peptides derived from *Arca*. Although this strategy allowed them to identify 17 candidate peptides, only three showed anticancer activity after *in vitro* validation, while others even promoted cell proliferation.

Similarly, Law *et al*. [29] applied a strategy combining programs such as ADP3, AMPfun, ANTICP, ProtParam, ToxinPred2, and ADMETlab2.0 to design and predict anticancer peptides derived from the fish *Anabas testudineus*. Despite using multiple tools, only one peptide exhibited significant cytotoxicity against the MDA-MB-231 cell line.

In both cases, the combination of different tools enabled a more exhaustive selection of peptides with anticancer potential. Although this procedure does not guarantee bioactivity in *in vitro* tests, it does improve the selection of peptides with bioactive potential. Furthermore, to control peptide dynamics and aggregation *in silico*, structures could be designed in which hydrophobic and polar amino acids are strategically positioned [25].

4. Conclusions

Peptides identified *in silico* from rainbow trout proteins as potential anticancer agents did not exhibit the predicted activity after synthesis and *in vitro* evaluat*ion*. This finding reinforces the importance of complementing *in silico* searches with experimental assays that confirm or refute their bioactivity. It also highlights the value of simultaneously employing multiple digital tools to optimize peptide selection. Furthermore, the results underline the need to refine the predictive workflow by integrating complementary algorithms and structural evaluation techniques to reduce false-positive hits. While this approach does not guarantee the presence of biological activity, it does increase the likelihood of identifying bioactive peptides with pharmacological potential, key aspects in the search for new therapies against diseases.

A key limitation of this study is that the selected peptides were short, low-hydrophobicity peptides that lacked structural optimization, which likely reduced their stability and capacity to interact with cancer cells. These factors should be addressed in future selection and validation strategies, with a particular emphasis on strengthening the integration of *in silico* and *in vitro* tools to enhance the accuracy and efficiency of peptide-based drug discovery.

Author Contributions

Conceptualization, M.B., F.G., and L.O.; methodology, F.E., C.J., J.V., and P.S.; data curation, F.E., A.C., C.A., and F.G.; investigation, C.J., J.V., M.B., A.C., C.A., and L.O.; validation, C.J. and J.V.; writing—original draft preparation, F.E.; writing—review and editing, C.J., P.S., and L.O.; supervision, C.J., J.V., and L.O.; resources, P.S.; project administration, P.S. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

Not applicable.

Data Availability Statement

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

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary materials

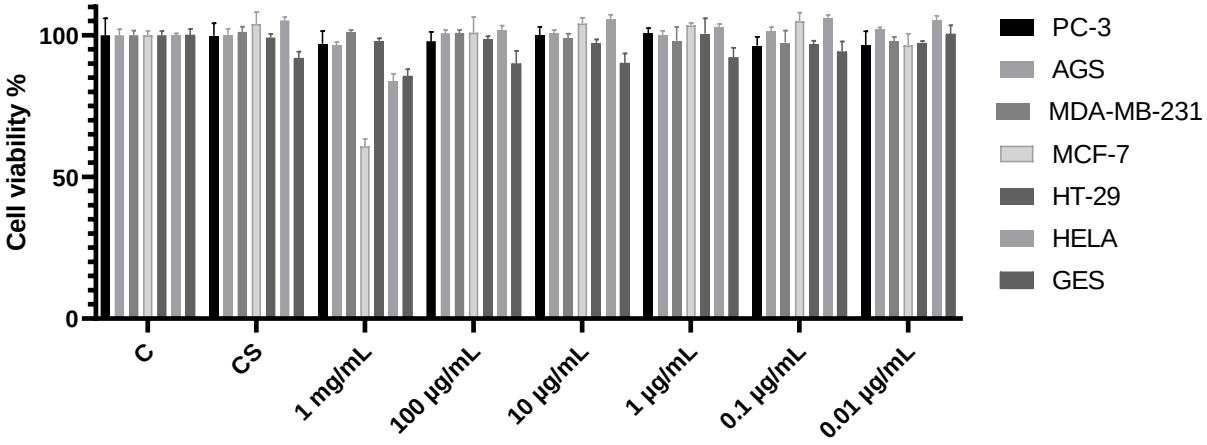

Figure S1. Percentage of viability of cell lines exposed to the CG peptide.

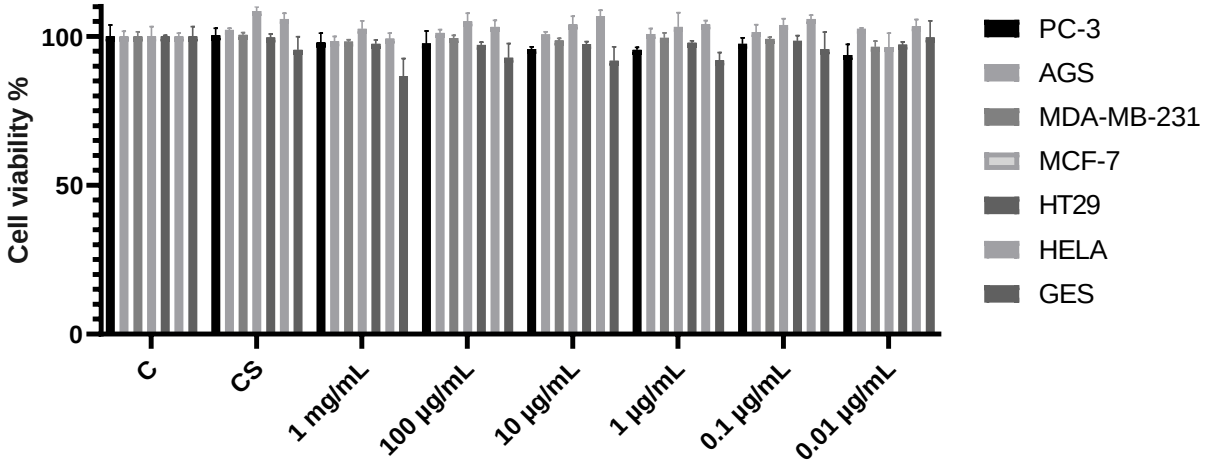

Figure S2. Percentage of viability of cell lines exposed to the LKK peptide.

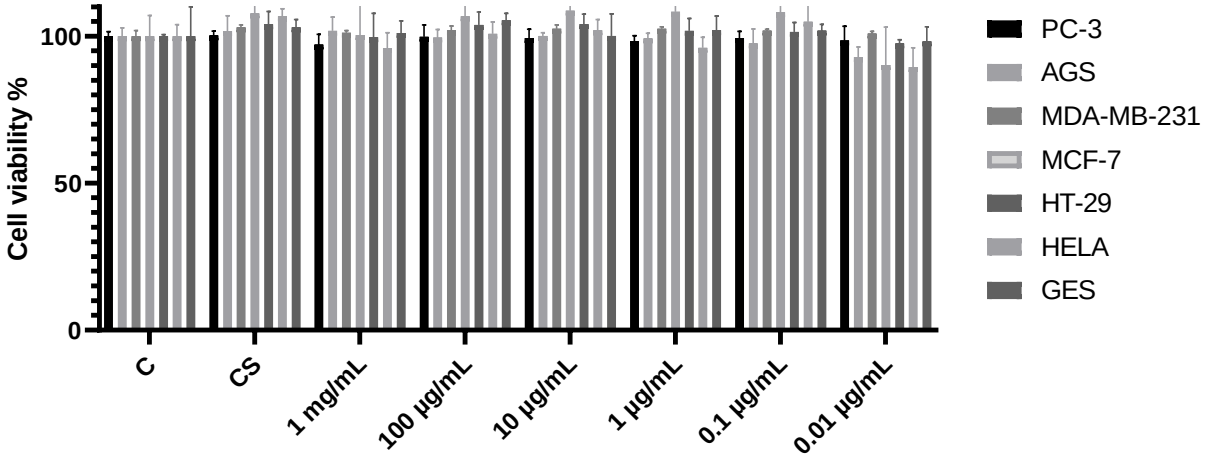

Figure S3. Percentage of viability of cell lines exposed to the RPK peptide.

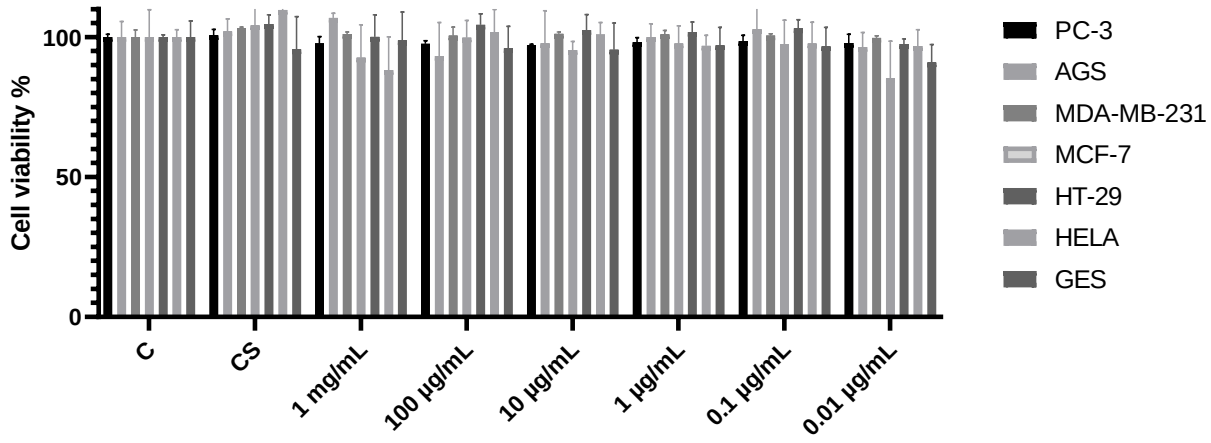

Figure S4. Percentage of viability of cell lines exposed to the YK peptide.

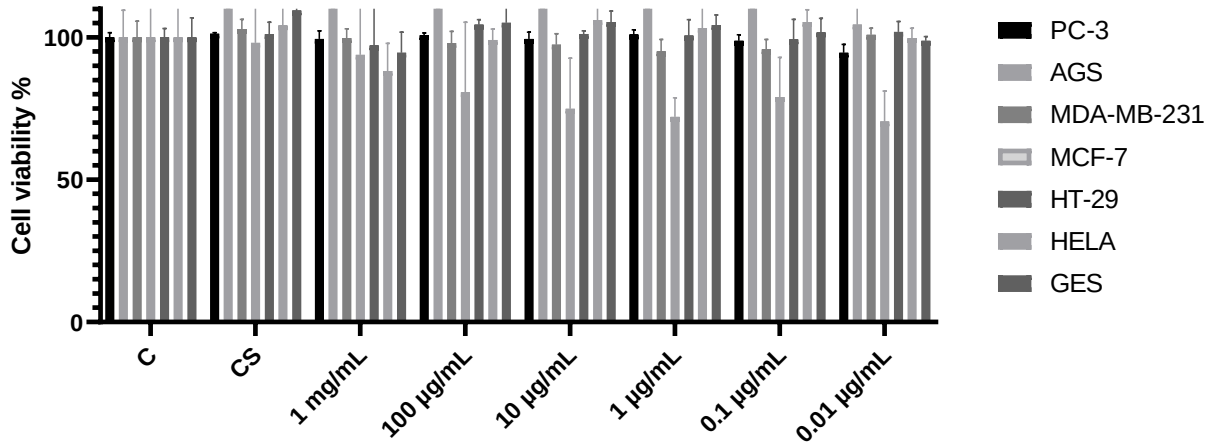

Figure S5. Percentage of viability of cell lines exposed to the VVV peptide.