

In Silico Study of Physicochemical Characteristics and Anticancer Potential of Peptides Derived *from Salvia Hispanica*

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Abstract

Peptides derived from *Salvia hispanica*'s protein fraction < 1 kDa, were identified and analyzed for the prediction of their physicochemical properties. The characterized peptide sequences were then submitted to a multi-criteria decision analysis to identify the peptides that possess the characteristics to potentially exert anticancer activity. Through molecular docking analysis, the potential anticancer activity of the Potentially Anticancer Peptide (PAP)-1, PAP-2, PAP-3, PAP-4 and PAP-5, was estimated by their binding interactions with cancer and apoptosis related molecules. All five evaluated PAPs exhibited strong binding interactions (<100 kcal/mol); however, PAP-3 showed the lowest binding free energies with several of the targets. Thus, PAP-3 shows potential to be used as a nutraceutical or ingredient for functional foods that adjuvates in cancer treatment. Further *in vitro* and *in vivo* studies are required to validate the predictions obtained by the *in silico* analysis, and to establish the mechanism of action and the clinical potential of the peptide.

1. Introduction

Cancer is one of the leading causes of death worldwide. In 2020, approximately 19.3 million new cases of cancer and 10 million deaths due to this disease were registered. As for 2040, 28.4 million new cases are estimated, representing an increase of 47% (Sung et al., 2021). Conventional cancer therapies, such as surgery, radiotherapy, and chemotherapy, are not selective to cancer cells and are highly toxic, which results in serious side effects on cancer patients and a negative impact on their quality of life (Rejhová et al., 2018). Due to the increase of new cases and deaths, and the low efficacy of conventional therapies, the need for complementary treatments that improve the patient's response to cancer therapy is of high importance. Bioactive peptides could be potentially used as a complementary therapy for this disease since they have reported selective anticancer activities (Quintal-Bojórquez & Segura-Campos, 2021). Peptides with anticancer activities share some common characteristics, such as: net charge from +2 to +9, amphipathic structure, amino acid sequence from 5 to 20 residues with presence of lysine and arginine and hydrophobicity from 30–50% (Felício et al., 2017; Hoskin & Ramamoorthy, 2008; Oelkrug et al., 2015). These peptides are generally identified from natural sources with high protein content, such as *Salvia hispanica*, whose seeds contain up to 25% of protein (Das, 2017), making it a potential source for the production of bioactive peptides.

Currently, the experimental identification and development of peptides is time-consuming, labor-intensive, and expensive. Therefore, the use bioinformatic tools that provide *a priori* knowledge of characteristics and potential activities of bioactive peptides, becomes indisputable (Liscano et al., 2020). The prediction of physicochemical characteristics of peptides is not only important for the prognosis of their activity, but also for the definition of their potential mechanism of action (Jakubczyk et al., 2020).

Molecular docking is an *in silico* technique that estimates the binding modes and affinities of the peptides to the binding sites of target molecules (Ferreira et al., 2015). This tool generally consists of four main strategies: peptide selection and preparation, ligand preparation, docking and analysis of results, thus, allowing the search of new anticancer peptides in a faster, more efficient, and low-cost manner (Tu et al., 2018). For most docking evaluations for anticancer peptides potential, the molecular targets studied are related to apoptosis and/or cancer development. Gupta et al. (2013) evaluated the binding properties of the peptide INSAYKLKYARG (1383.62 Da) in a p53 mutant protein, which is present in more than 80% of cancer cases. Through a docking analysis, the peptide reported potential cooperation for the degradation of the mutant protein. In another study, Kostrzewa et al. (2018) reported four peptides, all with molecular weight lower than < 1 kDa, that bound to two proteins PTP1B (protein tyrosine phosphatase 1B) and SHP2 (protein tyrosine phosphatase 2) and showed a decrease on their enzymatic activity. (Mishra & Dey, 2019) reported that the peptide RLGDGCTR (858.9 Da) was able to induce apoptosis and inhibit the action of protein kinases when its binding potential was evaluated on proteins related to apoptosis (procaspase 3, procaspase 7 and caspase 9, TRAIL, Survivin and MMP-2) and cancer (EGFR, VEGFR, p38 and MMP-9). In 2020, Bakare et al. (2020) evaluated the binding potential of the peptide "B001" and reported its ability to aid in the prediction of invasion and metastasis of cancer cells.

In a previous investigation (Quintal-Bojórquez et al., 2021) identified a protein fraction of < 1 kDa, from *S. hispanica* seeds, that reported cytotoxic effects on four cancer cell lines (MCF-7, Caco2, PC-3 and HepG2). Accordingly, the peptides present in this fraction were identified. However, the physicochemical properties of the peptides obtained, and their potential anticancer activities have not yet been analyzed.

For that matter, the objective of this study is to predict and evaluate the physicochemical characteristics and potential anticancer activity of the peptides identified from *S. hispanica* protein fraction < 1 kDa, through the use of *in silico* tools.

2. Materials And Methods

2.2 Amino acid sequence identification

The peptides analyzed were identified from *Salvia hispanica* seeds as described in the preliminary study by (Quintal-Bojórquez et al., 2021). The seeds were degummed, defatted, ground, and sieved in order to obtain a high protein chia flour, which was then enzymatically hydrolyzed and ultrafiltered to be separated in three protein fractions < 1, 1–3 and 3–5 kDa. The protein fraction < 1 kDa reported cytotoxic activity in four cancer cell lines (MCF-7, Caco2, PC-3 and HepG2) and no effects on a normal cell line of fibroblasts (hFB). The peptides identification of the < 1 kDa fraction was performed through an EasyLC nano liquid chromatography system and an Orbitrap Velos Mass Spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK). The sequences were analyzed through PEAKS Studio X+ (Bioinformatic Solutions Inc, Waterloo, Ontario, Canada) and searched against the Uniprot databases (release 2020_03). All compounds are > 95% pure by HPLC.

2.3 Prediction of physicochemical characteristics of the peptides

The physicochemical characteristics of the peptides were determined with *in silico* tools available as free web servers: the net charge was determined with ExPASy ProtParam (<https://web.expasy.org/protparam/>) and Innovagen (<https://pepcalc.com>), the hydrophobicity with Peptide 2.0 (https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php), and the allergenicity and toxicity with AllerTOP (<https://www.ddg-pharmfac.net/AllerTOP/index.html>) and ToxinPred (<https://webs.iitd.edu.in/raghava/toxinpred/design.php>), respectively. The length and molecular weight of the peptides were retrieved from the LC-MS/MS spectrometry analysis.

2.4 Multi-criteria decision analysis of amino acid peptide sequences

Once the physicochemical characteristics of the peptides were calculated, and their toxicity and allergenicity predicted, the 1186 sequences were placed in a pool of peptides for their evaluation through a multi-criteria decision analysis to identify the ones with the highest potential anticancer activity. The analysis was performed as described by (Quintal-Bojórquez et al., 2021), with a hybrid method using AHP (Analytic Hierarchy Process) and TOPSIS (Technique for Order Preference by Similarity to Ideal Solution). The criteria established for the identification of the peptides with potential anticancer activity were net charge from + 2 to + 9, amphipathic structure, amino acid sequence from 5 to 20 residues with presence of lysine and arginine and hydrophobicity from 30–50% (Felício et al., 2017; Hoskin & Ramamoorthy, 2008; Oelkrug et al., 2015). From this analysis, the top five Potentially Anticancer Peptides (PAPs) that best met the established criteria, were selected for further analysis.

2.5 Modeling of peptides

The peptides identified from the multi-criteria analysis with the highest potential anticancer activity (PAP-1, PAP-2, PAP-3, PAP-4 and PAP-5) were built on the software Discovery Studio (DS4.0, Accelrys Inc., San Diego, CA, USA). The structures were minimized, with the OPLS-AA force field. Subsequently, the model was saved as a PDB file and converted to PDBQT file in the MGLtools software (<http://mgltools.scripps.edu/downloads>).

2.6 Docking of peptides with anticancer molecular targets

The *in silico* molecular docking tool plays an important role in the identification of peptides with potential anticancer activity since it is used as a screening method based on the binding energy between the peptides and a molecular target (Mishra & Dey, 2019).

The docking studies were carried out between the peptides (PAP-1, PAP-2, PAP-3, PAP-4, PAP-5) and the apoptosis and cancer-related targets described in Table 1. These were retrieved from Protein Data Bank (<https://www.rcsb.org>). Docking calculations were conducted with HDock (<http://hdock.phys.hust.edu.cn>) and fixed with the most important residue of the site action of each target, which are also mentioned in Table 1. Finally, the pose was selected by the best score value. The visualization was achieved with PyMOL (The PyMOL Molecular Graphics System, Version 1.0, Schrödinger, LLC.) and with Molecular Operating Environment (MOE, Chemical Computing Group Inc.: Montreal, QC, Canada, 2016).

Table 1
Apoptosis and cancer related targets for molecular docking studies.

Molecular target	Description	Relevant residue of site of action	PBD ID	Reference
PTP1B	Protein tyrosine phosphatase 1B	Arg221	5K9V	(Kostrzewa et al., 2018)
SHP2	Protein tyrosine phosphatase 2	Thr108	5EHR	
IKK α	Inhibitory- κ B Kinase alpha	Glu729	3BRT	(Jabeen et al., 2015)
IKK β	Inhibitory- κ B Kinase beta	Glu729	3BRV	
Cadherin	-	Ser8	4ZTE	(Bakare et al., 2020)
EGFR kinase	Epidermal growth factor receptor	Ala719	2GS2	(Mishra & Dey, 2019)
VEGFR2 kinase	Vascular endothelial growth factor receptor 2	Asn923	1VR2	
P38	Mitogen-activated protein kinase	Arg68	4MYG	
PBK	Protein kinase B	Arg274	1GZN	
MMP-2	Matrix metalloproteinase-2	Gly394	1CK7	
MMP-9	Matrix metalloproteinase-9	Glu130	1L6J	
Procaspase 3	-	-	4JQZ	
Procaspase 7	-	-	1K88	
Caspase 9	-	-	2AR9	
TRAIL	TNF-related apoptosis-inducing ligand	-	1D2Q	
Survivine	-	Glu40	1XOX	
p53	p53 protein "mutant type"	Asp228	2J1X	(Nguyen & Nguyen, 2016)
p53	p53 protein "wild type"	His179	2OCJ	(Gupta et al., 2013)

3. Results And Discussions

3.1 Amino acid sequences and their physicochemical characteristics

From the peptide identification of the < 1 kDa protein fraction, 1186 peptides were identified. The peptide sequences were searched against the UniProtKB databases to determine if any of the sequences had been previously reported; these sequences were titled *reported* peptides for future references. From the total 1186 peptides, 119 *reported* peptides were identified in other organisms (Supplementary Information Table 1), along with their physicochemical characteristics and the prediction of toxicity and allergenicity. The *reported* peptides were identified in the sequence of uncharacterized proteins derived from *Salvia splendens*. This plant belongs to the same Family (*Lamiaceae*) and Genus (*Salvia*), as *Salvia hispanica*. *S. splendens*, is native from Brazil and it's mostly used as a garden ornamental, different to chia, which is known for its seeds' benefits to human health (Narayan et al., 2015).

The other remaining 1067 peptides are considered as *de novo* sequences since they haven't been identified in other organisms; these peptides are presented in Supplementary Information Table 2, along with their physicochemical characteristics. Additionally, the average local confidence (ALC) of each *de novo* sequence presented is a percentage that ranges from 0 to 100% and it indicates how likely the complete sequence of the peptide is correct; thus, it represents an average of the confidence of the assignment of each individual amino acid residue on each peptide (Ma et al., 2003; O'Bryon et al., 2020). In accordance with (Tran et al., 2016),

peptides with a low ALC (< 50%) indicate a large number of incorrect amino acid residues in each sequence. Consequently, the *de novo* peptide sequences identified in this study with an ALC < 50%, were removed from the list.

Peptides with low molecular weight (< 10 kDa) have the advantage of being able to efficiently penetrate into tumors (Liu et al., 2019). The density of the stroma and the high pressure of the interstitial fluid of tumors makes the penetration of higher molecular weight compounds, such as antibodies, disadvantageous (Scodeller & Ascitto, 2020). Particularly, a large percentage of peptides that have reported *in vivo* and *in vitro* anticancer activity possess a molecular weight lower than < 3 kDa. Regardless of the peptides identified in this study from the < 1 kDa protein fraction of *S. hispanica* seeds, their molecular weight range between 200 to 2050 Da, which is within the range suggested for a high tumor penetration rate. The difference between the resulting range of molecular weight and the membrane's cut off used in the ultrafiltration is most likely attributed to the fact that membrane manufacturers characterize their ultrafiltration membranes where 90–95% of the proteins are rejected and the remaining 5–10% can pass through it, allowing higher molecular weight peptides to be part of the permeate (Kadel et al., 2019; Yammine et al., 2019).

Most of the anticancer peptides that have been identified are short in length, contain 5 to 50 amino acid residues, and have positively charged residues such as lysine and arginine (Felićio et al., 2017; Hoskin & Ramamoorthy, 2008; Oelkrug et al., 2015). From the 1186 peptides identified in this study, 99.4% were constituted by 5 to 20 amino acid residues, and only 21.6% do not contain a positively charged residue in their sequence. The amino acid composition of peptides is relevant for the determination of their physicochemical properties such as net charge, hydrophobicity, and structural folding, all of which are important to enable its interaction with lipid membrane of cancer cells (Shoombuatong et al., 2018).

Peptides with anticancer activity are generally cationic in nature with a net charge, at neutral pH, that ranges from + 2 to + 9 (Quintal-Bojórquez & Segura-Campos, 2021). The *reported* peptides' net charge ranges from – 3.9 to + 2, from which only two peptide sequences met the net charge parameter to potentially present anticancer activity. As for the *de novo* peptides, their net charge ranges from – 6 to + 11, from which 109 peptide sequences met this characteristic. The positive net charge allows the peptides to interact with the anionic cell membrane of the cancer cell and thus, selectively kill cancer cells. The anionic nature of the lipid membrane of cancer cells is given by the presence of phospholipids with anionic heads, such as phosphatidylserine and phosphatidylethanolamine, on the extracellular layer of the membrane. These phospholipids are usually not present in regular cells; thus, cationic peptides can selectively bind to the anionic cancer cell through electrostatic interactions between the lateral chains of the amino acid residues and the heads of the phospholipids (Sánchez Acosta, 2016).

Hydrophobic interactions play a fundamental role in the folding mechanisms of the peptides since they are essential for the stabilization of their structure (Popp et al., 2014). Peptides with anticancer activity mainly adopt an α -helix and rarely a β -sheet structure (Shoombuatong et al., 2018). Most of the anticancer peptides adopt a conformation prior to engaging with the cancer cell membrane, to then penetrate intracellular spaces and lead to the destruction of the lipidic bilayer through pore formation and/or changes in the cell membrane charge, resulting in the final interference with necrotic and/or apoptotic cell death pathways (Gaspar et al., 2013). Also, Huang et al. (2011) reported a correlation between the peptide hydrophobicity and its hemolytic activity, a parameter important to predict the peptides toxicity. The trend observed is that the higher the hydrophobicity, the stronger the hemolysis against human red blood cells. Thus, anticancer peptides' hydrophobicity ranged from 30–50% (Hoskin & Ramamoorthy, 2008) in which anticancer activity has been reported with low or no toxicity. From the total of the *reported* and *de novo* peptides, hydrophobicity ranged from 0 to 91.67%, being 568 peptides within the suggested hydrophobicity threshold.

3.2 Multi-criteria decision analysis (MCDA)

The MCDA techniques are widely used to help make a decision when more than one criterion is relevant (Arslan et al., 2021). In this study, the hybrid of AHP and TOPSIS were applied. The AHP technique allows the assignment of a value to each criterion, which represents a preference degree (Chai et al., 2013). The criteria established that the peptides with anticancer potential should meet were assigned with the same value of preference, since they are all considered to be equally important for the peptide to exert the expected activity. On the other hand, the TOPSIS approach aims to evaluate the alternatives by simultaneously measuring their distances to the Positive Ideal Solution (PIS). The resulting rank of the alternatives is built according to the relative closeness to the PIS (Dehghani Soufi et al., 2015) and a value of 1 is assigned to the peptide sequence that best met the criteria. According to (Nayeb et al., 2016) ranking values higher than 0.8 indicate a very good factor analysis, higher than 0.5 is moderate and lower than 0.5 is unacceptable. For purposes of this study, peptide sequences with a ranking value lower than 0.5 were excluded; the peptides

with a value higher than 0.5 are presented in Supplementary Information Table 3. In the same Table, it is possible to observe that the peptides that made the cut due to values higher than 0.5, are only *de novo* sequences. This is consistent with the properties predicted from the *reported* peptides since they mostly own neutral, negative, or very low positive net charges. Some of the analyzed peptides resulted in the same rank number due to their coincidence in their physicochemical properties.

The top 5 peptides from the rank were named as Potentially Anticancer Peptides (PAPs) and were selected to continue with further analysis. PAP-1, PAP-2, PAP-3, PAP-4 and PAP-5 and their physicochemical characteristics are presented below in Table 2.

Table 2. Top 5 PAPs (Potentially Anticancer Peptides) and their physicochemical properties obtained from the multicriteria decision analysis of the peptides' sequences derived from the *Salvia hispanica* protein fraction < 1 kDa.

PAP	Sequence	Molecular weight	Lenght	Net charge	Hydrophobicity (%)	ToxinPred prediction	Allergenicity
PAP-1	KLKKNKL	742.5065	6	+ 3	33.33	Non-toxin	Probable allergen
PAP-2	MLKSKR	761.98	6	+ 3	33.33	Non-toxin	Probable non-allergen
PAP-3	KKYRVF	839.5017	6	+ 3	33.33	Non-toxin	Probable non-allergen
PAP-4	FRTKKK	806.5126	6	+ 4	16.67	Non-toxin	Probable non-allergen
PAP-5	SVVAKAPVGKR	1110.687	11	+ 3	54.55	Non-toxin	Probable allergen

On Table 2 it is possible to observe how the peptides meet or not, the characteristics established to potentially exert anticancer activities. The reason wherefore peptides from the top 5 may not be within the range suggested for anticancer peptides is because the MCDA weighs all the characteristics and calculates the best combination that delivers the peptide closer to the PIS. However, all the selected PAPs sequences are within the established range of number of aminoacidic residues and net charge. PAP-1, PAP-2, PAP-3, PAP-4 and PAP-5 were selected to continue the prediction of their anticancer activity by molecular docking analysis.

3.3 Docking of PAPs to target molecules

Docking of small molecules, such as peptides, has been applied for the prediction of the binding potential and affinity of a compound with a specific target (Pantsar & Poso, 2018). This bioinformatic tool utilizes scoring functions to provide a fast estimation of the binding affinity within the protein-ligand complex (Kitchen et al., 2004); such scoring functions are directly related to the Gibbs energy of binding (Liao et al., 2017).

PAP-1, PAP-2, PAP-3, PAP-4, and PAP-5 were docked on the 3D structures of cancer and apoptosis related molecules and the results are presented in Table 3. These predictions of binding affinities allow to estimate the peptide's capability to bind with the anticancer targets and the potential mechanism of action of the PAPs. The binding free energy was used as the parameter of energy needed for the bonding between the ligand and target molecule. Low binding free energies represent increased strength or affinity of bonding. The 2D possible binding interactions of the PAPs with the highest binding free energy to the target molecules are presented in Table 4.

Table 3
Binding free energies (ΔG , kcal/mol) between PAPs and target molecules.

Molecules related to cancer	PAP-1	PAP-2	PAP-3	PAP-4	PAP-5
PTP1B	-128.27	-159.32	-162.80	-140.01	-140.70
SHP2	-117.82	-173.90	-173.03	-168.72	-159.60
IKK α	-114.05	-119.53	-121.98	-124.74	-116.48
IKK β	-100.72	-125.37	-117.18	-124.76	-113.89
Cadherine	-111.62	-141.78	-135.39	-135.63	-139.50
EGFR	-136.43	-147.17	-167.62	-159.83	-143.57
VEGFR2	-124.07	-143.65	-155.32	-165.86	-176.33
P38	-147.46	-140.24	-169.26	-173.29	-154.39
PBK	-121.33	-139.15	-145.07	-143.18	-137.27
MMP-2	-146.05	-179.72	-191.66	-191.04	-166.38
MMP-9	-143.42	-154.32	-174.04	-160.37	-156.42
Procaspase 3	-118.75	-156.35	-159.65	-138.05	-149.56
Procaspase 7	-146.11	-156.73	-175.94	-168.12	-157.81
Caspase 9	-141.47	-165.34	-183.56	-152.73	-167.07
TRAIL	-156.80	-171.27	-215.76	-179.35	-174.47
Survivine	-141.38	-151.45	-153.77	-158.31	-130.87
P53 mutant type	-134.63	-145.04	-164.10	-162.35	-180.05
P53 wild type	-120.13	-130.28	-133.66	-133.77	-143.52

Table 4. Types of binding between the PAP with the highest binding energy and the molecular target's site of action.

simulation was correlated to the decrease of the enzymatic activity and the cellular viability of a breast cancer cell line. As shown in Table 3, all PAPs presented strong binding energies to the allosteric site of the target molecule. PAP-3 reported the strongest binding energy with a ΔG of -162.8 kcal/mol; its potential to allosterically bind to PTP1B and exert anticancer activities through the inhibition of the enzyme is established. As shown in Table 4, although the binding of the PAPs was fixed to Arg221 for the docking analysis, PAP-3 did not report binding interactions with it. However, PAP-3 did bind to other residues found in the allosteric site of PTP1B such as Glu115, Lys116, Lys120 and Gln262; the former being hydrogen bonds and the latter polar interactions.

On the other hand, SHP2 is a phosphatase ubiquitously expressed that transduces mitogenic, pro-survival and pro-migratory signals from several growth factors, cytokines, and extracellular-matrix receptors. Active mutants of SHP2 have been identified in patients with juvenile myelomonocytic leukemia, acute myelogenous leukemia, and certain types of solid tumors, such as lung and colon cancer (H.-C. Wang et al., 2014). Recent studies have explored the inhibition mechanism of this enzyme and interactions with certain amino acid residues from its allosteric site 1, allosteric site 2, P-loop and Q-loop are speculated to be key to lead to the auto-inhibition conformation of SHP2 (Song et al., 2014; Tripathi & Ayyannan, 2021). The residue Thr108 belongs to the allosteric site 1, to which the PAPs evaluated in this study were fixed into to obtain potential binding simulations. As shown in Table 3, PAP-2 reported the strongest bond with the target molecule SHP2 with a ΔG of -173.9 kcal/mol, however, PAP-3 reports a similar ΔG at -173.03 kcal/mol, both proving to be promising bioactive compounds for the inhibition of SHP2. According to Table 4, PAP-2 does not spontaneously bind to the residue Thr108; however, it did report binding to other residues found in the allosteric site including: Arg11 through a basic contact to its side chain, and Gln256 and Gln257 through polar contacts to its side chains.

3.3.2 Inhibitory- κ B kinases (IKKs)

The Inhibitory Kappa B Kinases (IKKs) are well recognized as key regulators of the nuclear factor kappa B (NF- κ B), which transduces pro-inflammatory and growth stimulating signals that assist in the pathogenesis of several diseases (Gamble et al., 2012; Prescott & Cook, 2018). Numerous kinases have reported to be overactivated in cancer and even influence the development of therapy resistance; thus, targeting these molecules has been of interest for improving patient's outcome by overcoming chemotherapy resistance (Colomer et al., 2020). Therefore, the inhibition of NF- κ B signaling has potential use for the prevention and treatment of cancer. IKK α and IKK β are the catalytic subunits of the IKK complex and were set as targets in the docking studies of the PAPs.

Molecular docking was conducted to simulate the interaction of PAPs with IKK and consequent inhibition of NF- κ B. As shown in Table 3, all evaluated PAPs exhibited strong bonding to both kinases; thus, the binding analysis supports the fact that all the five PAPs could be effective inhibitors of the kinases. Particularly, PAP-4 reported the lowest binding free energy with a ΔG of -124.64 kcal/mol with IKK α , and PAP-2 with a binding free energy of -125.37 kcal/mol with IKK β . From the key residues found in the active site of both kinases, Glu729 was chosen to be set as the fixed residue for the docking analysis. As presented in Table 4, PAP-4 exhibited binding to IKK α through the residue Glu729 by a polar interaction with its backbone; thus, indicating potential interaction with the target molecule and further inhibition of NF- κ B. PAP-2 did not exhibit binding interaction with the residue Glu729; however, it did report polar interaction to another key residue of the active site: Gln730. The multiple polar and non-polar interactions along with strong binding energy, are possible explanations of how peptides would exhibit anticancer activity to both IKKs (Jabeen et al., 2015).

3.3.3 Cadherins

Cadherins are transmembrane glycoproteins that are responsible for cell-cell adhesion and maintenance of tissue integrity. Epithelial cadherin (E-cadherin) has been identified in many malignancies, among them pancreatic, breast and gastric cancers, since it has demonstrated to be involved in cell migration, invasion, and tumor progression (Kaszak et al., 2020). E-cadherin is usually inactive or blocked during carcinogenesis, allowing the development and progression of cancer and/or metastasis (Bruner & Derksen, 2018). The increased upregulation of E-cadherin in cancer has been linked to the suppression of malignant cells; thus, making these proteins a relevant target for cancer therapy. Recently, Bakare et al. (2020) suggest the use of five peptides as biomarkers for the identification of E-cadherin as a prognostic indicator of patients that receive cancer therapy, since all five reported strong binding energies to E-cadherin on docking studies.

The amino acid residues found in the active site of E-cadherin are Val3, Ile4, Pro5, Pro6, Ile7, Ser8, Leu21, Val22, Gln23 and Lys25 (Siahaan et al., 2020). All five PAPs reported strong bonding values to the target molecule. As reported in Table 3, PAP-2 exhibited

the lowest ΔG at -141.78 kcal/mol, followed closely by PAP-5, PAP-4, PAP-3 and PAP-1 with ΔG s of -139.5, -135.63, -135.39 and -111.62 kcal/mol, in that order. On Table 4 it is possible to visualize the binding predictions of PAP-2 with the active site of E-cadherine. PAP-2 binds with key residues of the target molecule such as Leu21 through a non-polar contact, and Lys25 through a hydrophobic interaction.

3.3.4 Protein kinases

Protein kinases (PTKs) are enzymes that regulate proteins' biological activities by phosphorylation of specific amino acid residue, using ATP as the source of phosphate, resulting in the induction of a conformational change and the activation of the protein (Avendaño López & Menendez, 2015).

Epidermal growth factor receptor (EGFR) is a transmembrane protein with a cytoplasmic kinase unit that is responsible for the transduction of growth signals that regulate cell proliferation, differentiation, motility, and metabolism (da Cunha Santos et al., 2011; Du & Lovly, 2018). EGFR is found to be overexpressed in over 60% of non-small cell lung carcinomas; thus, becoming an important therapeutic target for the treatment of these tumors. Also, EGFR is found to be overexpressed in 50% of triple-negative breast cancer (TNBC) and is an indicator of poor prognosis (Mishra et al., 2014).

Vascular endothelial growth factor receptor 2 (VEGFR2) exhibits a significant impact on the process of angiogenesis, which is defined as the formation of new blood vessels from existing vasculature; tumor growth and metastasis directly depend on this process (Modi & Kulkarni, 2019). After activation by vascular endothelial growth factors (VEGF), VEGFR2 undergoes autophosphorylation that leads to the proliferation of endothelial cells, tumor angiogenesis, tumor growth and metastasis; thus, an overexpression of VEGFR2 is observed in a several cancers such as breast cancer, cervical cancer, non-small lung cancer, hepatocellular carcinoma, and others (Yan et al., 2015). The activity of these kinases must be closely regulated and accurately balanced to mediate their normal cellular physiological functions. Signal attenuation and downregulation provides important implications in cancer therapies (Du & Lovly, 2018). The binding interaction of the PAPs with EGFR and VEGFR2 were simulated through docking analysis. All five PAPs evaluated exhibited strong bonds to both kinases. As for EGFR, PAP-3 exhibited the strongest affinity, reporting a ΔG of -167.62 kcal/mol. As shown in Table 4, PAP-3 displayed forming hydrogen bonds with Lys721 and Asp831. These hydrogen bonds have been reported previously by (Sangande et al., 2020). Particularly, the residue Asp831 is part of the DFG (Asp-Phe-Gly) motif which plays a key role in ATP binding and is relevant for the increased inhibitory activity of the receptors (Peng et al., 2013).

PAP-5 exhibited the strongest binding affinity with VEGFR2, with a binding free energy of -176.33 kcal/mol. Since Asn923 is found in the ATP-binding site of VEGFR2, the overall high affinity values of the PAPs indicate that they could potentially inhibit its activity¹⁴. As shown in the 2D representation of the binding interactions in Table 4, PAP-5 did not bind to the fixed residue for the docking analysis. However, it did bind to other residues such as Glu850, Arg1080 and Thr864, through hydrogen bonds and polar interactions, which aided the peptide to be positioned in the active side of VEGFR2 (Rampogu et al., 2019).

p38 is part of the mitogen activated protein (MAP) kinases that mediate cellular behaviors that respond to extracellular stimuli, and it serves as a link for signal transduction mediator and other biological processes such as inflammation, cell death, cell cycle, cell growth, cell differentiation and senescence (Zarubin & Han, 2005). In humans, p38 has shown to be upregulated in tumors boosting cell survival, migration, resistance to stress and chemotherapeutic agents, and metastasis (Wagner & Nebreda, 2009). Abundant evidence shows the function of p38 as pro-oncogenic in various types of cancer opening the possibility of potential cancer therapies based on its inhibition (Martínez-Limón et al., 2020). PAP-4 reported a binding free energy of -173.29 kcal/mol with the target molecule, exhibiting the strongest binding affinity, closely followed by PAP-3 (-169.26 kcal/mol). However, the remaining PAPs also reported strong affinity values (<-145 kcal/mol). The binding interactions between PAP-4 and the target molecule p38 are presented in Table 4. Among the interactions reported are hydrogen bonds with the residues Asp113, Asp150 and Arg68, and polar and non-polar interactions with Gln115 and Met107, respectively.

Protein kinase B (PKB, also known as Akt) is a crucial regulator of cell proliferation and survival; its oncogenicity is due to the activation of proliferative and anti-apoptotic signaling, promotion of cell invasiveness and angiogenesis (Hill & Hemmings, 2002). Moreover, PKB is involved in the regulation of cell metabolism, protein synthesis and immune cell function (Henderson et al., 2015). Mutations on this kinase are one of the most frequent alterations found in human cancers such as breast, thyroid, and lung cancer

(Miricescu et al., 2020; Szymonowicz et al., 2018). Since PKB is involved in almost all aspects of cancer, its hyperactive conformation in malignant tumors is clinically relevant to the progression and outcome of cancer therapy (Szymonowicz et al., 2018). The interaction between the PAPs and PKB reported strong binding values (<-120 kcal/mol), being PAP-3 the one with the highest affinity displaying a binding free energy of -145.07 kcal/mol. These values indicate the potential of all five PAPs as PKB inhibitors (Mishra & Dey, 2019). The possible binding interactions between PAP-3 and the target molecule are presented in Table 4 and occur on common amino acid residues of PKB such as Asp275, Glu315, Thr119, and Lys277. The strong binding energy between the peptide and the molecule indicate its possible role as an inhibitor.

3.3.5 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases, which include MMP-2 and MMP-9. These proteases are either secreted or associated to the plasma membrane and are responsible for the degradation of components, such as collagen, fibronectin, and plasminogen, all of which conform the extracellular matrix (ECM) and the basement membrane (BM). Once activated, MMP-2 and MMP-9 degrade collagen IV and V in the ECM and BM disturbing their ability to avoid tumor cell movement. Thus, an overexpression of MMP-2 and MMP-9 in human cancers is strongly associated with metastasis and survival rates since they play key roles in the degradation of the extracellular matrices and promoting tumor invasion and metastasis (H. Li et al., 2017; Martínez-Limón et al., 2020). Additionally, a high expression of MMP-2 and MMP-9 has also been reported to influence cancer development and progression due to their participation in cell apoptosis, proliferation, and angiogenesis (Jiang & Li, 2021).

From the PAPs evaluated, all five had strong binding affinity values with of MMP-2 and MMP-9. However, PAP-3 exhibited the lowest binding free energies in both proteases with a ΔG of -191.66 kcal/mol for MMP-2 and -174.04 kcal/mol with MMP-9, indicating strong binding affinities. Despite PAP-3 showed the strongest binding affinity for both molecular targets, the possible types of interaction with each one are different which include hydrogen bonds and polar interactions, as shown in Table 4. The binding free energies obtained for both proteases indicate the possibility of PAPs, particularly of PAP-3, to inhibit the activity of MMP-2 and -9 and therefore decrease the metastatic process.

3.3.6 Caspases

Caspases are a family of cysteine proteases that play a crucial role in the initiation and execution of apoptosis. These proteases exist as inactive zymogens in the cells known as pro-caspases (F. Wang et al., 2014). Caspases are grouped into two subfamilies, initiators, and effectors, categorized according to their position in the apoptotic signaling cascade. The initiator caspase group is formed by caspase-1, -2, -4, -5, -8, -9, -10, -11, -12, and the effector group by caspase-3, -6 and -7 (Boice et al., 2022). Among these, caspase-3 is a key effector caspase that plays an essential role in the induction of cell death through apoptosis (Yadav et al., 2021). Caspase-3 exists in the cell as a low activity zymogen, procaspase-3, which requires activation by proteolysis, to then enter the nucleus to induce the programmed cell death pathway (F. Wang et al., 2014). Overexpression of procaspase-3 has been reported in a variety of human tumors, including colon cancer (Putt et al., 2006), lung cancer (K?epela et al., 2004), melanoma (Fink et al., 2001), hepatoma (Persad et al., 2004) and breast cancer (O'Donovan et al., 2003). Caspase-7, as caspase-3, is an effector caspase whose low cleavage/activation levels have been associated with unfavorable clinical outcome in cancer patients (Lidner et al., 2018). For that reason, bioactive compounds that specifically increase the conversion of procaspase-3 and procaspase-7 to its active form, caspase-3, and caspase-7, may represent a selective cancer therapy that is not toxic to normal cells.

On the other hand, Caspase-9 is the most studied initiator caspase since it has a major role in the intrinsic or mitochondrial apoptotic pathways. An unsuccessful activation of caspase-9 by the apoptosome has demonstrated negative physiological and pathological outcomes, including the development of cancer (P. Li et al., 2017). Owing to the relevant function of the caspase-9 of converting a death signal into the first proteolytic event that leads to the activation of executioner caspases, the activation of caspase-9 is a promising therapeutic goal for the treatment of cancer.

As described in Table 4, the caspases evaluated in the present study were not fixed to a specific amino acid residue for the molecular docking analysis, since no description was found in literature about the specific amino acid residues on the molecules' active sites. However, through random docking simulations, strong binding affinities were exhibited by the PAPs to the caspases.

The docking analysis reported that PAP-3 had the strongest binding affinity values with procaspases-3 and -7 reporting ΔG s of -159.65 and -175.94 kcal/mol, respectively. However, the other four PAPs also reported high affinity values with ΔG that range

from -118.75 to -156.35 kcal/mol for procaspase-3, and from -146.11 to -168.12 kcal/mol for procaspase-7. Also, PAP-3 reported a strong binding energy with caspase-9 with a ΔG of -183.56 kcal/mol, closely followed by PAP-5, PAP-2, PAP-4 and PAP-1 with ΔG s of -167.07, -165.34, -152.73 and -141.47 kcal/mol, respectively. These strong binding energies and binding interactions presented in Table 4, suggest that the PAPs, particularly PAP-3, could participate in the conversion of procaspases to their active form, and therefore, favor the clinical outcome of cancer patients.

3.3.7 Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)

The induction of apoptosis in tumor cells is a promising anticancer treatment strategy. There are two principal pathways through which apoptosis can occur, the intrinsic and extrinsic pathways. The extrinsic apoptosis pathway is triggered by the death receptor activation by ligands such as TRAIL (Yuan et al., 2015). Due to its ability to induce apoptosis in tumor cells but not in normal cells, TRAIL has become a promising target for cancer therapy (Kojima et al., 2011). PAP-3 revealed strong binding affinity with towards TRAIL with a ΔG of -215.76 kcal/mol, establishing its possible role mediating apoptotic cell death. As for PAP-1, -2, -4 and -5, they also exhibited strong binding affinities by ΔG s < -150 kcal/mol. As reported in Table 4, the type of binding predicted between PAP-3 and TRAIL is through the residue Glu271 with an acid contact.

3.3.8 Survivin

Survivin is the smallest of the inhibitor apoptosis proteins (IAPs) family, and it performs important roles in cell cycle, apoptosis, angiogenesis, and cancer development and progression (Warrier et al., 2020). In cancer cells, it is found in different cellular compartments, mainly in the nucleus, cytoplasm, mitochondria, and extracellular space (Duffy et al., 2007). As for its role in cancer evolution, studies have reported that an increased expression of survivin in the G2/M phase of cell cycle aids cell division and mitosis (Mita et al., 2008) and that its ability to enhance VEGF transcription, synthesis, and release, is key to the advancement of angiogenesis (Sanhueza et al., 2015). Moreover, survivin was recently found to be involved in DNA damage repair and immune response (Gil-Kulik et al., 2019). The clear correlation between the increased expression of survivin to aggressive and invasive tumors, reduced response to cancer therapies, poor prognosis, and recurrence of the disease (Altieri, 2013, 2015), make this protein an exceptional target for cancer therapeutics. From the binding simulation of the PAPs with the survivin protein, all five peptides reported high binding affinities below -130 kcal/mol, being PAP-4 the peptide with the strongest bond since it reported a docking score of -158.31 kcal/mol. The potential binding interactions between PAP-4 and survivin are presented in Table 4. PAP-4 reported a hydrogen bond with the amino acid Glu40, which is found to be a participant residue for the possible inhibitory activity of the anticancer peptides on the protein (Mishra & Dey, 2019). Other relevant interactions reported include hydrogen bond with Glu94 and Lys15, and a non-polar interaction with Phe86, which are found in a cavity of the molecule (Quispe et al., 2019).

3.3.9 Tumor suppressor p53

p53, considered as the “guard of the genome”, is a tumor suppressor protein and the principal responder to cellular stress signals such as oncogene activation, DNA damage, hypoxia, reactive oxygen species, including others (Zhu et al., 2020). Among the responses p53 executes upon activation are cell cycle arrest (to attempt restoration of DNA integrity), apoptosis or senescence (Levine, 2020). Mutations on gene TP53, which encodes for protein p53, are found in over half of human cancers, being the most common mutant human gene in cancer patients. The primary outcome of TP53 mutations is the loss of wild type p53 “normal” functions that result in responses that support tumor progression from mutant p53 (Mantovani et al., 2019).

For docking analysis purposes, two forms of the p53 protein were evaluated in this study: a mutant type and a wild type. PAPs exhibited strong binding affinities (<-103 kcal/mol) with the mutant type p53. As for the wild type p53, the five evaluated PAPs reported binding free energies below -115 kcal/mol, representing strong binding affinities. For the mutant and wild types of p53, PAP-5 reported the strongest binding affinities with ΔG s of -180.05 and -143.52 kcal/mol, respectively. The possible interactions that take place between PAP-5 and the amino acid residue of each type of p53, are presented in Table 4. Particularly, PAP-5 reported interaction with the amino acid Asp228 from the mutant type, which is described to be a key residue in the structure of the protein since it is found on the S7-S8 loop of the binding region, resulting on its stabilization, prevention of its displacement (Boysen et al., 2019; Hientz et al., 2016) and possible rescue of the mutant's DNA binding function (Raghavan et al., 2019). As for the fixed amino acid residue His179 for the wild type of p53, PAP-5 did not report binding interactions with it. However, it displayed polar interactions with Asn239 and Gln136, and a non-polar contact with Ala276.

As reported on Table 3, all five PAPs evaluated reported high binding energies (> 100 kcal/mol) with the target molecules either related to cancer or apoptosis. However, PAP-3 was the most representative peptide displaying the lowest binding free energies on several of the evaluated targets, indicating stronger affinities.

The results presented in Table 3, set evidence for the potential use of PAP-3 as an adjuvant therapeutic for cancer treatment. As shown in Table 2, the sequence of this potential anticancer peptide is composed by 6 aminoacids, including positively charged residues such as lysine and arginine, to which its cationic net charge (+ 3) can be attributed. The amino acid sequence of the peptide also contains hydrophobic residues (valine and phenylalanine) which account for the hydrophobic nature of the peptide that would allow the interactions with the molecular targets. Additionally, the toxicity of a therapeutic peptide has been related to its hydrophobicity; accordingly, the prediction of non-toxicity of PAP-3 could be associated to its 33.33% of hydrophobicity which is within the range of reported peptide safety (Felicio et al., 2017; Hoskin & Ramamoorthy, 2008; Oelkrug et al., 2015).

Currently, there is no consensus as for the structural factors that could aid in the experimental or computational identification of allergenic peptides (Dimitrov et al., 2013; James et al., 2018). However, the free available web server AllerTOP reports a highly sensible and specific prediction of allergenicity based on an alignment-independent method that evaluates the main physicochemical properties of peptides that aim to avoid an allergic reaction, such as number of residues, molecular weight, isoelectric point, net charge at pH 7, estimated water solubility and extinction coefficient (Dimitrov et al., 2013). According to this predictor, PAP-3 shows promise to be a non-allergen and therefore avoid an immune response.

The physicochemical characteristics of PAP-3 predicted by *in silico* tools, only confirm the potential use of this peptide as a nutraceutical or ingredient of functional food, that acts as an adjuvant for cancer therapy. The fundamental difference between functional foods and nutraceuticals is that the former offers, in addition to nutrition, health benefits by supplying one or more active ingredients within the food matrix and the latter refers to ingredients or components that have been isolated or concentrated and offer medical benefits such as the treatment of diseases (Girija, 2018). Either as nutraceuticals or ingredients of functional foods, it can be suggested that anticancer peptides, such as PAP-3, may help to decrease the number of new cases and mortality rates in the near future (Chiangjong et al., 2020).

Although the potential of peptides in cancer therapy is eminent, there are some limitations to their use, being the most relevant: a short plasma half-life and low bioavailability when administered orally (Liscano et al., 2020; Quintal-Bojórquez & Segura-Campos, 2021). To overcome these obstacles on both *in vitro* and *in vivo* studies, would mean to achieve peptide stability throughout gastrointestinal digestion, peptides would be able to safely reach the desirable sites of action (Bhandari et al., 2020; Chiangjong et al., 2020) and exert anticancer activities.

4. Conclusion

The prediction of the physicochemical characteristics of peptides and the estimation of their binding affinities through *in silico* tools allow a guided discovery and development of potential anticancer biomolecules, saving important resources such as time, cost, and labor. Through proteomic, bioinformatic and statistic tools, 1186 peptides were identified from *S. hispanica*'s < 1 kDa protein fraction and five peptides (PAPs) were identified that could possibly present anticancer activities due to their predicted physicochemical properties. Through molecular docking studies, the binding of the PAPs to target molecules of interest for cancer treatment was simulated. Despite all five peptides reporting strong binding affinities, PAP-3 exhibited the lowest binding free energies. Based on the results obtained from this study, the investigation group that sustains this manuscript has determined to continue with the development of the corresponding *in vitro* and *in vivo* evaluations of the PAPs to further determine their potential use as nutraceuticals or ingredient to functional foods that could aid cancer therapy.

Declarations

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Conflict of interest statement

The authors have no competing interests to declare that are relevant to the content of this article.

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