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In vitro apoptotic activity of *Lablab purpureus* (L.) Sweet low-molecular-weight peptides

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Abstract

Cancer prevalence is a rapidly growing concern globally. Traditional treatment protocols, radiotherapy, and chemotherapy are associated with adverse side effects on the human body. Therefore, there is a need for alternative therapies. Peptides from leguminous protein are known for exhibiting positive biological activity that could target cancer cells and minimize side effects. This study assessed the apoptotic ability of peptide fractions 2. The fraction was derived from the pepsin hydrolysate of *Lablab purpureus* that was fractionated by ultrafiltration and subsequently by reverse-phase high-pressure liquid chromatography. The markers of apoptosis (caspase-9 and -3, p53, and annexin V-PI) were then observed for efficacy. Caspase-9 and -3 showed activity to a greater degree in cancerous cell line A549 (2.3-fold and 1.1-fold) and MCF-7 (2.7-fold and 1.8-fold), respectively, compared to camptothecin (positive control). Expression of p53 on treated cancerous cell lines (A549 and MCF-7) demonstrated greater ability over the non-cancerous cell line (HEK293) with A549 showing the highest activity at 29.923 µg/mL. Finally, annexin V-PI staining sorted cells into phases of apoptosis. Treated A549 and MCF-7 cells detected 85.4% and 89.6% of cells undergoing apoptosis. Overall, Fraction 2 triggered apoptosis in cancer cell lines while minimally harming non-cancerous cells.

Keywords: *Lablab purpureus*; caspase-3; caspase-9; p53.

Practical Application: Peptides derived from L. purpureus have shown to exhibit anticancer potential against cancerous cell line A549 (Lung cancer cells) and MCF-7 (Breast cancer cells) while having limiting impact on healthy cells (HEK293). For practical application of these peptide, animal and clinical studies would need to be conducted.

1 INTRODUCTION

Globally, cancer affects approximately 19.3 million people and is one of the leading causes of death (Quintal-Bojórquez & Segura-Campos, 2021, Rayaprolu et al., 2017; WHO, 2020). Traditional cancer therapies, chemotherapy, and radiotherapy are effective in attacking cancer cells. However, side effects are experienced because the drugs also affect normal cells in the body (Chen et al., 2019, Huang et al., 2011; Orona-Tamayo et al., 2018; Quintal-Bojórquez & Segura-Campos, 2021). Alternative cancer treatment would be a solution to overcome this issue. An alternative anticancer agent should therefore have a targeted approach and be nontoxic to healthy cells (Sipahli et al., 2022). Apoptosis or programmed cell death is a normal process that occurs in the lifecycle of a healthy cell. Damaged cells evade this process resulting in the development of tumors (Alison, 2001; D'Arcy, 2019; Letai, 2017; Zivny et al., 2010).

Tumor suppressor protein, p53, plays a protective role in decreasing the development of cancers by initiating apoptosis in "suspicious" cells (Brown and Attardi, 2005, Letai, 2017; Marqus et al., 2017; Xu et al., 2019). Subsequently, the expression of pro-apoptotic proteins is induced (Bowne et al., 2007). Caspases from the group of cysteinyl aspartate-specific proteases are responsible for the disassembly of cells during apoptosis (An et al., 2019; D'Arcy, 2019). Annexin V, from the phospholipid-binding annexin family, can bind to a marker of apoptosis, phosphatidylserine (PS); therefore, Annexin V-PI staining determines apoptotic cells by their ability to bind to PS (Logue et al., 2009). Nutraceuticals have been studied for their ability to induce apoptosis in cancer cells (Gupta and Bhagyawant, 2021). Bioactive peptides derived from legumes have been associated with lower-risk cancers (Barman et al., 2018; Orona-Tamayo et al., 2018). A study conducted by Tak et al. (2021) suggested that bioactive peptides can arrest cancer development by hindering specific pathways by inhibiting inflammation and cell proliferation, thereby inducing apoptosis. *Lablab purpureus* is an underutilized crop cultivated in Africa. The legume has a protein content of 18–25% and a good balance of essential and non-essential amino acids (Cheng et al., 2019; Naiker et al., 2020).

Enzymatic hydrolysis is commonly preferred for the extraction of bioactive peptides. Toxic secondary metabolites are not produced during hydrolysis. The process also stimulates gastrointestinal digestion, subsequently allowing for a shorter reaction time (Quintal-Bojórquez & Segura-Campos, 2021). A previous study by Sipahli et al. (2022) provided the basis for this study. The authors showed that *L. purpureus* hydrolysates derived from pepsin hydrolysis exhibited potential chemopreventative activity. The lung cancer cell line, A549, showed 2.5-fold greater cytotoxicity compared with the chemopreventative agent, camptothecin (Sipahli et al., 2022). The pepsin hydrolysate was also found to be more effective in initiating apoptosis in the A549

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and MCF-7 cancer cell lines. Annexin V-PI staining showed that 41.5% (A549) and 33.4% (MCF-7) of cells were undergoing early apoptosis, while the positive control (camptothecin) showed 31.6%. The healthy cell line (HEK293) showed 77.2% of cells in early apoptosis, indicating that healthy cells were least affected by the pepsin hydrolysate (Sipahli et al., 2022). This study assesses the apoptotic activity of the pepsin hydrolysates from *L. purpureus* that have been fractionated using ultrafiltration and reverse-phase high-pressure liquid chromatography (RP-HPLC).

2 MATERIALS AND METHODS

2.1 Sample preparation

Lablab purpureus (L.) Sweet samples were collected in Durban (KZN, South Africa), with seeds dehulled, dried, milled, and sieved (180 µm sieve). The flour prepared from the seeds was then defatted with hexane at a 1:5 (w/v) ratio for 16–24 h. Excess hexane was removed, and the flour was allowed to dry under a fume hood overnight and stored at 4°C until analysis.

2.1.1 Protein extraction

Protein isolates derived from *L. purpureus* were prepared using defatted flour reconstituted in distilled water (1:5; w/v) and adjusted to pH 10 (He et al., 2013). The solution was then stirred at 37°C for 2 h and centrifuged (Eppendorf, Hamburg, Germany) at 10,000x*g* (45 min at 4°C). The supernatant was then adjusted to pH 5 and centrifuged, and the pellet was resuspended in distilled water (pH 7). The protein isolate was subsequently freeze-dried (Virtis Sentry 2.0, Pennsylvania, USA) and stored in a biofreezer at -80°C until required.

2.1.2 Preparation of protein hydrolysates and fractions

Protein isolate was reconstituted in distilled water (1:20; w/v), adjusted to pH 2, and preincubated for 20 min at 37°C. Then, pepsin (Sigma Aldrich (Missouri, USA) — 561 U/mg; pH 2) was added at 5% of the substrate (dwb) (Tang et al., 2009). The substrate-enzyme solution was then incubated at 37°C for 4 h, with the pH maintained at 2. Then, to stop the reaction, the solution was heated (100°C; 10 min), cooled on ice to 37°C, and centrifuged at 10,000x*g* (30 min at 4°C). The supernatant was fractionated by ultrafiltration stirred cell Amicon 8400 using <1, 3, 5, 10, and >10 kDa filters (Merck Group, Darmstadt, Germany). Collected fractions (<1, 3, 5, 10, and >10 kDa) were freeze-dried (Virtis Sentry 2.0, Pennsylvania, USA), with samples stored in a biofreezer at -80°C until required for analysis.

2.1.3 Cell culture

The cell lines used in this study [human embryonic kidney (HEK293), breast cancer (MCF-7), and human lung cancer (A549)] were grown at 37°C in a 5% CO_2 incubator using Dulbecco's modified Eagle's medium (DMEM) (Dwarka et al., 2017).

2.1.3.1 Cytotoxicity assay

Cytotoxicity was measured using the MTT assay with 50 µL of cells (1x10^6 cells/mL) in DMEM seeded in a 96-well flat-bottom plate and incubated for 24 h at 37°C in 5% CO_2. Cells were then treated with 50 µL of the sample prepared in 5% dimethyl sulfoxide (DMSO) (1,000-7.8 µg/mL) with camptothecin used as a positive control with untreated cells serving as the negative control and incubated for 24 h. An aliquot of MTT solution (20 µL; 5 mg/mL) was then added to cells and incubated for 4 h at 37°C in 5% CO_2. A volume of 100 µL of DMSO was then added, and the absorbance was read at 570 nm (Multiscan Go, Thermo Scientific, Waltham, MA, USA). Percentage viability was determined using Equation 1, with the most active ultrafiltration fraction further purified by RP-HPLC.

$$\%\ Cell\ viability = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells} \times 100 \qquad [1]$$

2.1.4 Peptide fractionation using reverse-phase high pressure liquid chromatography

RP-HPLC (Shimadzu Corporation, Kyoto, Japan) coupled with a photodiode array detector was used to further fractionate the selected sample. The sample was passed through a Zorbax 300SB-C8 column (9.4x250 mm ID, 5 µm particle size, and 300 Åpore size) (Agilent Technologies, Santa Clara, CA, USA). Mobile phase A contained 0.1% trifluoroacetic acid (TFA) in water, while mobile phase B contained 0.1% TFA in acetonitrile. The flow rate was maintained at 1 mL/min with a fixed column temperature of 32°C. Separation was performed with a stepwise gradient, 10–60% of mobile phase B (increasing 10% every 10 min) over 60 min. Fractions at each major peak were collected, freeze-dried, and stored at -20°C until required for analysis.

2.1.5 Caspase-3 and -9 ELISA assay

The Human Caspase-3 and -9 ELISA kits (Cat No. E-EL-H0663 and E-EL-H0017, Elabscience, Wuhan, China) were used according to the manufacturer's protocol. Briefly, cells were seeded (1x10^6 cells/mL) into a T25 cell culture flask and incubated at 37°C in 5% CO_2 for 24 h. The selected fraction and camptothecin were then used to treat cells at IC_{50} concentrations determined by the MTT assay and incubated at 37°C in 5% CO_2 for 24 h. Treated cells were lysed using the freeze-thaw method and analyzed according to the manufacturer's protocol with the absorbance read at 450 nm.

2.1.6 p53 ELISA assay

The p53 Human SimpleStep ELISA kit (Lot no. GR3221726-1, Abcam, Cambridge, UK) was used according to the manufacturer's protocol. Cells were seeded (1x10^6 cells/mL) into a T25 cell culture flask, incubated at 37°C in 5% CO_2 for 24 h, and subsequently treated with the selected fraction and camptothecin at IC_{50} concentrations determined by the MTT assay and incubated at 37°C in 5% CO_2 for 24 h. Treated cells were lysed using the extraction buffer and analyzed according to the manufacturer's protocol with the absorbance read at 450 nm.

2.1.7 Annexin V-PI assay

The FITC Annexin V apoptosis detection kit I (Cat no. 556547, BD Bioscience, Franklin Lakes, NJ, USA) assay was conducted according to the manufacturer's specifications. Cells were seeded into a 24-well flat bottom plate ($1x10^6$ cells/mL), incubated overnight, treated with 100 µL of the selected fraction for each of the cell lines, and analyzed using flow cytometry (BD LSRFortessa, BD Bioscience, Franklin Lakes, NJ, USA).

2.1.8 Reverse-phase liquid chromatography mass spectroscopy

The purity and molecular weight of the selected fraction were analyzed by RP-HPLC. The Prominence-i liquid chromatography-mass spectroscopy (LCMS) coupled with a Refractive Index Detector (RID-20A) (Shimadzu Corporation, Kyoto, Japan) was used with a Phenomenex Yarra 3 µm SEC 3000 (300x7.8 mm) column (Torrance, CA, USA). The mobile phase comprised 50 mM Na phosphate buffer with 300 mM NaCl (pH 6.8) at a flow rate of 1 mL/min and a fixed column temperature of 30°C. Molecular weight standards were used to achieve a standard curve that was then used to calculate the unknown molecular weight.

2.1.9 Statistical analysis

Data were analyzed using one-way and two-way ANOVA (GraphPad Prism software, San Diego, CA, USA) with all analyses in triplicate and the results were presented as mean±standard deviation. Inhibitory concentrations (IC_{50}) were also determined using GraphPad Prism.

3 RESULTS AND DISCUSSION

3.1 Cell viability (MTT Assay) of peptide fractions

An effective anticancer agent should inhibit cancer cells at the lowest inhibitory concentration (IC_{50}) while leaving healthy cells relatively unharmed. The MTT assay confirms that the ultrafiltration fractions do not have a cytotoxic effect on the cells (Figure 1). The IC_{50} values were then established to determine the concentration at which 50% of the cells were inhibited. Low-molecular-weight fractions showed better inhibition of cancer cells as seen by the low IC_{50} values while minimally affecting healthy cells as observed by the higher IC_{50} values (Table 1).

Compared with the positive control, camptothecin, the 3 kDa fraction exhibited lower IC_{50} values in cancer cells, A549 (13.6 µg/mL) and MCF-7 cells (20.48 µg/mL). At a concentration of 15.63 µg/mL, the viability for the cells was 55.49% (A549) and 56.11% (MCF-7). The non-cancerous cell line, HEK293, at a concentration of 145.10 µg/mL, was 2.5-fold greater than camptothecin. The cell viability was 84.31% and 68.16% at concentrations of 15.63 and 125 µg/mL, respectively. Chen et al. (2019) corroborated that lower-molecular-weight peptides showed improved anticancer activity. Their <4 kDa derived from black soybean had the highest anticancer activity compared to their larger factions (4–6 and >6 kDa). The 4 kDa fraction displayed 2.28-, 5.91-, and 1.96-fold inhibition on HepG2, MCF-7, and HeLa cells, respectively (Chen et al., 2019). This study selected the 3 kDa fraction for further purification by RP-HPLC because it showed the best fit for each of the cell lines.

3.2 Cell viability (MTT) RP-HPLC Fractions

Cells treated with Fraction 2 (the 3 kDa fraction by RP-HPLC) exhibited IC_{50} values for A549 and MCF-7 of 141.80 and 250.00 µg/mL, respectively (Table 2). The A549 cell line showed a significant ($p<0.05$) increase compared to the positive control, camptothecin (Figure 2). The healthy cell line, HEK293, exhibited an IC_{50} of 179.00 µg/mL compared to camptothecin which was 126.80 µg/mL. The purified kla-TAT conjugated peptide showed the lowest IC_{50} value on the A549 cell line, while the highest IC_{50} was observed on the healthy cell line HaCat (Chen et al., 2019). This supports the findings in this study as the cancerous cell line was more sensitive to the peptide and had lower toxicity compared to the healthy cell line (Chen et al., 2019). Fraction 2 fractionated by RP-HPLC was selected for further analysis because the results showed the best fit for each of the cell lines. After RP-HPLC, collected fractions were run on SDS-PAGE, and no bands were visible in the lanes with fractions. The low molecular weight of the peptides could have run out of the gel (Taniya et al., 2020).

3.3 Caspase-3 and -9 assay

When caspase-9 is triggered, caspase-3 is activated, thereby signaling a caspase cascade and ultimately leading to apoptosis (Jan and Chaudhry, 2019). Cells treated with Fraction 2 were analyzed for their caspase-3 and -9 activity by ELISA (Table 3) at concentrations of 141.80 µg/mL (A549), 250 µg/mL (MCF-7), and 179 (HEK293). Caspase 3, the executioner caspase, showed the highest caspase activity in MCF-7 cells (0.137±0.0015 ng/mL). Cell lines, A549 and MCF-7, showed 2.3- and 2.7-fold greater activity compared to camptothecin. Conversely, camptothecin showed 0.63-fold greater activity than the healthy cell line (HEK293). Analysis of caspase-9 activity observed a similar trend by HEK293 cells as they exhibited 0.9-fold lower activity. It can therefore be deduced here that Fraction 2 did not harm the cells to a great degree. Cancer cells, MCF-7 and A549, showed 1.8-fold (0.205 ng/mL) and 1.1-fold (21.966 ng/mL) greater caspase-9 activity. Comparatively, purified peptides from kidney beans were also able to induce apoptosis in MCF-7 cells via the extrinsic pathway. This involved the upregulation of caspase-3 and -9 in cells (Rao et al., 2018).

3.4 Annexin V-PI assay

Based on the results, cell death induced by Fraction 2 was studied for apoptotic activity by observing PS translocation using the annexin V-FITC/PI assay. Cells were treated at concentrations of 141.80 µg/mL (A549), 250 µg/mL (MCF-7), and 179 (HEK293). Quadrants (Figure 3) are labeled as viable (lower left), early apoptosis (lower right), late apoptosis/dead (upper right), and necrotic (upper right). Cancerous cell lines, A549 and MCF-7, treated with peptide Fraction 2 reported 85.4% and 89.6% of cells in early apoptosis, respectively. Early apoptosis is

distinguished by the translocation of PS from the inner layer of the plasma membrane to the outer surface (Kwan et al., 2016).

Cells in late apoptosis were 14.2 and 10.4%, respectively, for each of the cancer cell lines. The healthy cell line comparatively showed a larger population in early apoptosis of 93.9%; however, 0.3% of the cells were still viable. The cancer cell lines did not show any viable cells. When compared to camptothecin, the cell lines showed an equal or better ability than the peptide Fraction 2. The cell lines did not observe necrosis, thereby indicating that the peptide Fraction 2 and camptothecin were not toxic to the cells.

Table 1. Inhibitory concentrations (IC_{50}) of A549, MCF-7, and HEK293 cell lines treated with *Lablab purpureus* fractions*.

Fraction	A549 (µg/mL)	MCF-7 (µg/mL)	HEK293 (µg/mL)
<1 kDa	20.20[a]	16.45[a]	181.60[a]
3 kDa	13.60[a]	20.48[a]	145.10[a]
5 kDa	15.27[a]	14.29[a]	36.30[a]
10 kDa	14.12[a]	15.54[a]	19.76[a]
>10 kDa	18.45[a]	51.40[a]	168.2[a]
Camptothecin	19.73[a]	44.60[a]	40.51[a]

*Data represent mean±SD (n=3). Values with different superscripts are significantly different (p<0.05).

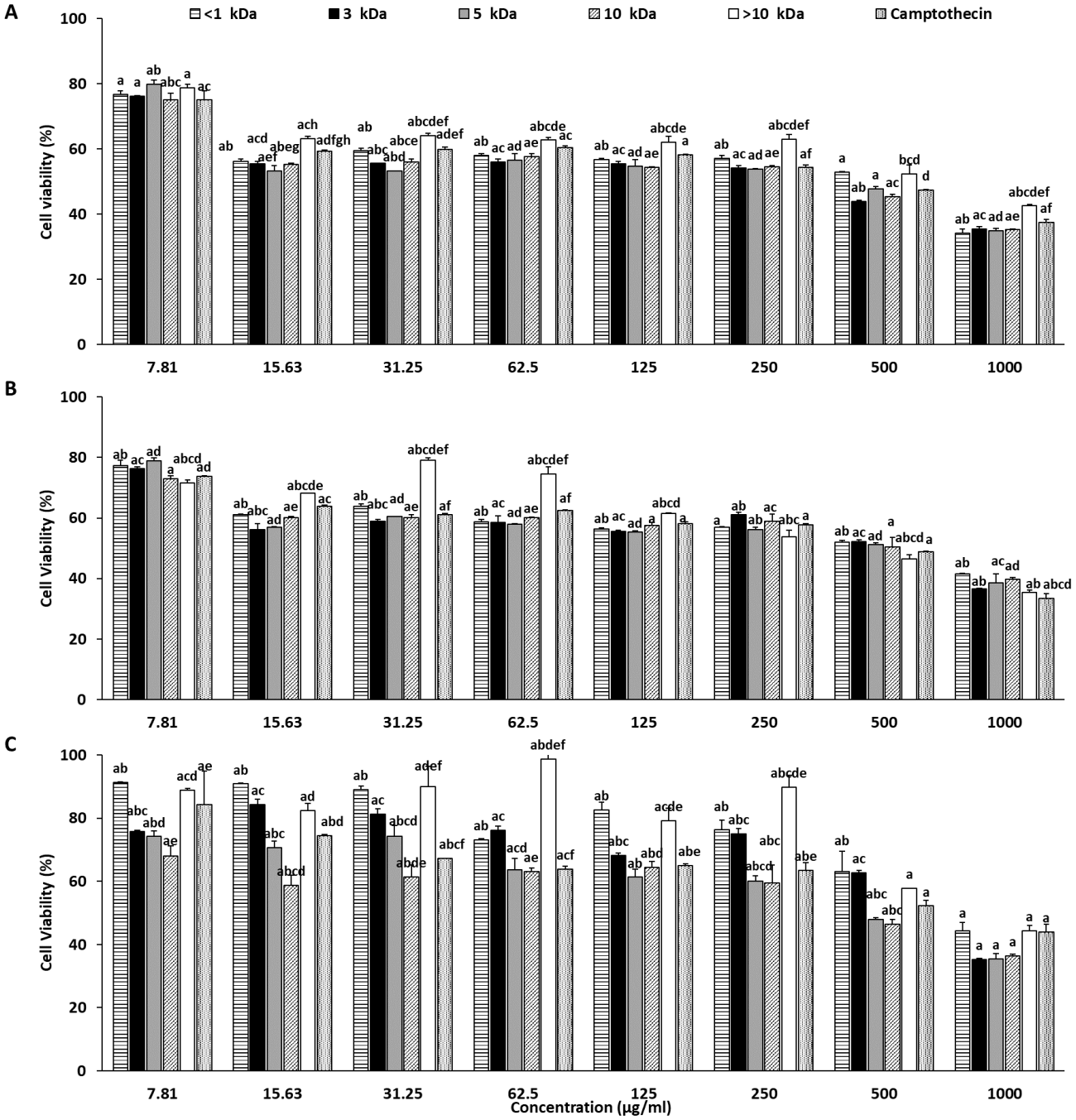

Figure 1. Cell viability of (A) A549, (B) MCF-7, and (C) HEK293 cell lines treated with *Lablab purpureus* fractions. Data represent mean±SD (n=3). Values with different superscripts are significantly different (p<0.05).

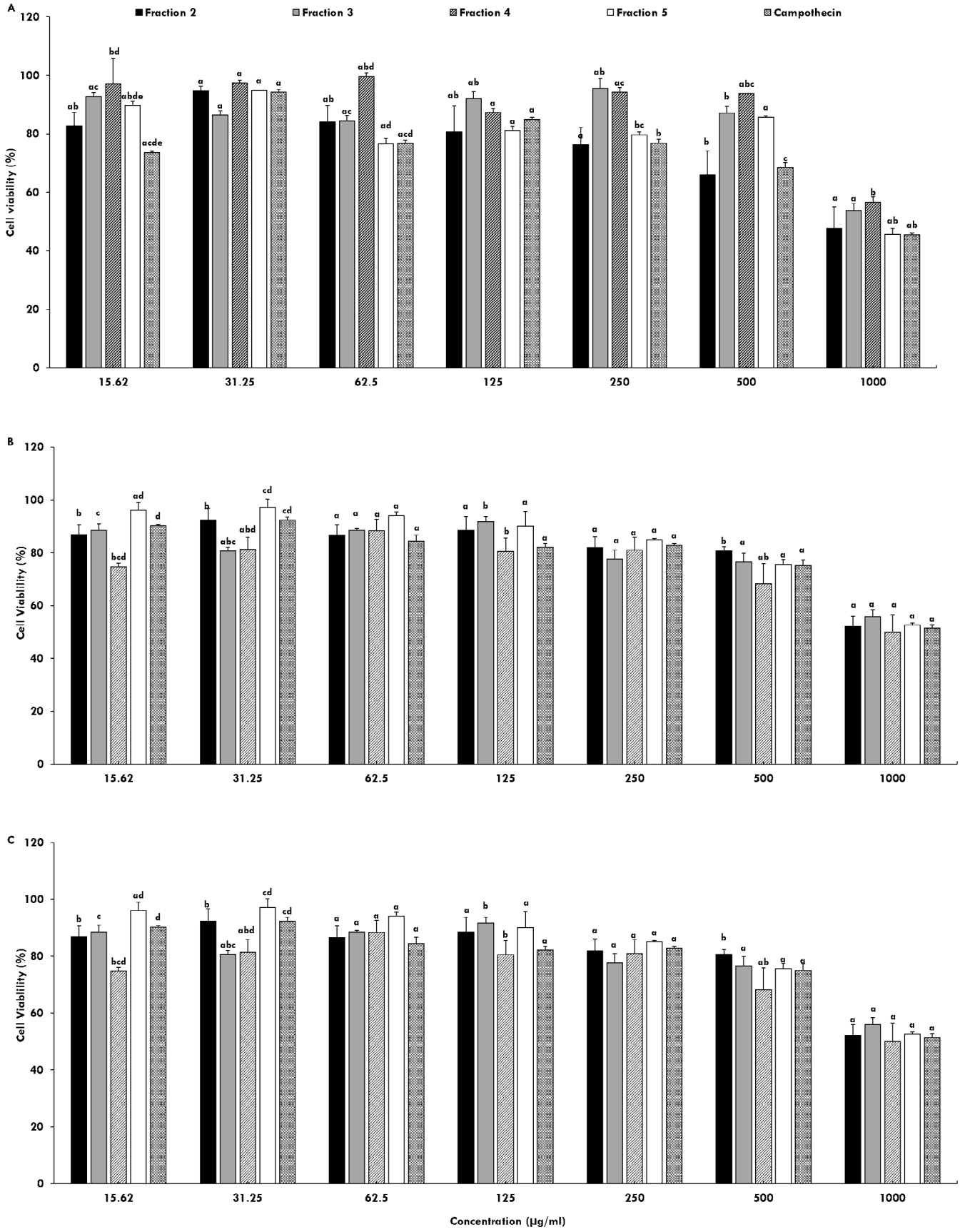

Figure 2. Cell viability of (A) A549, (B) MCF-7, and (C) HEK293 cell lines treated with *Lablab purpureus* RP-HPLC fractions. Data represent mean±SD (n=3). Values with different superscripts are significantly different ($p<0.05$).

3.5 p53 assay

The p53 ELISA kit was used to verify the apoptotic ability of the fractions and determine whether the peptide sequence can specifically bind to p53 (Xue et al., 2015). When p53 becomes activated in cancer cells, apoptosis is also activated by inducing and activating the expression of pro-apoptotic proteins (Bowne et al., 2007). Bioactive compounds have been reported to reduce p53 degradation and assist in the activation of p53 (Xue et al., 2015). The synthetic peptide derived from chickpeas exhibited an increase in p53 protein compared to its control (Xue et al., 2015). This study also observed higher p53 expression levels in cells treated with the peptide fraction compared to the positive control, camptothecin, and negative control, untreated cells. MCF-7 and A549 showed a 1.03- and 1.97-fold increase, respectively, when compared with camptothecin. Under normal conditions, p53 levels are low because of ubiquitin-dependent proteolysis. However, levels increase when cells undergo stress and eventually lead to apoptosis (Marqus et al., 2017). The results show a similar trend. Therefore, the increase of p53 could regulate signaling

Table 2. Inhibitory concentrations (IC_{50}) of A549, MCF-7, and HEK293 cell lines treated with *Lablab purpureus* RP-HPLC fractions*.

Sample	A549 (μg/mL)	MCF-7 (μg/mL)	HEK293 (μg/mL)
Fraction 2	141.80^{a}	250.00^{a}	179.00^{a}
Fraction 3	542.60^{a}	235.00^{a}	299.90^{a}
Fraction 4	712.70^{a}	265.00^{a}	190.70^{a}
Fraction 5	373.30^{a}	350.90^{a}	174.30^{a}
Camptothecin	255.20^{a}	239.40^{a}	126.80^{a}

*Data represent mean±SD (n=3). Values with different superscripts are significantly different ($p<0.05$).

Table 3. Caspase-3 and -9 activity on A549, MCF-7, and HEK293 cells treated with *Lablab purpureus* RP-HPLC Fraction 2 derived from pepsin hydrolysate*.

Sample	MCF-7		A549		HEK293	
	Caspase-3 (ng/mL)	Caspase-9 (ng/mL)	Caspase-3 (ng/mL)	Caspase-9 (ng/mL)	Caspase-3 (ng/mL)	Caspase-9 (ng/mL)
Treated	0.137^{ab}±0.015	0.205^{a}±0.065	0.067^{a}±0.032	21.966^{a}±0.302	0.076^{a}±0.003	0.191^{a}±0.064
Camptothecin	0.051^{ab}±0.002	0.112^{a}±0.014	0.029^{a}±0.017	20.486^{a}±0.172	0.121^{ab}±0.015	0.220^{a}±0.036
Untreated	0.041^{a}±0.002	0.155^{a}±0.020	0.016^{a}±0.007	20.148^{a}±0.496	0.012^{ab}±0.010	0.249^{a}±0.050

*Data represent mean±SD (n=3). Values with different superscripts are significantly different ($p<0.05$).

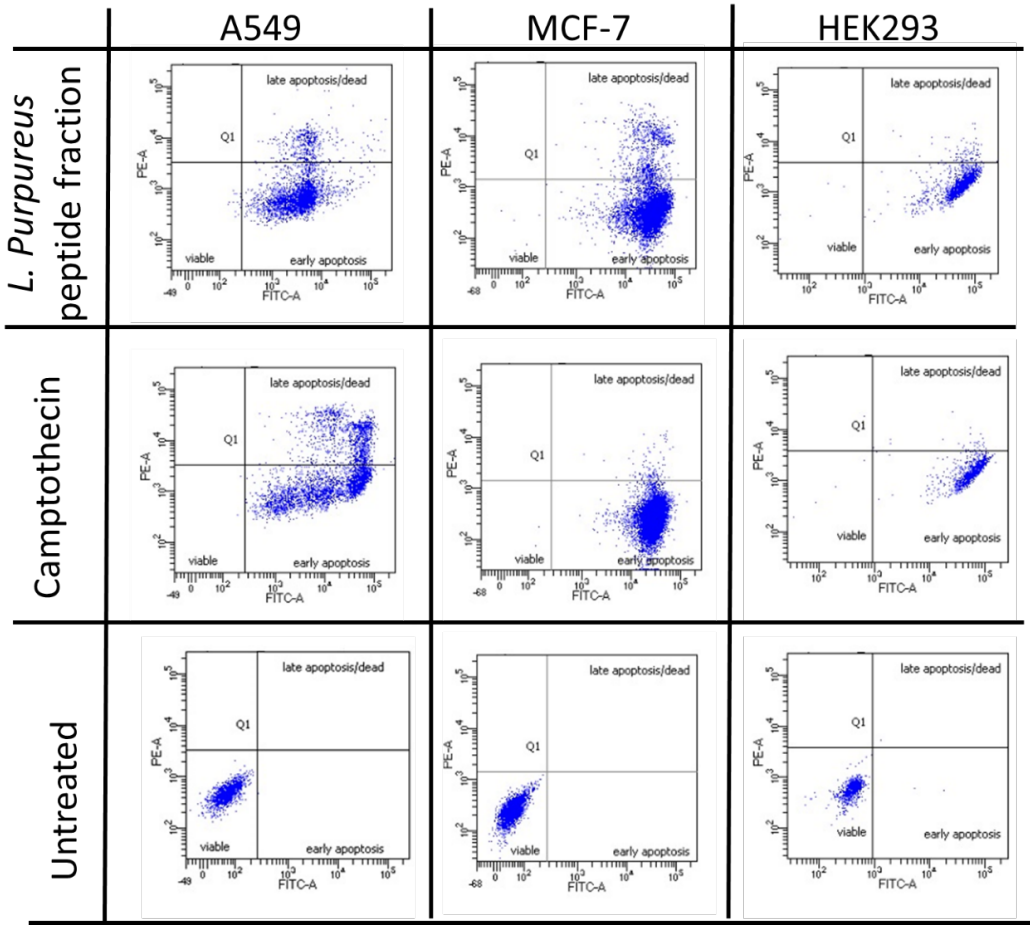

Figure 3. Flow cytometry analysis showing the externalization of phosphatidylserine in A549, MCF-7, and HEK293 cells treated with *Lablab purpureus* peptide Fraction 2, camptothecin, and untreated cells.

pathways, resulting in targeted apoptosis and mitochondrial oxidative phosphorylation (Quintal-Bojórquez & Segura-Campos, 2021). Healthy cells (HEK293) observed low p53 levels (Table 4).

3.6 RP-LCMS

The purity of Fraction 2 was determined by passing the sample through RP-LCMS (Figure 4). The fraction seems to be fairly pure as there is one large peak that appears on the chromatogram at a retention time of 11.85 min when detected at 214 nm. Fraction 2 was achieved by further purifying the 3 kDa fraction by RP-HPLC. The determined molecular weight of the largest peak was 13.01 Da. Further analysis would be required to determine the peptide sequence and structure.

4 CONCLUSIONS

The development of a new class of anticancer agents that lack toxicity to healthy cells is of utmost importance. Particularly, food-derived peptides have become desirable as nutraceuticals because of their enhanced bioactive abilities and their low toxicity. The peptide fraction derived from *L. purpureus* has demonstrated the ability to induce apoptosis. Caspase-3 and -9 are markers of apoptosis, which were observed by the cancerous cell line A549 and MCF-7. The healthy cell line, HEK293, showed lower caspase-3 and -9 activities. Similar findings were observed for the presence of p53. Finally, annexin V-PI staining confirmed that Fraction 2 does induce apoptosis in the cancer cells. Although this fraction can induce apoptosis in cancer cells (A549 and MCF-7) and has minimal impact on the non-cancerous cell line, further analysis is required to determine the peptide sequence and the structure of this peptide.

Table 4. Activity of human p53 in A549, MCF-7, and HEK293 cells treated with *L. purpureus* RP-HPLC Fraction 2 derived from pepsin hydrolysate*.

Sample	MCF-7 (ng/mL)	A549 (ng/mL)	HEK293 (ng/mL)
Treated	$9.021^{a}\pm0.024$	$29.923^{ab}\pm1.661$	$0.762^{a}\pm0.216$
Camptothecin	$8.697^{a}\pm0.252$	$15.195^{ab}\pm0.128$	$0.650^{a}\pm0.152$
Untreated	$6.776^{a}\pm0.080$	$21.888^{ab}\pm0.735$	$0.538^{a}\pm0.008$

*Data represent mean±SD. Values with different superscripts are significantly different ($p<0.05$).

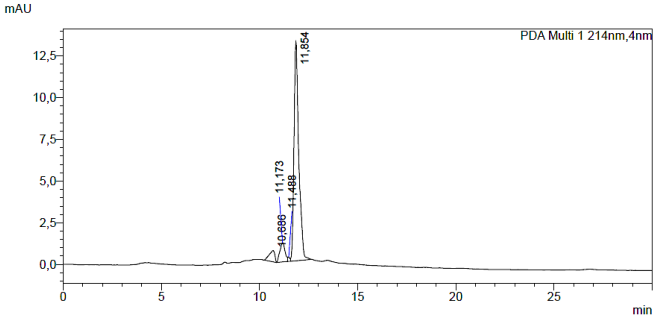

Figure 4. LCMS chromatogram of Fraction 2 from *Lablab purpureus* purified by RP-HLPC.

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