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Stability of an anticancer peptide isolated from Flathead by-products during in vitro gastrointestinal digestion

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ABSTRACT

Background: Several peptides from seafood have shown effective anticancer activities. Nonetheless, one of the most significant challenges in developing fish peptides as functional food ingredients is proving their efficacy as anticancer agents. This study was aimed to evaluate the anticancer capacity and stability of a purified peptide (H. Met-Gly-Pro-Pro-Gly-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg.OH) during a simulated gastrointestinal (GI) digestion.

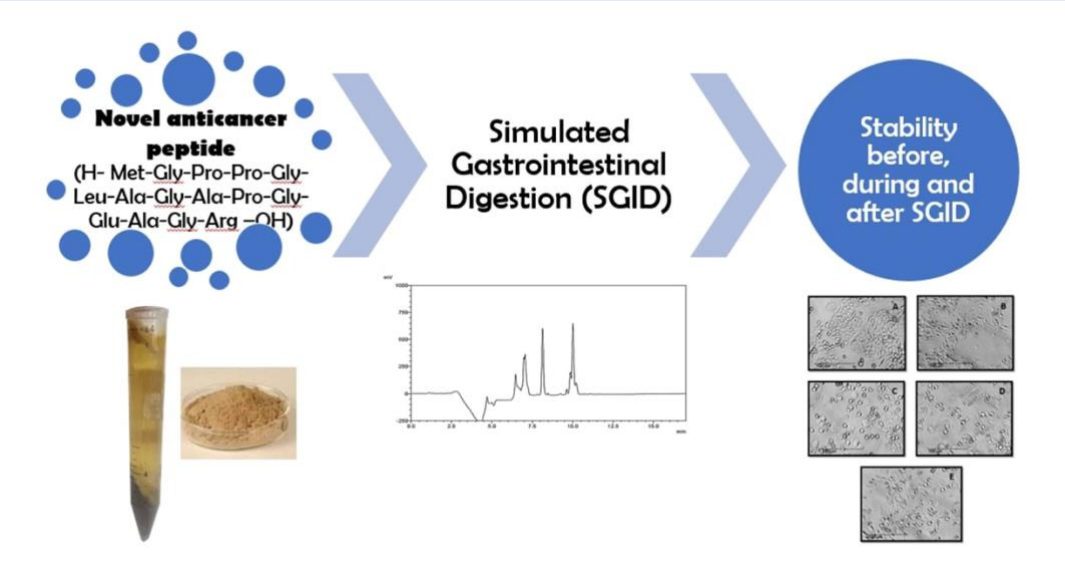
Methods: The anticancer activity of the peptide(s) before, during, and after GI digestion was analyzed against colon cancer cells (HT-29). Changes in cell morphology were assessed using an inverted microscope, while the degree of apoptosis was observed using a Muse Cell Analyzer.

Results: Results showed little or no hydrolysis of the bioactive peptide by pepsin was observed, indicating the peptide was resistant to digestion in gastric conditions. The growth of HT-29 cells was significantly inhibited ($P < 0.05$) by the un-digested peptide and peptide(s) present in the digesta that was yielded by gastric and gastrointestinal digestion up to

28.89%, 29.68%, and 38.3%, respectively. HT-29 cells treated with pepsin and pancreatin digested peptides showed the highest cell death (3.54±2.30%).

Conclusion: Overall, the findings showed that the purified peptide has the potency to be used in cancer therapy via oral administration and/or incorporation in food(s) applications for the treatment of specific cancer.

Keywords: bioactive peptide; digestion; fish by-products; hydrolysis



INTRODUCTION

Colorectal cancer is the second most diagnosed cancer in females and third in males, with an estimated 1.1 million cases and 551,269 deaths occurring in 2018 worldwide [1]. The increasing incidence of colorectal cancer is associated with an unhealthy diet, obesity, and smoking habits [2]. While the most common treatment for colorectal cancer is surgery combined with chemotherapy by cytotoxic drugs and radiation, this therapy is just moderately successful, especially in the late stages of cancer. Furthermore, such treatments often are associated with deleterious effects caused by drug-induced damage to healthy cells and tissue [3]. Thus, discovering new safe cancer drugs from natural products becomes an important goal of research in biomedical sciences.

Recently, an increasing number of new anticancer compounds have been identified in the marine environment [4]. Furthermore, protein hydrolysates and/or peptides from seafood also showed effective anticancer activities [5, 6]. Kannan *et al*. [7] reported that peptide fractions extracted from shrimp by-products inhibited the growth of human colon epithelial cancer cell line Caco-2 by 60% after 72 hours of exposure. Low molecular weight (< 3kDa) peptide fractions isolated from Flathead (*Platycephalus fuscus*) by-products were reported to show strong antioxidant and anticancer activities against HT-29 cell lines [8]. The purification and identification stage revealed a novel peptide with 15 amino acid residues (H.Met-Gly-Pro-Pro-Gly-Leu-Ala-Gly-

Ala-Pro-Gly-Glu-Ala-Gly-Arg.OH; Mw = 1337.51Da), predicted to have anticancer properties based on AntiCP prediction. In the present study, therefore, the intent was to establish the potency of this peptide as an anticancer agent.

One of the most significant challenges in developing fish peptides as functional food ingredients is proving their efficacy as bioactive components such as anticancer agents. The potential effect of the peptides depends on their capacity to reach the target organs. Gastrointestinal (GI) tract conditions may influence the primary structure and the intended functions of the peptides before they reach the required target sites [9]. Several fish bioactive peptides with ACE inhibitor and antioxidative properties have been assessed for their stability during GI digestion [10] [11]. Still, there is not much information on the stability of fish anticancer peptides during GI digestion. The objective of this study, therefore, was to evaluate the impact of simulated GI digestion on peptide structure by means of reversed phase high performance liquid chromatography (RP-HPLC) and to analyse the anticancer capacity of the peptides before and after GI digestion.

METHODS

Materials: The peptide (H- Met-Gly-Pro-Pro-Gly-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg -OH) was synthesized by Mimotopes (Clayton, VIC, Australia) at >95% purity. Staurosporine solution (from *Streptomyces* sp), trifluoroacetic acid (TFA), and pepsin (from porcine gastric mucosa) were obtained from Sigma-Aldrich Pty. Ltd. (Castle Hill, Australia). Pancreatin Amylase and Protease were purchased from U.S. Pharmacopeia (Rockville MD, USA). Acetonitrile was purchased from Merck (Darmstadt, Germany). All other chemicals used were of analytical reagent grade.

Stability of peptide against gastrointestinal (GI) protease: The peptide stability against the gastrointestinal proteases was assayed using an *in vitro* gastrointestinal model system [12]. Exactly 30 mg of the peptide was diluted in 10 mL KCl-HCl buffer. The pH was adjusted to 2.0 with a drop-wise addition of 1 M HCl. Pepsin was then added (E/S 1:35 w/w), and the mixture was incubated in an incubator shaker (Innova 4200, New Brunswick Scientific GmbH, Germany) for 60 min at 37□C. At the end of this period, an aliquot (2 mL) was sampled to establish the effects of pepsin digestion on the peptide stability and its anticarcinogenic properties. The pH of the solution was then adjusted to 5.3 with 1 M $NaHCO_3$ solution and further to pH 7.5 with 1 M NaOH. Afterward, pancreatin (E/S 1:25 w/w) was added, and the mixture was further incubated with continuous shaking for 3 hours at 37□C. The digestion was terminated by submerging the solution in boiling water for 10 min. As soon as the GI digest was cooled to room temperature, it was centrifuged at 12 000 *x g* for 25 min (Eppendorf Centrifuge 5415C, Crown Scientific Pty Ltd, Moorebank, NSW, Australia). The supernatant was then collected for analysis.

Peptide profiling by reversed-phase high performance liquid chromatography (RP-HPLC): The stability of anticancer peptide to digestive proteases in a stimulated GI digestion was assessed using RP-HPLC. 4 mg synthetic (undigested) peptide was dissolved in Milli Q water (1 mL) prior to centrifugation. All peptide solutions were centrifuged at 8161*g* for 30 min at room temperature. Peptides were separated on a Shimadzu HPLC system (Shimadzu Model LC-2030, Shimadzu Corporation, Kyoto, Japan) equipped with the Vydac Everest C18 column (Grace Davison Discovery Sciences, Rowville, VIC, Australia). The peptides were separated by a linear gradient of 0% to 100% of solvent B (0.1% TFA in acetonitrile) in solvent A (0.1% TFA in deionized water)

over 25 min. The flow rate was maintained at 0.75 $mL \cdot min^{-1}$, and eluted peptides were detected at 215 nm.

Anticancer effects of peptides: The colon cancer cell lines (HT-29) were grown and cultured in RPMI 1640 medium with 10 % Fetal Bovine Serum (FBS) and incubated at 37□C under 5% CO_2. HT-29 cells were purchased from American Type Culture Collection (Manassas, VA. USA). Passage 16-20 was used in cancer cell cytotoxic experiments.

The anticancer activity of peptides on HT-29 was determined using an MTS assay based on the previously published method [13]. Briefly, viable HT-29 cells (2.0 x 10^3) were seeded into each well of a 96-well plate containing 100 □L of the medium and incubated at 37 □C in 5% CO_2 for 24 h. After incubation, the spent media was removed, and cells were then treated with peptide samples (at 0.25 $mg \cdot mL^{-1}$). Unstimulated cells were used as a negative control, while Staurosporine (at 0.25 µM) was used as a positive control. After 72 h of cell exposure to the peptide samples, MTS reagent (20 □L) was added to the wells and incubated for an additional 1 h under the same conditions. The absorbance was measured at 490 nm using a microplate reader (iMark Bio-rad, Bio-Rad Laboratories Pty., Ltd., New South Wales, Australia). The experiments were repeated three times.

Morphology of the cells: The changes in morphology of the cells after treatments were assessed by an inverted microscope (Motic AE2000 Trinocular, Motic Incorporation Ltd, Hong Kong). Briefly, viable HT-29 cells (2.0 x 103) were seeded into each well of a 96-well plate as described previously. After 72 h incubation, the plate was mounted to the microscope and viewed with a magnification of 40x. The cells were photographed via a Moticam attached to the microscope. The images of cells were analyzed using software Motic Image Plus 2.0.

Extent of cell apoptosis: The degree of apoptosis of cell population is an essential parameter of cell health. A Muse Cell Analyzer (Merck Millipore, Australia) was used for the apoptosis experiment. In brief, HT-29 cells were plated in 12-well plates at a density of 2.0 x 10^3 containing 500 □L medium and incubated at 37 □C in 5% CO_2 for 24 h. After 24 hours of incubation, the spent media were removed and replaced with peptides solution or Staurosporine. The cells were incubated for 72 hours before being harvested for the apoptosis experiment. The final concentration of harvested cells used in the assays was between 1 x 10^5 to 1 x 10^7 cells/mL. The staining and assay protocol was performed based on the manufacturer's guide.

Statistical analysis: Data presented are the means ± SD of results from a minimum of three independent experiments with similar patterns unless otherwise mentioned. Statistical analysis was performed using Minitab 20, in which the one-way ANOVA was employed. Duncan's post-hoc test was used to verify the significant differences between the mean values ($P < 0.05$).

RESULTS

Stability of peptide against gastrointestinal (GI) protease: After oral administration, peptides must survive a series of possible hydrolysis by gastrointestinal protease before reaching target sites and becoming functionally active. In this work, the identified peptide Met_1-Gly_2-Pro_3-Pro_4-Gly_5-Leu_6-Ala_7-Gly_8-Ala_9-Pro_{10}-Gly_{11}-Glu_{12}-Ala_{13}-Gly_{14}-Arg_{15} was subjected to *in vitro* digestion using pepsin and pancreatin proteases. The peptide digestion during simulated gastric and intestinal phases of in *vitro* GI model was followed by peptide profiling by RP-HPLC (Fig 1 A-C).

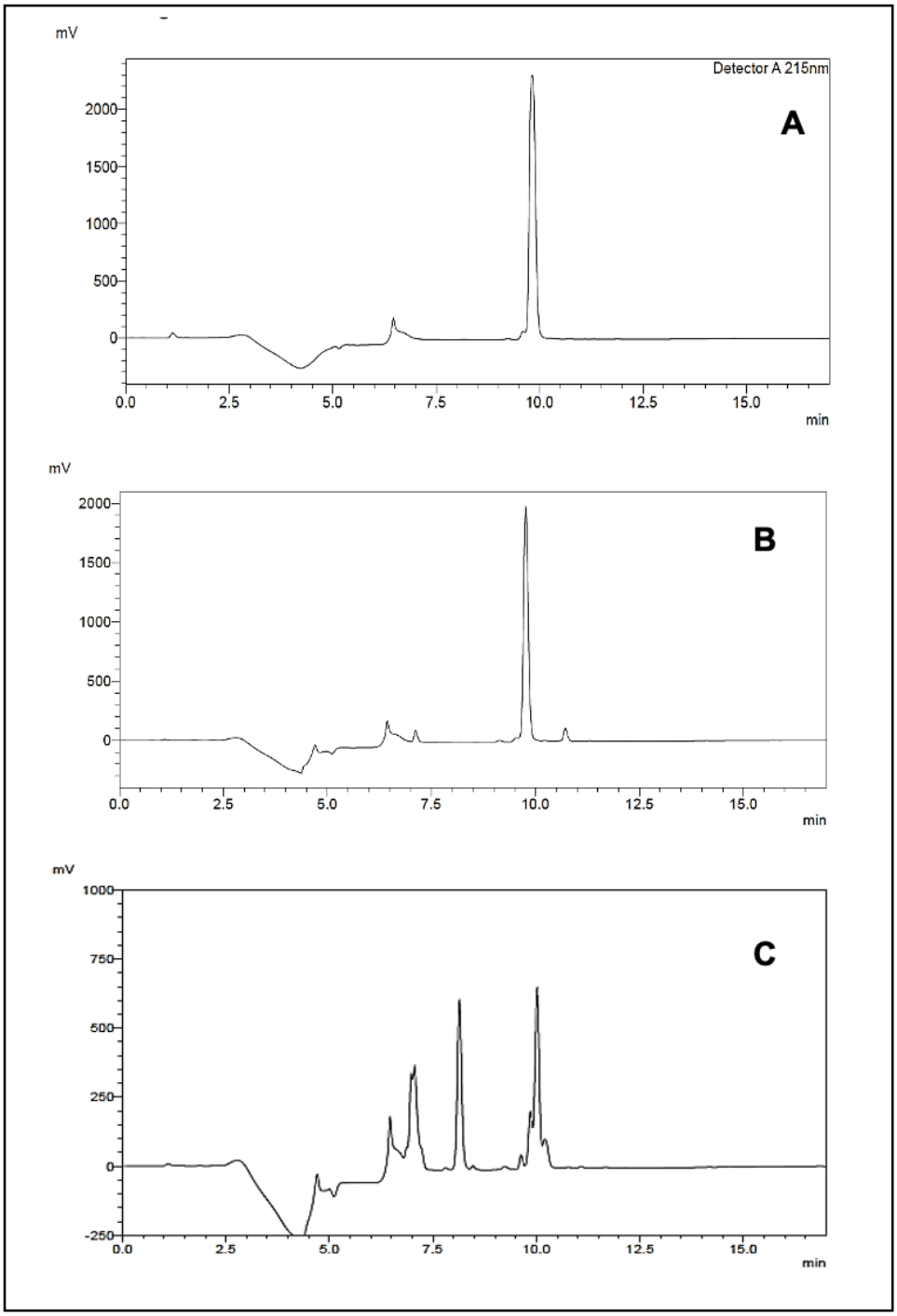


Fig 1. Reverse-phase HPLC profile of undigested peptide (A); pepsin digested peptide (B) and pepsin and pancreatin digested peptide (C).

Anticancer activity of purified peptide: Many research papers have reported the anticancer activity of purified peptides isolated from fish by-products [6]. In the current study, all peptides, including Staurosporine, showed anticancer activity by inhibiting the growth of HT-29 during 72 h incubation. Staurosporine, isolated from *Streptomyces staurospores*, showed very strong anticancer activity by reducing the cell viability to 19.4 %. In addition, the undigested and pepsin digested peptides (at 0.25 mg·mL^{-1}) were found to have a similar anticancer activity (Fig 2).

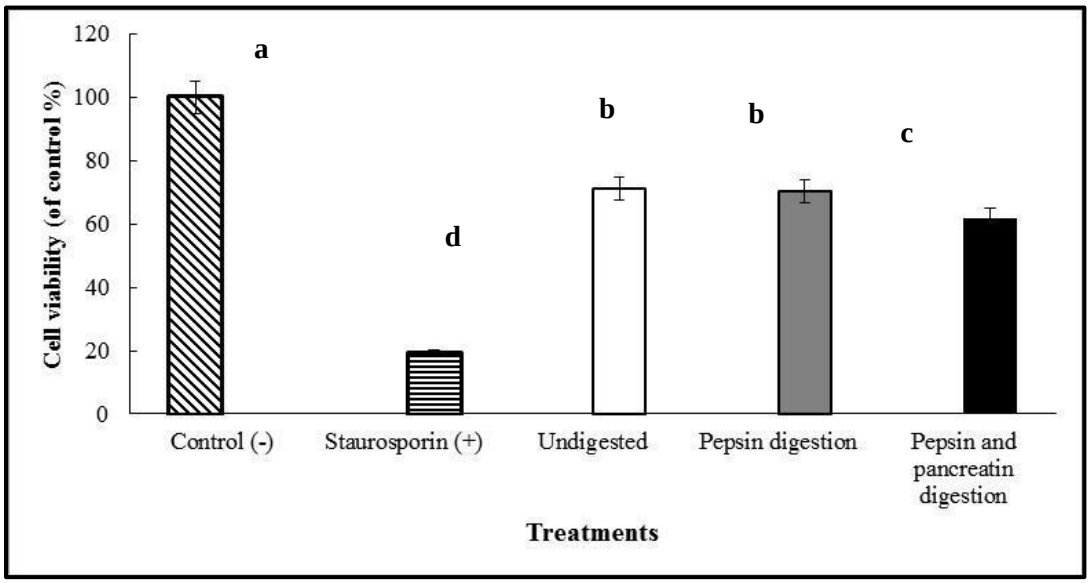


Fig 2. Anticancer activity expressed as cell viability, as a percentage of control, of HT-29 colon cancer cells treated with undigested and GI digested peptides (at 0.25 mg·mL^{-1}). Staurosporin (at 0.25 µM) was used as a positive control. Values with the different lowercase letters (a, b, c, d) indicate a significant difference ($P < 0.05$)

Assessment of cell morphological changes: In the present study, observation using an inverted microscope revealed that peptide treatments induced apoptosis in HT-29 cells (Fig 3). Staurosporine (Fig 3B) and digested peptide solution treatments (Fig 3C; 3D; 3E) caused substantial apoptotic morphological changes in HT-29 cells compared to the control (Fig 3A). The morphological features of apoptosis observed included apoptotic bodies and cell shrinkage (arrow).

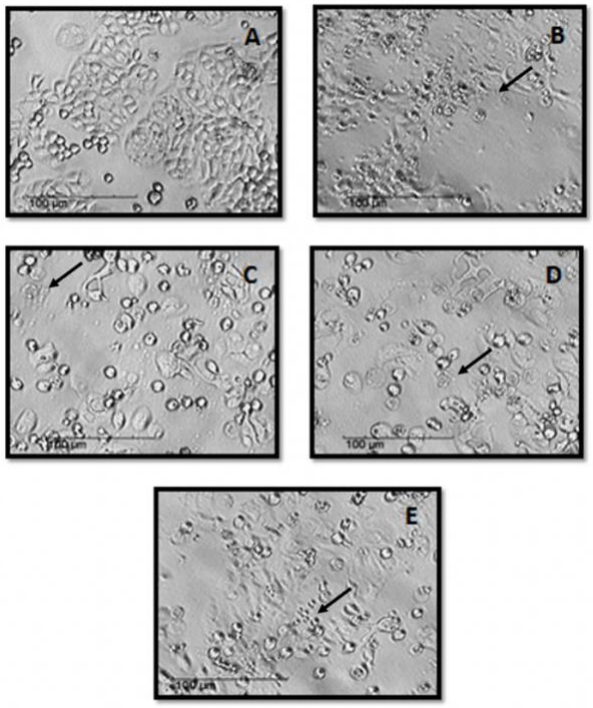
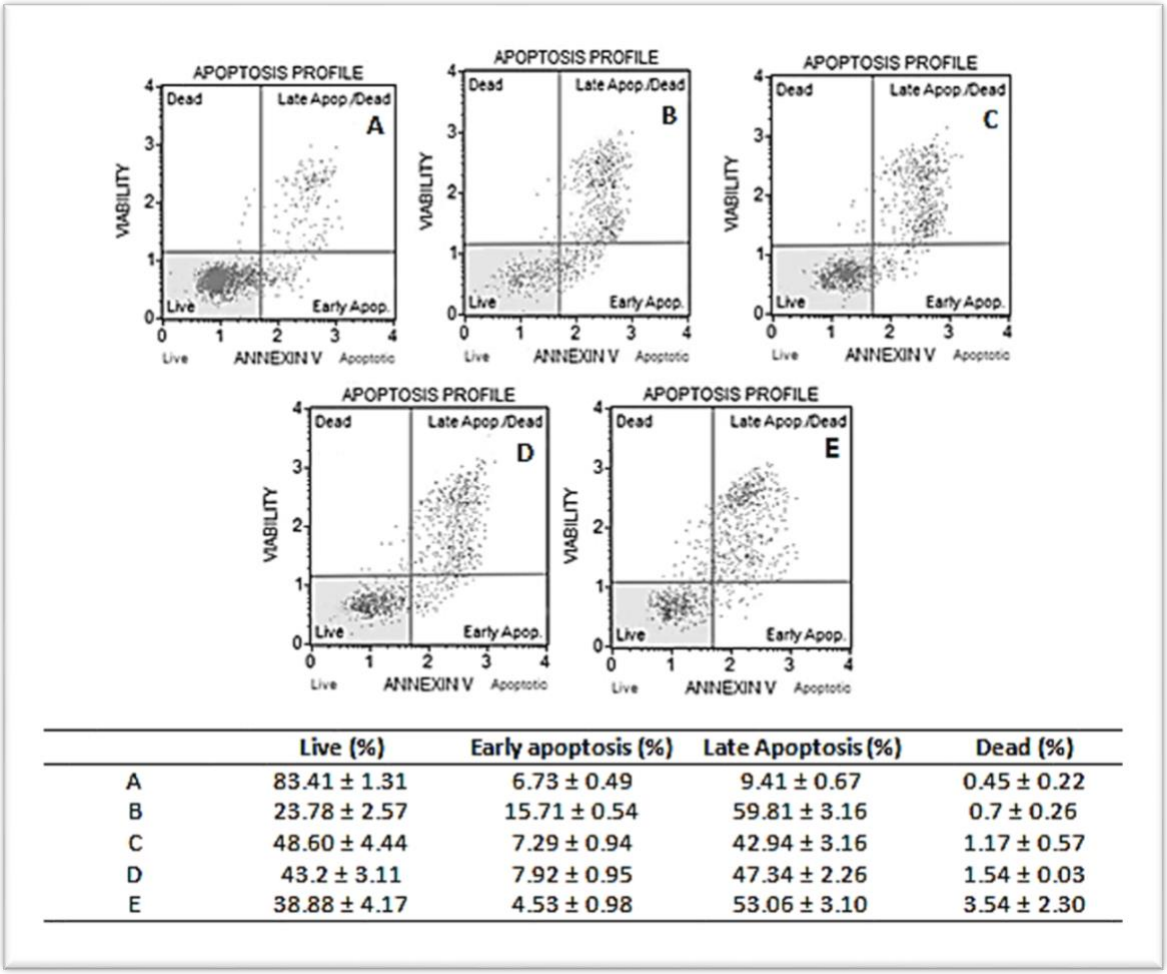


Fig 3. Morphological changes of HT-29 cancer cells after 72 hours of incubation with media/control (A); staurosporine (B); undigested peptide (C); pepsin digested peptide (D); and pepsin and pancreatin digested peptide (E).

Degree of apoptosis of HT-29 colon cancer cell line: To quantitatively illustrate the apoptotic process of HT-29 cells induced by peptides, Muse Annexin Viable & Dead Cell Assay was carried out to detect phosphatidylserine (PS) on the surface of apoptotic cells (Fig 4).



	Live (%)	Early apoptosis (%)	Late Apoptosis (%)	Dead (%)
A	83.41 ± 1.31	6.73 ± 0.49	9.41 ± 0.67	0.45 ± 0.22
B	23.78 ± 2.57	15.71 ± 0.54	59.81 ± 3.16	0.7 ± 0.26
C	48.60 ± 4.44	7.29 ± 0.94	42.94 ± 3.16	1.17 ± 0.57
D	43.2 ± 3.11	7.92 ± 0.95	47.34 ± 2.26	1.54 ± 0.03
E	38.88 ± 4.17	4.53 ± 0.98	53.06 ± 3.10	3.54 ± 2.30

Figure 4. Apoptosis profile of HT-29 treated with media/control (A); Staurosporine (B); undigested peptide (C); pepsin digested peptide (D); and pepsin and pancreatin digested peptide (E) for 72 hours. All data are presented as the mean ± SD of triplicate results.

DISCUSSION

Little or no hydrolysis of the peptide in the gastric phase digestion was observed (Fig 1B), which indicated that the bioactive peptide was resistant to proteolysis by pepsin. The resistance of peptide to pepsin hydrolysis could be attributed to the primary amino acid sequence of the peptide and the physio-chemical parameter of the reaction medium (e.g. pH, ionic strength etc.), which may have influenced structure of substrate and structure/activity of enzyme. It is also known that proteolytic enzyme, such pepsin has the highest preference for Phe and Leu in the P1 position, followed by Met, but also hydrolyses peptide bonds involving Tyr, Trp, Cys, and Glu in the P1 position. The amino acid in P1' position does not have as strong an influence on pepsin specificity as the P1 position, although aromatic residues (Tyr, Phe, and Trp) and, to a lesser extent, Ile and Val are preferred. Conversely, the presence of His, Lys, Arg, and

Pro in the P1 position strongly disfavors pepsin activity. Pepsin also discriminates against Pro residues in the P2 position and, to a lesser extent, in the P2' and 3' positions. In addition, Arg, Lys, or His residues are disfavored in the P3 position [14]. Three out of fifteen residues in the identified peptide were proline residues, which are likely responsible for its resistance to pepsin digestion. A desktop-based enzymatic digestion exercise was performed on the above-identified peptide as substrate, using the online tool "PeptideCutter". PeptideCutter tool identified one potential pepsin cleavage site, namely Leu_6-Ala_7.

Further digestion using pancreatin, which mimics the digestion process in the intestine, resulting in the hydrolysis of parent molecule yielding smaller peptides (Fig 1C). Pancreatin preparation contains numerous enzymatic activities, including amylase, lipase, and proteases [15]. In addition to trypsin (cleaved peptide bonds such as Lys-X and Arg-X residues), pancreatin also contained chymotrypsin, which is known to hydrolyze peptide bonds involving hydrophobic amino acid residues such as Tyr, Trp, Phe, and Leu. Once again, the digestion of the identified peptide was attempted using PeptideCutter tool, but with the selection of enzymes such as chymotrypsin and trypsin. This exercise yielded two potential hydrolysis sites on the peptide, namely Met_1-Gly_2 and Leu_6-Ala_7.

Figure 2 showed that the growth of HT-29 cells was significantly inhibited by up to 28.89% and 29.68% by undigested and pepsin digested peptides, respectively. At the same concentration, the peptide digested with both pepsin and pancreatin showed higher HT-29 growth inhibition activity, resulting in lower cell viability (61.7 %). These results indicated that not only the identified parent peptide but the smaller peptides also showed similar activity and may, in fact, possess higher anticancer activity. Umayaparvathi *et al.* [16] reported that Leu-Ala-Asn-Ala-Lys (515.29 Da), a purified peptide from oyster hydrolysate, had a strong cytotoxic activity on the HT-29 colon cancer cells with IC_{50} = 60.21 ± 0.45 $\mu g \cdot mL^{-1}$. The size of the peptide and its amino acid composition obviously may affect its anticancer activity. However, the authors did not test the resistance and behavior of the smaller peptides produced during the GI digestion of the parent peptide.

Apoptosis, a form of programmed cell death, normally occurs in multicellular organisms, enabling them to control cell numbers and eliminating cells that threaten their survival. This mechanism is characterized by morphological changes such as chromatin condensation, cell shrinkage, membrane blabbing, packing of organelles, the formation of apoptotic bodies, inter-nucleosomal DNA fragmentation and the eventual cell death [17]. Morphologically, undigested and digested peptides showed high impact on HT-29 cells (Fig. 3). Wang *et al.* [18] reported that cell selectivity and susceptibility to lysis were determined by the composition of cell membrane bilayers and the distribution of phospholipids. The amount of phosphatidylserine (PS) located in the outer leaflets of cancer cells is 3–7 times higher than that in the inner leaflets of normal cell membranes [19]. The identified peptide is composed of several hydrophobic amino acids, including Met, Gly, Pro, Leu, and Ala, which could lead to enhanced interactions between the peptide and the outer leaflets of the cancer cell membrane bilayers that have high anionic phospholipid contents. Huang *et al.* (2011) [20] stated that the hydrophobic properties of peptides likely play important roles in their anticancer activities and may support the selectivity properties of the identified peptide.

As depicted in Fig. 4., the ratio of apoptotic cells of the treated group (B, C, D, E) was higher than that of the control (A), confirming that peptides induced apoptosis of HT-29 cells. Similarly, Sabbione [21], reported a significant increase of early apoptotic and late

apoptotic/necrotic HT-29 cells compared to untreated ones. HT-29 cells treated with Staurosporine (B) showed the lowest number of live cells (23.78 ± 1.31%), followed by treatment E (pepsin and pancreatin digested peptide), D (pepsin digested peptide) and C (undigested peptide). The HT-29 cells treated with pepsin and pancreatin digested peptides showed the highest number of dead cells (3.54 ± 2.30%). The similar ratio of live, apoptotic and dead cells observed for treatments C and D. After 72 h of exposure, treated HT-29 cells were mainly in the late apoptotic stage, indicating early apoptotic stage might occur earlier. The effect of the different exposure periods, therefore, would be the subject of further research.

CONCLUSIONS

Bioactive peptide, Met_1-Gly_2-Pro_3-Pro_4-Gly_5-Leu_6-Ala_7-Gly_8-Ala_9-Pro_{10}-Gly_{11}-Glu_{12}-Ala_{13}-Gly_{14}-Arg_{15}, isolated from Flathead hydrolysate showed anticancer activity. The parent bioactive peptide was found to be resistant to the gastric phase digestion by pepsin. Digesta resulting from GI digestion, containing both parent bioactive peptides and its smaller breakdown products, was found to have higher anticancer activity against HT-29 colon cancer cells. This observation clearly showed that the smaller fragments of the parent peptide might even be more potent anticancer active lead(s). The identity of these smaller fragments of the parent bioactive peptide were not elucidated in the present study, but this work could be taken up in any future work undertaken on the topic. It will also be interesting to evaluate the anticancer properties of the parent bioactive peptide and of the smaller peptide resulting from intestinal phase digestion against different cancer cell lines before any animal trial is undertaken. The bioactive peptide, and smaller peptides produced from the same, with further characterization, including animal and human clinical trials, could potentially be developed into an ingredient for personalized food applications for cancer patients and/or pharmaceutical preparation.

List of Abbreviations: GI, Gastrointestinal; RP-HPLC, Reversed-phase High Performance Liquid Chromatography; FBS, Fetal Bovine Serum; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]

Author Contributions: R.N., T.V., conception and design, acquisition of data, drafting the article; T.K.S., and O.N.D., analysis and interpretation of data, revising it critically for important intellectual content; A.P. and T.S.K, reviewing and revising article, funding acquisition; All authors gave final approval of the version to be published.

Competing Interests: The authors declare that they have no conflicts of interest.

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