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Assessment of antiproliferative and toxic effects of a peptide from *Momordica dioica* using in vitro and in vivo studies

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ABSTRACT

Inflammation occurs during a cascade reaction to cause damage to the tissues. Increased oxidant and cytokine expression were observed in the damaged tissues. Inflammatory bowel disease (IBD) may be a chronic and lethal disease of inflammation in gastro enteric tissue characterized by intestinal inflammation. The objective of the study was to investigate the effects of the peptide from *Momordica dioica* in treating ulcerative colitis. The isolated protein was digested with trypsin enzyme and the hydrolyzed peptide were analyzed using LCMS where the peak obtained at 678.67Da at 17mins and MALDI-TOF analysis which showed similar peaks at 3388.7Da. The results from the MTT assay showed the IC_{50} value of the peptide at 100µg/mL. Similarly, the acridine orange staining showed decreased green-fluorescent nuclei cells than red fluorescent acidic vesicular organelles indicating autophagy-dependent cell death. The peptide displayed a cell viability effect on Colo-205 cells at 100 µg/mL when compared to the control. In the acute toxicity test, the mice were orally receiving peptide at a dose of 50-250 mg/kg BW for 14 days. The significant adverse effects were evident at a dose of 250mg/kg BW indicating that the LD_{50} value is lesser than 250 mg/kg. Based on the results obtained from this study, the peptide with a molecular mass of 3388.7Da exhibited an increased rate of cytotoxicity and autophagy induction in cancer cells at the concentration of 100 µg/mL. Therefore, the peptide can be further used for the formulation of anticancer drug for treating ulcerative colitis conditions.

INTRODUCTION

Inflammatory bowel disease (IBD) is chronic inflammation of the gastrointestinal tract and termed for certain conditions such as Crohn's disease [1], ulcerative colitis, and other conditions [2,3]. Although both the conditions have similar characteristics, they differ in the position and nature of the inflammation. Crohn's disease is observed with inflammation in the gastrointestinal tract, while ulcerative colitis is shown by inflammation localized to the large intestine [4]. The intestinal inflammation of the mucosa in IBD is characterized by pain in the abdominal region, frequent bloody and watery stools, loss of weight, and the intrusion of neutrophils and macrophages that are responsible for cytokine production, proteolytic enzymes, and free radicals that result in swelling and ulceration [5]. In contrast, ulcerative colitis occurs in periodic patterns and involves the rectum and entire colon. The pathological condition of ulcerative colitis is in the submucosa and mucosa crypt abscesses [6]. Inflammation is a reaction that causes damage to tissues resulting in increased expression of cytokines in the tissues. Peptide from various sources could be used as therapies to treat inflammation. An alternate treatment supported by the utilization of bioactive peptide as medicinal drug agents is being developed [7]. The peptide, in relation to little molecules, possess higher efficiency and property for their targets, primarily owing to their biological diversity [8]. Bioactive peptides have a sequence range of 5-20 amino acids. The major anti-inflammatory effect of the peptide is due to the high presence of hydrophobic and positively charged amino acids. The biological activity of the peptide mainly depends

on the length, the molecular weight of the peptide, and the residues present at both ends of the peptide chain [9]. The proteins known as autophagy-related (ATG) are necessary for the autophagy process, and some of which belong to the Atg8 (autophagy-related 8) family. The proteins of the Atg8 family have all been linked to a variety of pathways, including cellular trafficking, autophagy, and cancer [10]. Improper functioning of these related proteins or dysfunction leads to various disease conditions like inflammation, homeostasis of colonic inflammation, and other disorders [11].

MATERIALS AND METHODS

Materials

Roswell Park memorial Institute medium 1640 (RPMI 1640),50X Antibiotic-Antimycotic solution-A002, Fetal bovine serum (FBS)-RM10432,1X Trypsin-EDTA solution-TCL007 was purchased from HIMEDIA Laboratories. Acridine orange, MTT, Trypsin enzyme, sodium dihydrogen phosphate, and disodium hydrogen phosphate were obtained from Sisco Research Laboratories. COLO 205 (human epithelial, colon cancer) cell lines were obtained from NCCS, PUNE, and cultured in RPMI Medium containing 5% FBS and 1% antibiotic solution at 370C maintained in a 5% CO2 Incubator.

Protein extraction

The seeds of *Momordica dioica* were shade dried and powdered. 10g of powdered seed was dissolved with 0.2M phosphate buffer solution (pH- 6.6) and incubated overnight in a shaker at 37^0C. The solution was filtered through the muslin cloth and the filtrate was centrifuged at 12000 rpm for 10 min. Then the supernatant collected was lyophilized and stored for further analysis [12].

The lyophilized powder was digested with trypsin enzyme for 2.5h at 37^0C with a substrate to enzyme ratio of 100:1 w/w in 0.1M sodium phosphate buffer, then the enzyme was inactivated by heating the solution at 90^0C for 5min. The digested solution was centrifuged at 12,000 rpm for 10min, and the supernatant was lyophilized and stored for future use [13]. The digested solution of *Momordica dioica* seed solution was fractionated through the 2KDa dialysis membrane against water for 24h in a magnetic stirrer overnight. The dialysate was then further lyophilized and stored at -20^0C for further analysis.

HPLC-MS analysis of partially purified peptide

The Mass spectrometry of the peptide mixture was analyzed using Impact HD, Bruker Germany. The column used was the zorbax eclipse C18 column with 3.5-micron Agilent, USA. The mobile Phase A: 0.1%formic acid and mobile phase B: 0.1% FA in 80% acetonitrile the mobile phase B is operated at a gradient range of 2 to 35%, then increased linearly to 80%. The flow rate of the system is maintained at 0.3 mL/min. The range was set to 200-2000 m/z [14,15].

MALDI-TOF analysis

The MALDI-TOF analysis was performed using MALDI TOF (UltrafleXtreme II) BRUKER Germany. The mobile phase was prepared by mixing 100mg of α-cyano-4-

hydroxycinnamic acid in 10 mL of 1:1 acetonitrile and TFA. The sample for analysis was then mixed with the CHCA solution for analysis [16].

Cell viability assay

The viability of the cells was measured using MTT [17]. The colo205 cells were seeded at 5x 10 cells /well in 96 well plates and incubated at 37^0C for 24h. After 24 h the cells were treated with different concentrations of the peptide ranging from (25-125 µg/mL) and incubated for 24h. After incubation, the overnight grown cells were treated with MTT(5mg/mL) in PBS and incubated for 3h. The formazan crystals formed were dissolved using DMSO (100 µl) solution. The absorbance was measured at 570 nm using a microtiter plate.

Staining of autolysosomes by acridine orange

The colo205 cells were seeded in 24 well plates at 5×10^3 cells/well treated with LPS(1mg/mL) and incubated for 24 h. After 24 h the LPS-induced cells were washed with 1x PBS with different concentrations of peptide ranging from (25-125 µg/mL) and cultured for 24 h growth period. Then the cells were stained with acridine orange dye for 30 min, which was then examined using a fluorescence microscope [18].

Toxicity evaluation of peptide in-vivo

The experimental BALB/c mice about 8 weeks of 20-25g were purchased from TANUVAS with IAEC Approval No. SAF/200711/04. The acute toxicity study was conducted to determine the adverse effects of the peptide according to the (OECD) guideline 420 [19]. The mice in Group II-VI (n=5) received peptide concentrations of (50,100,150,200 & 250) mg/kg BW respectively, while the control group (n = 5) received no treatment. The experimental animals were maintained under observation for 14 days. On day 15th, the mice were anesthetized using ketamine and xylazine and sacrificed using cervical dislocation. For histopathological examination, the tissues were fixed with 10% formalin for 6-12 h.

In designing the experiments for the acute toxicity study, the animals were divided into six groups with five animals each. Group I (control group): received water at an oral dose for 14 days. Group II: Animals received peptide at an oral dose of 50 mg/kg BW. Group III: Animals received peptide at an oral dose at dose 100 mg/kg BW. Group IV: Animals received peptide at an oral dose of dose 150 mg/kg BW. Group V: Animals received peptide at an oral dose of 200 mg/kg BW. Group VI: Animals received peptide at an oral dose of 250 mg/kg BW. All dose ranges were studied for 14 days.

Statistical analysis

Statistical analysis was performed using Graph-Pad Prism 6 software. The data obtained for the study in finding differences among the control and treatment groups were analyzed using one-way ANOVA analysis. All data were expressed as mean ± SD. Statistical significance **** was set at $P < 0.0005$.

RESULTS

Characterization of the peptide from *Momordica dioica* using HPLC-MS and MALDI-TOF analysis

The HPLC-MS analysis of peptide was shown in (Figure 1A) which represents the peaks obtained after 15 min indicating late eluting hydrophobic peptide at 17 min. The peaks in the chromatogram (Figure 1B) represent the molecular mass analysis of the peptide which was found to be 678.67 Da which confirms the tryptic digestion of peptide.

The peptide from the hydrolyzed sample was identified using MALDI-TOF, and the results are shown in (Figure 2) which indicates peaks obtained are found in the range of 3388.67 Da.

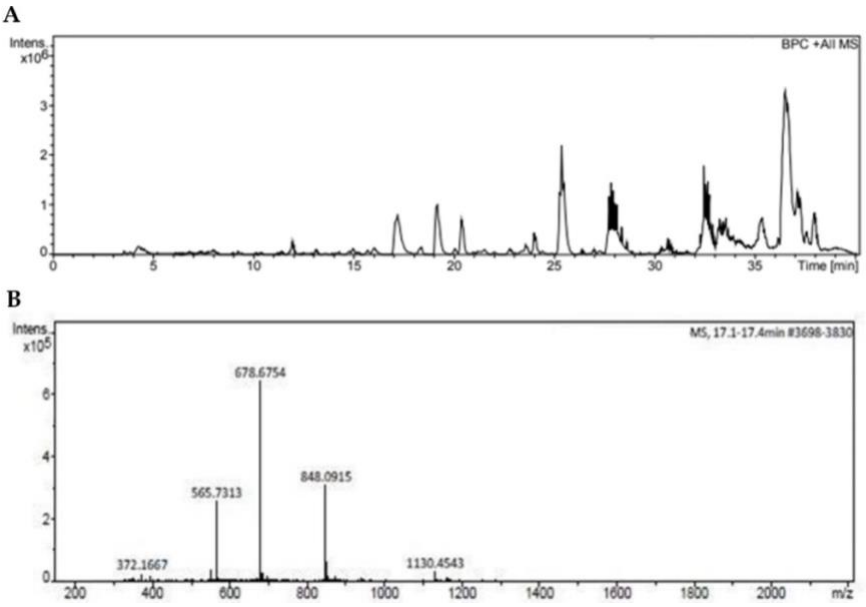

Figure 1. A) HPLC analysis of peptide from the seeds of *Momordica dioica* showing peaks at a later elution time after 15 min, indicates hydrophobic peptide. B) LC-MS analysis of the peptide from the seeds of *Momordica dioica* at 17 min depicts the molecular mass of 678.67Da.

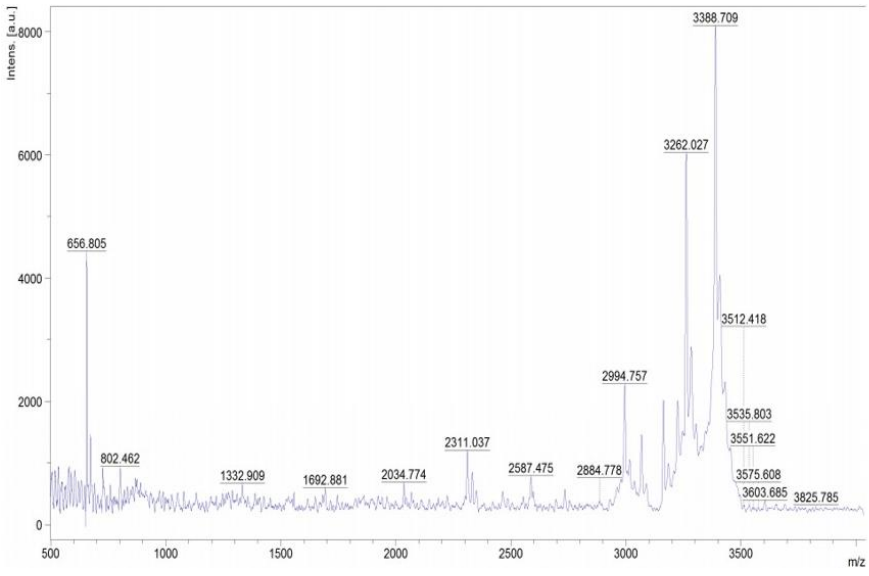

Figure 2. MALDI-TOF analysis of peptide from seeds of *Momordica dioica* shows the molecular weight of the peptide at 3388.7Da.

Anti-proliferative effect of peptide from *Momordica dioica* on Colo-205 cells

The MTT assay was performed to check the cell viability of the cells before and after drug treatment strategies. The peptide from *Momordica dioica* significantly inhibited the viability of the cells in a dose-dependent manner. When compared to control cells, a substantial decrease in cell viability was observed with the increasing concentrations of 25 to 125 µg/mL of peptide with an IC_{50} value of 100 µg/mL (Figure 3).

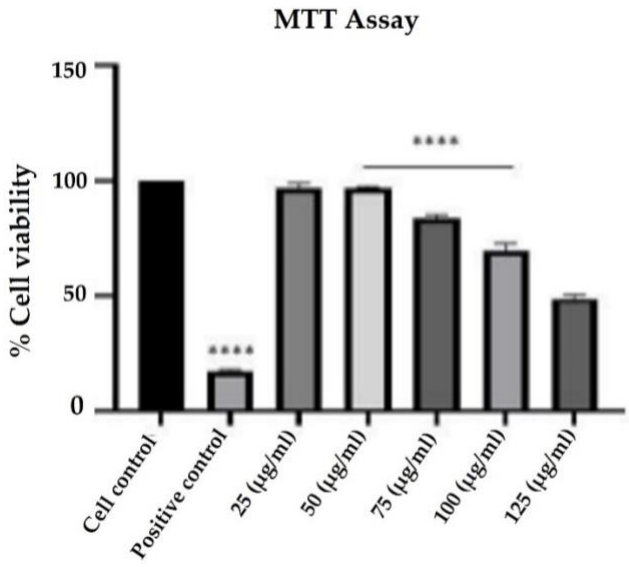

Figure 3. Antiproliferative effect of peptide on the viability of colon cancer cells. Results are represented as mean ± SE for significant differences ($P< 0.001$) among treatment and cell control (untreated).

Autophagy induction in peptide-treated Colo-205 cells

Colo 205 cells were treated with 25-125 µg/mL of peptide for 24 h, and the results shown in Figure 4 indicated that the cells treated at different concentrations showed significant morphological autophagic changes. The nuclei are shown in green fluorescence, whereas the autolysosomes are shown in red fluorescence. The colo205 cells treated with 100 µg/mL represent a significant decrease in the green nuclei cells compared to control cells indicating higher autophagy of the cancer cells.

Histological examination of in-vivo samples treated with peptide

Histological evaluations of the kidneys, liver, and colon of mice showed no changes when compared to the control. The peptide treated groups with 50-200mg/kg respectively showed no signs of toxicity or injury in colon (Figure 5), kidney (Figure 6), and liver (Figure 7) compared to the control group. However, the liver tissue with peptide treatment (250mg/kg), examined mild periportal inflammation (Figure 7). Table 1 revealed no significant differences for behavioral changes between the peptide treatment group (II-V) while a significant difference in body weight was observed for the treatment group (VI) compared to control groups.

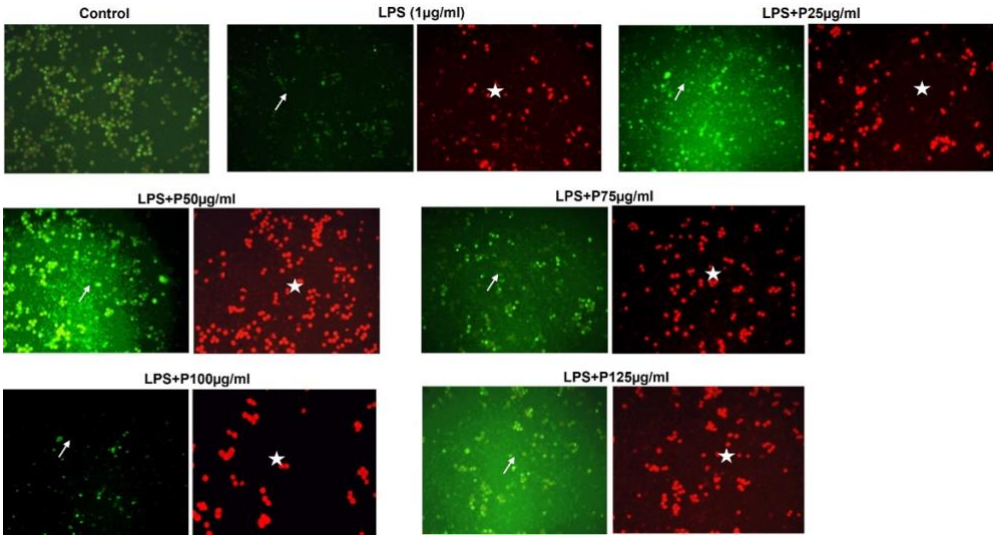

Figure 4. Fluorescence 40X microscopic images of Colo205 cells stained with acridine orange. Control, LPS 1 µg/mL (Positive control), LPS+P25µg/mL, LPS+P50µg/mL, LPS+P75µg/mL, LPS+P100µg/mL, LPS+P125µg/mL. Magnification: 10X, P: Peptide, LPS: Lipopolysaccharide, arrow indicates nuclei; star indicates acidic vesicular organelles.

Table 1. Behavioral responses and general parameters observed in mice treated with different doses of peptide.

Groups	Bodyweight (g)	Rectal bleeding observation	Stool observation	Animal behavior observation	Secretion
GI (Control group)	25.5 ± 1.2	Not observed	Normal	Normal	Not observed
GII (50 mg/kg BW)	26.16 ± 1.76	Not observed	Normal	Normal	Not observed
GIII (100mg/kg BW)	29.05 ± 1.15	Not observed	Normal	Normal	Not observed
GIV (150mg/kg BW)	27.07 ± 1.83	Not observed	Normal	Normal	Not observed
GV (200mg/kg BW)	29.76 ± 3.84	Not observed	Normal	Normal	Not observed
GVI (250mg/kg BW)	24.02 ± 1.22[a]	Not observed	Normal	Normal	Not observed

The significant difference ($P<0.05$) was represented as [a] compared with peptide treatment and control group according to Dunnett test.

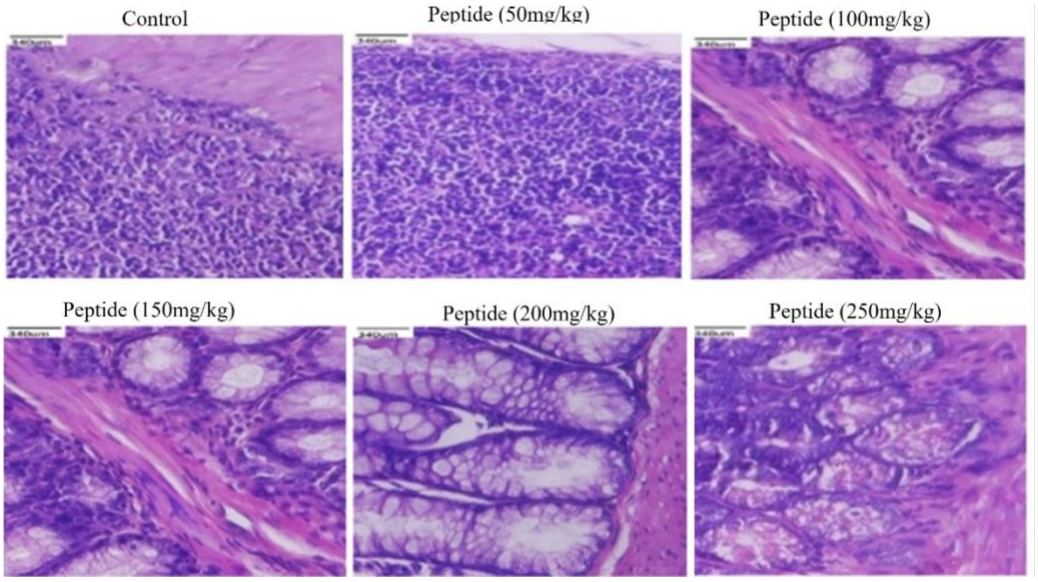

Figure 5. Photomicrograph of H&E staining 40X of peptide treated colon tissue (control, peptide-50mg/kg, peptide-100mg/kg, peptide-150mg/kg, peptide-200mg/kg, peptide-250mg/kg) with normal histological appearance.

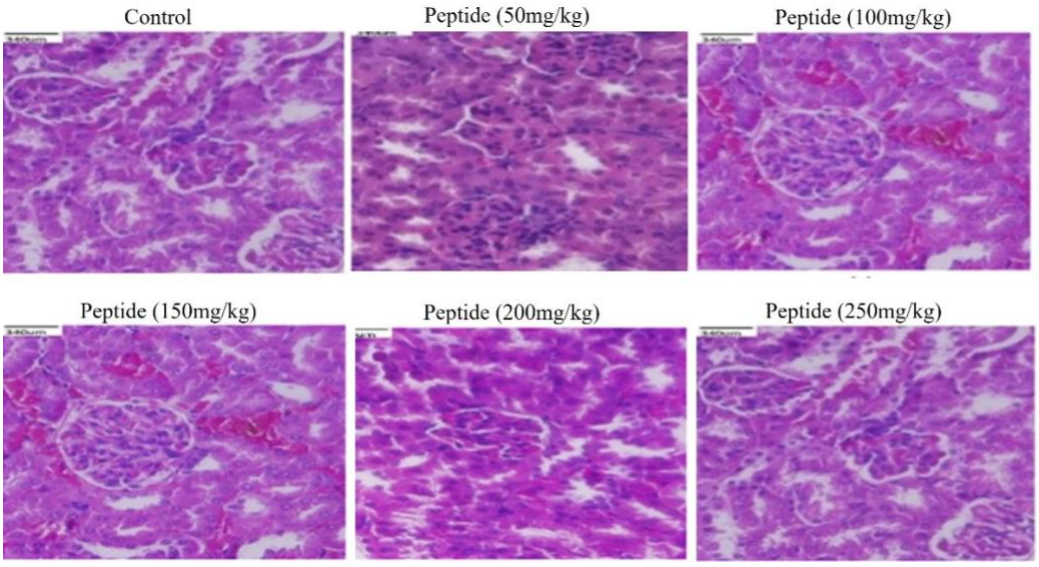

Figure 6. Photomicrograph of H&E staining (40X) of peptide treated kidney tissue (control, peptide-50 mg/kg, peptide-100 mg/kg, peptide-150 mg/kg, peptide-200 mg/kg, peptide-250 mg/kg) with conserved tubular and glomerular structure.

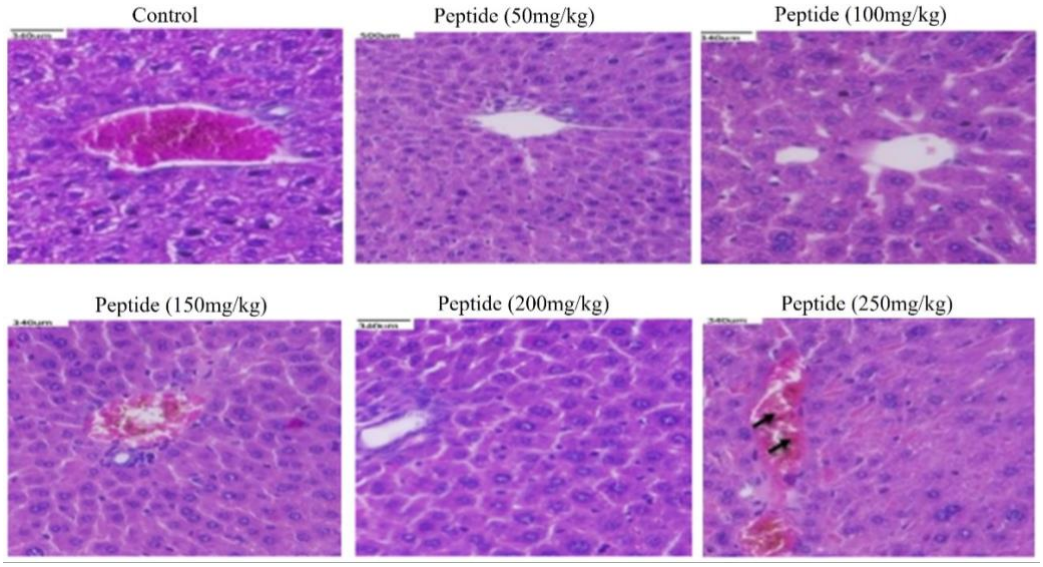

Figure 7. Photomicrograph of H&E staining 40X of peptide treated liver (control, peptide-50 mg/kg, peptide-100 mg/kg, peptide-150 mg/kg, peptide-200 mg/kg, peptide-250 mg/kg) with normal microscopic appearance and black arrow indicates central and portal vein congestion and mild periportal inflammation.

DISCUSSION

The current study demonstrated the effect of the isolated peptide dialysate on the cell viability of Colo205 cells induced with Lipopolysaccharide.LC-MS analysis of our peptide was found to be in the range of 1000Da. Cao *et al.,* [20] studied the trypsin hydrolysis of enzyme and obtained four peaks in the range of 3KDa with its structural characterization from the *Momordica* species. This study's results are in accordance with a previous study that discovered that peptide of 2000 Da exhibited a greater anti-inflammatory effect in protein hydrolysates made from a combination of proteases [21]. Previously reported by Xiang et.al., [22] showed hydrolysates of rice drew showed bioactive peptide with a less molecular weight greater than 1KDa.

MALDI-TOF analysis results showed bioactive peptide with 500-4000Da. Similar peaks with masses were found to be 1246.6 Da, 1723.8 Da, 1858.6 Da, 822.4 Da, 1435.7 Da, and 3279.2 Da after chymotryptic proteolysis using the *Citrullus colocynthis* a Cucurbitaceae plant family [23]. A similar study showed the MALDI-TOF of hydrolysates from *Tinospora cardifolia* produced by gastric enzyme cleavage with a mass of 1450.71 and 1023.51Da showed decreased level of oxidative stress parameters and inflammatory activity [24]. Peptide from egg white using pepsin hydrolysate showed the molecular weights of 3KDa which were shown to improve the antioxidant activity [25].

The MTT assay analyzed the cell viability of colo205 cells treated with an increasing dose of peptide dialysate concentration in which 75µg/mL was the concentration at which viability is maintained at 70%. A previous study reported a dose-dependent reduction in viability of leukemic cells of Molt-4 treated with hydrolysate of sesame seeds by pepsin enzyme [26]. Similar studies were carried out for the anticancer property of *Morinda Pubescens* peptide against human cancer cells A459 [27].

The autolysosome formation in the cells is a marker of autophagy stained with acridine orange dye [28]. The study reveals that the peptide in the increasing concentration shows autophagic cell death of colo205 cells at 75µg/mL. The morphological features of autophagy cells in earlier studies using PC-3 cells treated with oligopeptide QPK [29]

and HeLa cells treated with a hexapeptide with sequence Phe-Ile-Met-Gly-Pro-Tyr extracted from *Raja porosa* [30,31].

The *in vivo* acute toxicity and the behavioral changes were studied with the increasing dose (50-250mg/kg BW) of the peptide for the BALB/c mice. In this study, we observed a change in the morphology of the liver with the treatment of 250 mg/kg of peptide compared to the control group that received only water. This was in line with the study by Amare [32] who showed the presence of morphological change between the control group and mice treated with 200 mg/kg. A study similar to the current study showed that whey protein hydrolysate (200-600 mg/kg) in mice showed no signs of structural changes in the organ after histopathological examination [33].

CONCLUSION

In this study, the peptide of molecular mass 3388.7Da showed no signs of symptoms and toxicity in *in-vivo* study, which are evident from the microscopic observation of the tissues. Further, the peptide -induced cell death by showing the presence of acidic vacuolar organelles, and autolysosomes indicating autophagy induction. Therefore, the purified Peptide was shown to decrease the inflammation process through the upregulation of autophagic proteins in colon cancer cells.

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None.

AUTHOR CONTRIBUTIONS

RS designed the outline of the study and JS drafted the manuscript. JS performed the experiments and analyzed the data. JS wrote the initial draft of the manuscript. RS reviewed the scientific contents described in the manuscript. RS read and approved the final submitted version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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