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Synthesis of Linear and cyclic peptides analogues of Longicalycinin A and evaluation of toxicity effects on cancerous cells HepG2 and HT-29

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ABSTRACT

In this work, linear and cyclic peptides analogues of Longicalycinin A, known as a natural cyclopeptide anticancer agent, have been designed and successfully synthesized by solid phase peptide synthesis methodology of Fmoc/t-Bu. 2-Chlorotrityl chloride resin (2-CTC) was used as solid support. The synthesized linear (O*t*-Bu) Longicalycinin A analogues were cleavaged by the method of partial cleavage for the separation of the solid phase. The final deprotection was performed by treatment with TFA 95% containing scavengers to achieve deprotected linear Longicalycinin A analogues. Macrocyclization of deprotected cyclic Longicalycinin A analogues were characterized by different instrumental methods using FT-IR, LC-MS, ^{1}H-NMR and ^{13}C-NMR. Deprotected linear and cyclic analogues, synthesized as such, were evaluated for their toxic activity against cell lines of HepG2 (human liver cancer cell line) and HT-29 (human colorectal adenocarcinoma cell line) using MTT assay. The synthetic linear and cyclic analogues showed relatively good activity against cell lines of HepG2 and HT-29 with IC_{50} values from 8.76 to 17.2 µg/mL and 9.06 to 17.34 µg/mL, respectively, in comparison to standard drug 5-fluorouracil (5-FU). Safety profile of the synthesized liner and cyclic analogues of Longicalycinin A were also examined using skin Fibroblast cells. Among the linear peptides, linear compounds 1, 8, 12, 13 and 14, showed a considerable toxicity activity on cancer cell lines HT-29 than HepG2 along with a high safety on normal cells and among the cyclic peptides, compound 5, considering the property of toxicity action good enough on cancerous cell lines along with high safety profile on normal skin cells, can be good candidates for developing new anticancer agents.

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Introduction

Cancer is a disease marked by uncontrolled growth and division of cells, making tumors. If the tumor spread is not controlled, it can result in mortality. Cancer is caused by the both external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). [1] Cancer can occur in the breast, lungs, colon, blood cells and *etc*. Cancers are similar in some ways, but different in growth and spread routes.[2] Normal cells are divided in a regular routine order. They die when become damaged and new cells take their place.[3] When cancer cells spread in the body, it is called metastasis. Many new and effective therapies are currently being used to treat cancer. Among the new ways to battle cancer, chemotherapy based on peptides has attracted a great interest and considerable attention. This is due to the unique advantages of peptides, such as having low molecular weights, the ability to specifically target tumor cells, and low toxicity in normal tissues. During the last decade, peptides have gained a wide range of applications in medicine, drug delivery and biotechnology.[4]

Peptides, a class of compounds consisting of two or more amino acids linked by peptide bond, and are abundantly present in living organisms. A large number of peptides have been isolated from animals, plants and microorganisms. Based on their chemical configuration, peptides can be divided into linear and cyclic peptides.[5] Studies have shown that large numbers of peptides isolated from plants are cyclic peptides, so-called

cyclopeptides. Cyclopeptides compared with linear peptides, exhibit more potent biological activities, possibly due to the stable scaffolds provided by their cyclic structure. Pharmacy studies have proved that many peptides, including those isolated from plants, have a potential antitumor effect.[6-8] Peptides isolated from plants have a number of advantages over other chemical agents including relatively simple structure, their low molecular weight, lower antigenicity and fewer adverse actions, easy absorption, and a variety of routes of administration.[9] Recently, one of the most active areas of research is the search for natural components of plants with potent antitumor activity and low toxicity.[10] The inherent medicinal properties of cyclic peptides promoted scientists to isolate these compounds from natural sources. There are four possible ways that a peptide can be constrained as a macro cycle: head-to-tail (C-terminus to *N*-terminus), head-to-side chain, side chain-to-head, or side chain-to-side chain.[11] Cyclic peptides head to tail can easily be synthesized in good purity using standard procedures. However, the cyclization strategy is critical and depends upon the sequence of peptide, structural constraints, and the resulting ring size. A number of studies have been developed to improve this crucial strategy and to obtain cyclic peptides in good yield with minimum side reactions.[12]

One of the preferred methods for peptide synthesis is the synthesis on solid phase. Among the various resins employed for the synthesis of peptides is 2-chlorotrityl chloride resin. The trityl linker allows the protected compounds to be subjected to various chemical manipulations and consequently to afford pure compounds without employing numerous purification steps.[13-17] Oshima et *al* isolated a cyclic pentapeptide Longicalycinin A [cyclo-(Gly-Phe-Pro-Tyr-Phe)] from the plant Dianthus superbus *var*. Longicalycinus which has been used for treating carcinoma, diuretic and inflammatory diseases.[18-21] The aim of this study was to design and synthesize linear and cyclic pentapeptides analogues of Longicalycinin A that might have been expected to produce anticancer activity. The cytotoxic activities of synthesized peptides were evaluated against various human cancer cell lines including, HepG2 and HT-29.

Experimental:

Materials and Instruments

All other commercially obtained reagents and solvents were used without further purification. Trifluoroacetic acid (TFA), Fmoc amino acids and coupling reagents O-(7-Azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HATU), (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBop), Solvents like acetonitrile (MeCN), Piperazine, N,N-diisopropylethylamine (DIPEA), Diethylether, Dichloromethane (DCM), N,N-dimethylformamide (DMF), and methanol (MeOH) were purchased from Merck Co. Germany. 2-chlorotritylchloride resin (1% DVB, 200-400 mesh, 1 mmol/g) was purchased from Aldrich, Switzerland. Commercially available chemicals were used without further purification unless otherwise stated. Flash column chromatography was carried out using silica Gel 60 (particle size 0.04–0.06 mm / 230–400 mesh). FTIR spectra were obtained on SHIMADZU recording and Nicolet Magna 550 spectrometers. The mass spectral measurements were performed on a 6410 Agilent LC-MS triple quadruple mass spectrometer with an electrospray ionization (ESI) interface, USA. 1H-NMR and 13C-NMR spectra were obtained with a bruker 300 MHz spectrometer.

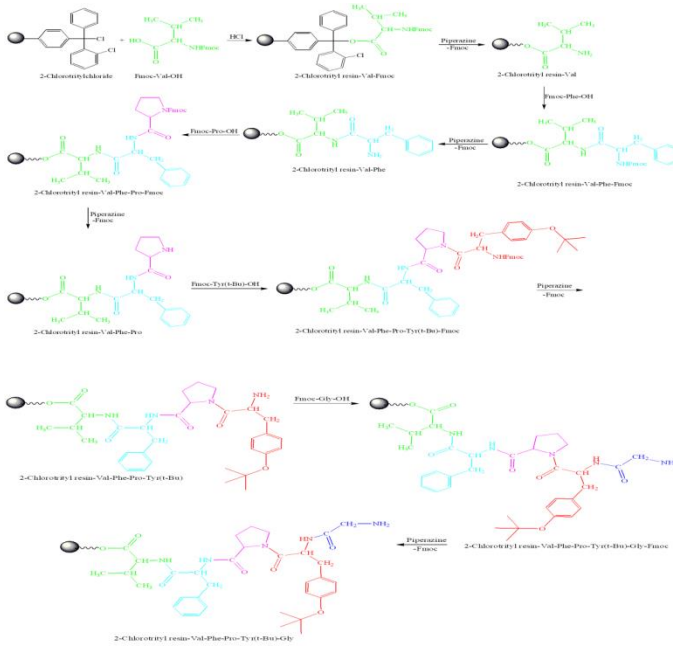
Designing linear peptides of Longicalycinin A analogues

We designed different amino acids for each position of diversities R1, R2, R3, R4 and R5 for the synthesis of eighteen Longicalycinin A analogues, based on conserving lipophilic characteristic of peptides (1-18). Thus, we chose Ala, Val and Gly for position R1, Phe and Ala for position R2, Pro for position R3, Tyr (t-Bu), Ser(t-Bu) and Thr(t-Bu) for position R4 and Gly, Leu and Phe for position R5 in the first category. Ala, Leu and Val for position R1, Phe and Ile for position R2, Val, Pro, Gly and Phe for position R3, Tyr(t-Bu) and Thr(t-Bu) for position R4 and Pro, Thr(t-Bu) and Val for position R5 in the second category. Gly, Val, Ala and Ile for position R1, Phe and Ala for position R2, Pro for position R3, Tyr(t-Bu), Ser(t-Bu) for position R4 and Ala and Phe for position R5 in the third category.

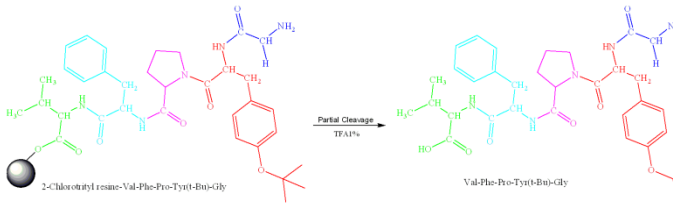
General procedure for the synthesis of linear pentapeptides (O*t*-Bu) Longicalycinin A analogues (Schemes 1 and 2)

The syntheses of linear pentapeptides (O*t*-Bu) Longicalycinin A analogues were carried out using 2-chlorotrityl chloride resin (1 mmol/g) following the standard Fmoc strategy. The first amino acid Fmoc-Val-OH (680 mg, 2 mmol) was attached to the 2-CTC resin using di isopropyl ethyl amine (DIPEA) (1 mL) in anhydrous di chloro methane and *N*, *N*- di methyl formamide (DCM:DMF) (30 mL, 1:1) at room temperature for 2h. After filtration, the remained tritylchloride groups were capped by a solution of DCM / CH_3OH / DIPEA (3:2.5:1.2) for 30 min. The mixture was filtered and washed thoroughly with DCM (10 mL) and DMF (2 × 20 mL). The resin-bound Fmoc-amino acid was treated with Piperazine 10% in DMF (100 mL) for 30 min and the resin was washed with DMF (4 × 20 mL). A solution of the second amino acid Fmoc-Phe-OH (780 mg, 2.01 mmol), HATU (650 mg, 1.7 mmol), and DIPEA (0.5 mL) in 10 mL DCM were added to the resin-bound free amine and shaken for 2h at room temperature. After completion of the coupling reaction, the resin was washed with DMF (2 × 10 mL). The resin-bound Fmoc-peptide was treated with Piperazine 10% in DMF (100 mL) for 30 min and the resin was washed with DMF (4 × 20 mL). Other protected amino acids, i.e., Fmoc-Pro-OH, Fmoc-Tyr(t-Bu)-OH and Fmoc-Gly-OH were added to the resin-peptide, sequentially, with the same procedure mentioned as above. In the all cases for detecting the presence or absence of free primary amino groups on the resin-peptide, chloranil test was used. The produced pentapeptide was cleaved from the resin by treatment of TFA 1% in DCM and neutralized with pyridine 4% in CH_3OH. The solvent was removed under reduced pressure and the residue was precipitated in water. The precipitate was filtered and dried. Other analogues were synthesized in the same way.

Scheme 1. The Solid-Phase Synthesis of Longicalycinin A analogue (1) via 2-chlorotrityl chloride resin



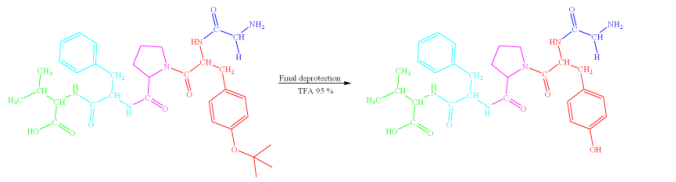
Scheme 2. The Solid-Phase Synthesis of protected Longicalycinin A analogue (1)



General procedure for the synthesis of deprotected linear Longicalycinin A analogues (Scheme 3)

A mixture of trifluoroacetic acid (1 mL), dichloromethane (1 mL), anisole (10 µL) and phenol (0.2 g) were added to the protected linear pentapeptides and stirred for 1h. Under such strong acidic condition, cleavage of side chain protecting groups of the peptides was achieved. Then, the excess TFA / DCM were removed under reduced pressure. The desired pentapeptides were precipitated in cold diethyl ether and collected by filtration.

Scheme 3. The Solid-Phase Synthesis of deprotected Longicalycinin A analogue (1)



General procedure for the synthesis of cyclic Longicalycinin A analogues (scheme 4)

All the Precipitates of linear (O*t*-Bu) Longicalycinin A analogues were dissolved in CH_3CN (100 mL) and treated with PyBop (2 eq) and DIPEA (4 eq). Obtained cyclic peptides were in good yield with minimum side reactions. Cyclic peptides were achieved by column chromatography (4:1, chloroform: methanol). Final deprotection on cyclic (O*t*-Bu) LongicalycininA analogues were done by treatment of TFA 95%, phenol and anisole as scavengers. The excess TFA / DCM were removed under reduced pressure. The desired peptides were precipitated in cold diethylether. The other cyclic analogues were synthesized in the same way.

Scheme 4. The Solid-Phase Synthesis of protected and deprotected cyclic Longicalycinin A analogue via 2-chlorotritylChloride resin

MTT assay

To determine the cytotoxicity of the linear and cyclic Longicalycinin A analogues, two human tumor cell lines were used, HepG2 and HT-29. Skin Fibroblast cell line was chosen as a safety control for normal cells. For the measurement of cell viability, succinate dehydrogenase (SDH) activity was assessed using the MTT test after 6h incubation of the cells with Longicalycinin A analogues (1.25, 2.5, 5, 10, 20, 50 and 100 µg/mL). The above-mentioned cells were cultured in RPMI1640 medium at 37℃ under 5% CO_2, 95% air, supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin and 100µg/mL streptomycin. The cells were seeded into 96-well plates with the concentration of 10^4cells/well and allowed to grow for 24h and then incubated with the increasing concentrations of each peptide compound for 6h. Cell activity was analyzed by using a MTT method which is based on the conversion of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) orange dye to purple formazan crystals by mitochondrial succinate dehydrogenase enzyme in living cells. At the end of each treatment period, MTT dye (10 µL, 5 mg/mL in phosphate-buffered saline) was added to each well and the micro plate was incubated at 37℃ for 4h. The medium containing MTT was removed and DMSO (10 µL) was added to each well to dissolve the formazan crystals. The plate was shaken for 30 min at 37℃ and the absorbance was read at 570 nm using a spectrophotometer plate reader (Infinite® M200, TECAN).[22] 5-Fluorouracil was also used as a positive control

and DMSO solvent as the blank for the test compounds. Data were presented as the mean of triplicate measurement of the number of living cells and their capacity to reduce MTT reagent. IC_{50} values were calculated by using Prism software.

Cell culture for LDH assay

For LDH assay, the HepG2 and HT-29 cells were maintained in cell culture flasks (75 cm^2) with RPMI 1640 cell culture medium containing 10% fetal bovine serum (FBS) in an incubator (37 °C, 5% CO_2, 95% air) and were passaged every 4 or 5 days. To passage cells, the cell culture flasks were washed with phosphate-buffered saline (PBS) three times and trypsin was added to detach cells from the bottom of the flasks.

LDH assay

LDH catalyzes the conversion of lactate to pyruvate, the forward reaction and the conversion of pyruvate to lactate, the reverse reaction. Lactate and NAD^+ are converted to pyruvate and NADH by the action of LDH. NADH strongly absorbs light at 340 nm, whereas NAD^+ does not. The rate of increase in absorbance at 340 nm is directly proportional to the LDH activity in the sample.[23] Cytotoxicity induced by peptides was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. The activity of LDH in the medium was determined using a commercially available kit from Sigma-Aldrich. To prepare samples for the LDH assay, the cancerous cells of passage numbers 11-20 were used. The cell samples (1mL, at a density of 10^7 cells/mL RPMI containing 10% FBS) were seeded in each well of 12-well plates and grown for 24hr before exposure to linear and cyclic Longicalycinin A peptide analogues. The cells were washed with PBS three times. Cell samples dosed with 10 µg/mL concentration of Longicalycinin A analogues for 6hr exposure in RPMI medium containing 1% FBS. The 12-well plates were shaken briefly to homogenize the released LDH in the cell culture medium and the medium was transferred to 1.5mL-microcentrifuge tubes and centrifuged at 1,000 ×g at 4°C for 15 min to remove any cell debris. The supernatant was separated and the absorbance at 340 nm was measured using ELISA reader. [24,25] The LDH assay of the samples was obtained by measuring optical density.

Flow cytometry analysis and Identification of apoptosis by PI staining

Flow cytometry analysis was determined by analytical DNA flow cytometry. In this work, HepG2 and HT-29 cells were harvested and adjusted to 10^4cells/mL (SPL, Korea) and then incubated for 6h with 10µg/mL of peptide samples. The cells were centrifuged at high speed (12,000 rpm) for 20 s. The pellet was washed with saline buffer, after a new centrifugation, resuspended in 0.2 mL of lysis buffer (0.1% sodium citrate and 0.1% Triton X-100) containing 50 μg/mL propidium iodide (PI) and stained with this reagent, a highly water-soluble fluorescent compound, at 37 °C for 15 min in the dark. The cells were then evaluated for the DNA fragmentation analysis using a FACScalibur flow cytometry equipment (Becton Dickinson, CA, USA) supplied with the flowing software 2.5.1.[26]

Determination of Lysosomal membrane integrity assay on HepG2 and HT-29

Lysosome damage using acridine orange (AO) as a probe was assayed in isolated lysosome obtained following HepG2 and HT-29 incubation with the peptide's samples. Aliquots of the cell suspension (0.5 mL) previously incubated with the peptide's samples (10 µg/mL) were stained with AO (5 µM) and precipitated from the incubation medium by 1 min centrifugation at 1000 rpm. The cell pellet was then suspended in 2 mL fresh incubation medium. This washing process was carried out twice to remove the fluorescent dye from the media. The AO redistribution in the cell suspension was then measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 495 nm excitation and 530 nm emission wavelengths. Lysosomal membrane damage was determined as the difference in redistribution of acridine orange from lysosomes into cytosol between treated cells and control cells at the time of preparation. [27,28]

Statistical analysis

Analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Statistical analysis among groups was performed using multiple comparisons by one way ANOVA followed by Tukey's post hoc test. All data are presented as arithmetic mean ± S.E.M of at least triplicate determinations. Significance was accepted at $P<0.05$.

Results:

The protected and deprotected linear Longicalycinin A analogues

Synthesis of Gly-Tyr-Pro-Phe-Val **(1)**

a) Synthesis of protected peptide

Yield: 75%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1677 (C=O COOH), 1641 (C=O amide), 1544 (C=C in amino acids Phenylalanine and Tyrosine), 1197 (C-O COOH), 1134 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (1a) 637.35, Found *m/z* = 635.9 (M-1), *m/z* = 638.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 75%; White solid; FTIR (KBr): ν (cm^{-1}) 3461.23 (OH), C=O amide under 1668.21 (C=O COOH) is also buried, 1546.85 (C=C in amino acids Phenylalanine and Tyrosine), 1192.63 (C-O COOH), 1131.96 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (1b) 581.29, Found *m/z* = 580.1 (M-1), *m/z* = 582.8 (M+1).

Synthesis of Leu-Tyr-Pro-Phe-Ala **(2)**

a) Synthesis of protected peptide

Yield: 77%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1677.95 (C=O

COOH), 1641.31 (C=O amide), 1541.02 (C=C in amino acids Phenylalanine and Tyrosine), 1199.64 (C-O COOH), 1137.92 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (2a) 665.38, Found *m/z* = 663.9 (M-1), *m/z* = 666.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 77%; White solid; FTIR (KBr): ν (cm^{-1}) 3461.23 (OH), C=O amide under 1670.13 (C=O COOH) is also buried, 1546.90 (C=C in amino acids Phenylalanine and Tyrosine), 1194.19 (C-O COOH), 1133.72 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (2b) 609.32, Found *m/z* = 608.9 (M-1), *m/z* = 610.8 (M+1).

ynthesis of Gly-Tyr-Pro-Phe-Ala **(3)***S*

a) Synthesis of protected peptide

Yield: 78%; Yellow oily liquid; FTIR (KBr): IR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1672.17 (C=O COOH), 1641.31 (C=O amide), 1546.80 (C=C in amino acids Phenylalanine and Tyrosine), 1197.71 (C-O COOH), 1134.07 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (3a) 609.32, Found *m/z* = 607.9 (M-1), *m/z* = 610.2 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 78%; White solid; FTIR (KBr): ν (cm^{-1}) 3461.23 (OH), 3278.32 (NH), C=O amide under 1672.77 (C=O COOH) is also buried, 1517.18 (C=C in amino acids Phenylalanine and Tyrosine), 1199.43 (C-O COOH), 1135.08 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (3b) 553.26, Found *m/z* = 551.9 (M-1), *m/z* = 554.7 (M+1).

of Ala-Tyr-Pro-Phe-Gly **(4)** *Synthesis*

a) Synthesis of protected peptide

Yield: 80%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1676.03 (C=O COOH), 1641.31 (C=O amide), 1546.80 (C=C in amino acids Phenylalanine and Tyrosine), 1201.57 (C-O COOH), 1135.99 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (4a) 609.32, Found *m/z* = 608.1 (M-1), *m/z* = 610.2 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 80%; White solid; FTIR (KBr): ν (cm^{-1}) 3461.23 (OH), C=O amide under 1667.76 (C=O COOH) is also buried, 1546.88 (C=C in amino acids Phenylalanine and Tyrosine), 1194.60 (C-O COOH), 1132.67 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (4b) 553.26, Found *m/z* = 551.9 (M-1), *m/z* = 554.8(M+1).

S*ynthesis of Phe-Ser-Pro-Phe-Gly* **(5)**

a) Synthesis of protected peptide

Yield: 80%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1668.31 (C=O COOH), 1641.31 (C=O amide), 1546.80 (C=C in amino acids Phenylalanine), 1195.78 (C-O COOH), 1134.07 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine); LC-MS (ESI) *m/z* Calcd for (5a) 609.32, Found *m/z* = 608.0 (M-1), *m/z* = 610.2 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 80%; White solid FTIR; IR (KBr): ν (cm^{-1}) 3499.87 (OH), C=O amide under 1673.86 (C=O COOH) is also buried, 1548.08 (C=C in amino acids Phenylalanine), 1194.60 (C-O COOH), 1130.81 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine); LC-MS (ESI) *m/z* Calcd for (5b) 553.26, Found *m/z* = 551.9 (M-1), *m/z* = 554.3 (M+1).

S*ynthesis of Val-Tyr-Pro-Ile-Ala* **(6)**

a) Synthesis of protected peptide

Yield: 74%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1670.24 (C=O COOH), 1641.31 (C=O amide), 1546.80 (C=C in amino acid Tyrosine), 1197.71 (C-O COOH), 1132.14 (C-O), 600-800 (out of plane bending vibration C-H in amino acid Tyrosine); LC-MS (ESI) *m/z* Calcd for (6a) 617.38, Found *m/z* = 616.1 (M-1), *m/z* = 618.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 74%; White solid; FTIR (KBr): ν (cm^{-1}) 3474.79 (OH), C=O amide under 1670.32 (C=O COOH) is also buried, 1546.10 (C=C in amino acid Tyrosine), 1191.37 (C-O COOH), 1132.54 (C-O), 600-800 (out of plane bending vibration C-H in amino acid Tyrosine); LC-MS (ESI) *m/z* Calcd for (6b) 561.32, Found *m/z* = 560.2 (M-1), *m/z* = 562.5 (M+1).

S*ynthesis of Pro-Tyr-Val-Phe-Ala* **(7)**

a) Synthesis of protected peptide

Yield: 77%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1679.88 (C=O COOH), 1641.31 (C=O amide), 1546.80 (C=C in amino acids Phenylalanine and Tyrosine), 1203.50 (C-O COOH), 1135.99 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (7a) 651.37, Found *m/z* = 650.0 (M-1), *m/z* = 652.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 77%; White solid; FTIR (KBr): ν (cm^{-1}) 3445.20 (OH), C=O amide under 1676.59 (C=O COOH) is also buried, 1547.59 (C=C in amino acids Phenylalanine and Tyrosine), 1198.25 (C-O COOH), 1135.86 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (7b) 595.3, Found *m/z* = 594.9 (M-1), *m/z* = 596.8 (M+1).

S*ynthesis of Phe-Tyr-Pro-Val-Gly* **(8)**

a) Synthesis of protected peptide

Yield: 79%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1679.88 (C=O COOH), 1641.31 (C=O amide), 1554.52 (C=C in amino acids Phenylalanine and Tyrosine), 1199.64 (C-O COOH), 1132.14 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (8a) 637.35, Found *m/z* = 635.9 (M-1), *m/z* = 638.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 79%; White solid; FTIR (KBr): ν (cm^{-1}) 3454.09 (OH), C=O amide under 1674.74 (C=O COOH) is also buried, 1547.55 (C=C in amino acids Phenylalanine and Tyrosine), 1194.60 (C-O COOH), 1132.42 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (8b) 581.29, Found *m/z* = 580.9 (M-1), *m/z* = 582.7 (M+1).

S*ynthesis of Phe-Tyr-Pro-Phe-Val* **(9)**

a) Synthesis of protected peptide

Yield: 75%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1681.81 (C=O COOH), 1641.31 (C=O amide), 1541.02 (C=C in amino acids Phenylalanine and Tyrosine), 1203.50 (C-O COOH), 1135.99 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (9a) 727.4, Found *m/z* = 726.0 (M-1), *m/z* = 728.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 75%; White solid; FTIR (KBr): ν (cm^{-1}) 3461.23 (OH), C=O amide under 1663.79 (C=O COOH) is also buried, 1547.13 (C=C in amino acids Phenylalanine and Tyrosine), 1194.60 (C-O COOH), 1129.62 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (9b) 671.33, Found *m/z* = 669.9 (M-1), *m/z* = 672.5 (M+1).

S*ynthesis of Phe-Tyr-Pro-Phe-Ala* **(10)**

a) Synthesis of protected peptide

Yield: 75%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1677.95 (C=O COOH), 1641.31 (C=O amide), 1544.88 (C=C in amino acids Phenylalanine and Tyrosine), 1203.50 (C-O COOH), 1135.99 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (10a) 699.37, Found *m/z* = 697.9 (M-1), *m/z* = 700.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 75%; White solid; FTIR (KBr): ν (cm^{-1}) 3467.04 (OH), C=O amide under 1669.47 (C=O COOH) is also buried, 1547.25 (C=C in amino acids Phenylalanine and Tyrosine), 1196.40 (C-O COOH), 1132.62 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (10b) 643.3, Found *m/z* = 642.9 (M-1), *m/z* = 644.9 (M+1).

S*ynthesis of Thr-Val-Pro-Phe-Ala* **(11)**

a) Synthesis of protected peptide

Yield: 76%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1681.81 (C=O COOH), 1641.31 (C=O amide), 1541.02 (C=C in amino acid Phenylalanine), 1203.50 (C-O COOH), 1135.99 (C-O), 600-800 (out of plane bending vibration C-H in amino acid Phenylalanine); LC-MS (ESI) *m/z* Calcd for (11a) 589.35, Found *m/z* = 588.0 (M-1), *m/z* = 590.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 76%; White solid; FTIR (KBr): ν (cm^{-1}) 3461.96 (OH), C=O amide under 1670.34 (C=O COOH) is also buried, 1547.07 (C=C in amino acid Phenylalanine), 1195.90 (C-O COOH), 1132.35 (C-O), 600-800 (out of plane bending vibration C-H in amino acid Phenylalanine); LC-MS (ESI) *m/z* Calcd for (11b) 533.29, Found *m/z* = 531.9 (M-1), *m/z* = 534.5 (M+1).

S*ynthesis of Phe-Tyr-Pro-Phe-Ile* **(12)**

a) Synthesis of protected peptide

Yield: 78%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1681.81 (C=O COOH), 1641.31 (C=O amide), 1541.02 (C=C in amino acids Phenylalanine), 1203.50 (C-O COOH), 1137.92 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine); LC-MS (ESI) *m/z* Calcd for (12a) 741.41, Found *m/z* = 740.0 (M-1), *m/z* = 742.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 78%; White solid; FTIR (KBr): ν (cm^{-1}) 3493.81 (OH), C=O amide under 1674.28 (C=O COOH) is also buried, 1546.97 (C=C in amino acids Phenylalanine and Tyrosine), 1196.18 (C-O COOH), 1133.05 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (12b) 685.35, Found *m/z* = 683.9 (M-1), *m/z* = 686.4 (M+1).

S*ynthesis of Val-Thr-Pro-Phe-Leu* **(13)**

a) Synthesis of protected peptide

Yield: 80%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1679.88 (C=O COOH), 1645.17 (C=O amide), 1542.95 (C=C in amino acid Phenylalanine), 1203.50 (C-O COOH), 1135.99 (C-O), 600-800 (out of plane bending vibration C-H in amino acid Phenylalanine); LC-MS (ESI) *m/z* Calcd for (13a) 631.4, Found *m/z* = 630.0 (M-1), *m/z* = 632.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 80%; White solid; FTIR (KBr): ν (cm^{-1}) 3461.23 (OH), C=O amide under 1670.07 (C=O COOH) is also buried, 1547.28 (C=C in amino acid Phenylalanine), 1194.60 (C-O COOH), 1132.01 (C-O), 600-800 (out of plane bending vibration C-H in amino acid Phenylalanine); LC-MS (ESI) *m/z* Calcd for (13b) 575.33, Found *m/z* = 573.9 (M-1), *m/z* = 576.5 (M+1).

S*ynthesis of Pro-Tyr-Phe-Phe-Leu* **(14)**

a) Synthesis of protected peptide

Yield: 72%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1679.88 (C=O COOH); 1637.45 (C=O amide), 1542.95 (C=C in amino acids Phenylalanine and Tyrosine), 1203.50 (C-O COOH), 1135.99 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (14a) 741.41, Found *m/z* = 740.0 (M-1), *m/z* = 742.4 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 72%; White solid; FTIR (KBr): ν (cm^{-1}) 3463.81 (OH), C=O amide under 1669.83 (C=O COOH) is also buried, 1546.08 (C=C in amino acids Phenylalanine and Tyrosine), 1191.50 (C-O COOH), 1132.97 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (14b) 685.35, Found *m/z* = 684.5 (M-1), *m/z* = 686.5 (M+1).

S*ynthesis of Pro-Tyr-Gly-Phe-Val* **(15)**

a) Synthesis of protected peptide

Yield: 73%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1679.88 (C=O COOH), 1641.31 (C=O amide), 1542.95 (C=C in amino acids Phenylalanine and Tyrosine), 1203.50 (C-O COOH), 1137.92 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (15a) 637.35, Found *m/z* = 636.0 (M-1), *m/z* = 638.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 73%; White solid; FTIR (KBr): ν (cm^{-1}) 3461.23 (OH), 3284.38 (NH amide), 1676.24 (C=O COOH), 1638.33 (C=O amide), 1546.48 (C=C in amino acids Phenylalanine and Tyrosine), 1200.21 (C-O COOH), 1133.74 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (15b) 581.29, Found *m/z* = 580.6 (M-1), *m/z* = 582.7 (M+1).

S*ynthesis of Phe-Thr-Pro-Phe-Val* **(16)**

a) Synthesis of protected peptide

Yield: 76%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1679.88 (C=O COOH), 1641.31 (C=O amide), 1541.02 (C=C in amino acids Phenylalanine), 1201.57 (C-O COOH), 1135.99 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine); LC-MS (ESI) *m/z* Calcd for (16a) 665.38, Found *m/z* = 664.0 (M-1), *m/z* = 666.2 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 76%; White solid; FTIR (KBr): ν (cm^{-1}) 3307.44 (OH), C=O amide under 1672.15 (C=O COOH) is also buried, 1535.11 (C=C in amino acids Phenylalanine), 1200.56 (C-O COOH), 1137.61 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine); LC-MS (ESI) *m/z* Calcd for (16b) 609.32, Found *m/z* = 608.1 (M-1), *m/z* = 610.1 (M+1).

S*ynthesis of Phe-Ser-Pro-Phe-Ala* **(17)**

a) Synthesis of protected peptide

Yield: 77%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1674.10 (C=O COOH), 1643.24 (C=O amide), 1541.02 (C=C in amino acids Phenylalanine), 1199.64 (C-O COOH), 1134.07 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine); LC-MS (ESI) *m/z* Calcd for (17a) 623.33, Found *m/z* = 621.9 (M-1), *m/z* = 624.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 77%; White solid; FTIR (KBr): ν (cm^{-1}) 3471.19 (OH), C=O amide under 1667.57 (C=O COOH) is also buried, 1547.01 (C=C in amino acids Phenylalanine), 1195.24 (C-O COOH), 1132.36 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine); LC-MS (ESI) *m/z* Calcd for (17b) 567.27, Found *m/z* = 565.9 (M-1), *m/z* = 568.7 (M+1).

S*ynthesis of Phe-Ser-Pro-Ala-Gly* **(18)**

a) Synthesis of protected peptide

Yield: 76%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1676.03 (C=O COOH), 1633.59 (C=O amide), 1546.80 (C=C in amino acid Phenylalanine), 1203.50 (C-O COOH), 1135.99 (C-O), 600-800 (out of plane bending vibration C-H in amino acid Phenylalanine); LC-MS (ESI) *m/z* Calcd for (18a) 533.29, Found *m/z* = 532.0 (M-1), *m/z* = 534.2 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 76%; White solid; FTIR (KBr): ν (cm^{-1}) 3309.47 (OH), C=O amide under 1673.18 (C=O COOH) is also buried, 1540.80 (C=C in amino acid Phenylalanine), 1199.65 (C-O COOH), 1136.13 (C-O), 600-800 (out of plane bending vibration C-H in amino acid Phenylalanine); LC-MS (ESI) *m/z* Calcd for (18b) 477.22, Found *m/z* = 475.9 (M-1), *m/z* = 478.1 (M+1).

S*ynthesis of Phe-Tyr-Pro-Phe-Gly (Linear Longicalycinin A)* **(19)**

a) Synthesis of protected peptide

Yield: 78%; Yellow oily liquid; FTIR (KBr): ν (cm^{-1}) 3407 (NH amide), broad peak 2400-3400 (OH COOH), 1676.03 (C=O COOH), 1641.31 (C=O amide), 1542.95 (C=C in amino acids Phenylalanine and Tyrosine), 1199.64 (C-O COOH), 1134.07 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (19a) 685.35, Found *m/z* = 684.0 (M-1), *m/z* = 686.3 (M+1).

b) *Synthesis of deprotected peptide*

Yield: 78%; White solid; FTIR (KBr): ν (cm^{-1}) 3323.98 (OH), C=O amide under 1673.08 (C=O COOH) is also buried, 1540.36 (C=C in amino acids Phenylalanine and Tyrosine), 1199.54 (C-O COOH), 1135.67 (C-O), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); LC-MS (ESI) *m/z* Calcd for (19b) 629.29, Found *m/z* = 627.9 (M-1), *m/z* = 630.7 (M+1).

3-2- The Cyclic Longicalycinin A analogues

Synthesis of [Cyclo-(Gly-Tyr-Pro-Phe-Val)] **(1)**

Yield: 75%; White solid; IR (KBr): ν (cm^{-1}) 3407 (NH amide), 1679.88 (C=O amide), 1622.02 (C=C in amino acids Phenylalanine and Tyrosine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 7.34-7.99 (m, CH-Ar), 8.11-8.27 (d, 1H, NHCO), 8.28-8.42 (s, br, 1H, NHCO), 8.47-8.81 (s, br, 3H, NHCO), 14.32-14.56 (s, br, OH, Tyrosine), ^{13}C NMR (DMSO-d_6, 75 MHz,) δ = 12.36 (CH_3), 16.64, 17.97 (CH_2 Proline), 23.66 (CH_2-N Proline), 25.62 (CH_2-Ar Phenylalanine), 25.72 (CH_2-Ar Tyrosine), 41.89 ($CH(CH_3)_2$), 44.97 (CH_2C=O), 46.02 (CH Tyrosine), 46.92 (CH Phenylalanine), 47.77 (CH Valine), 53.61 (CH Proline), 108.76, 109.69, 119.06, 120.37 (CH-Ar), 124.47 (C-Ar Tyrosine), 126.36 (CH-Ar), 127.49 (C-Ar Phenylalanine), 130.48 (C-OH Tyrosine), 137.06, 138.43, 141.00, 142.43, 142.88 (C=O) ; LC-MS (ESI) *m/z* Calcd for (1) 563.36, Found *m/z* = 564.10000(M+1).

Synthesis of [Cyclo-(Leu-Tyr-Pro-Phe-Ala)] **(2)**

Yield: 77%; White solid; IR (KBr): ν (cm^{-1}) 3407 (NH amide), 1679.88 (C=O amide), 1619.02 (C=C in amino acids Phenylalanine and Tyrosine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 6.69-6.79 (d, 2H, CH-Ar Tyrosine), 7.06-7.85 (m, CH-Ar), 7.90-8.06 (t, 1H, CH-Ar Phenylalanine), 8.14-8.27 (d, 1H, NHCO), 8.35-8.51 (s, br, 1H, NHCO), 8.56-8.83 (s, br, 2H, NHCO), ^{13}C NMR (DMSO-d_6, 75 MHz,) δ= 12.35, 16.63 (CH_3), 17.98 (CH_2 Proline), 23.64 (CH Leucine), 25.61 (CH_2 Proline), 25.73 (CH_2 Phenylalanine), 25.84 (CH_2 Tyrosine), 25.94 (CH_2 Leucine), 41.81 (CH_2-N Proline), 44.85 (CH Alanine), 45.93 (CH Leucine), 46.87 (CH Tyrosine), 47.75 (CH Phenylalanine), 53.55 (CH-N Proline), 108.78, 119.07, 120.37, 126.35 (CH-Ar), 127.28 (C-Ar Tyrosine), 127.47 (CH-Ar), 130.46 (C-Phenylalanine), 142.41 (C-OH Tyrosine) ; LC-MS (ESI) *m/z* Calcd for (2) 591.39, Found *m/z* = 592.40000(M+1).

[Cyclo-(Gly-Tyr-Pro-Phe-Ala)] **(3)** *Synthesis of*

Yield: 78%; White solid; IR (KBr): IR (KBr): ν (cm^{-1}) 3205.47 (NH amide), 1676.03 (C=O amide), 1610.80 (C=C in amino acids Phenylalanine and Tyrosine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 7.45-7.55 (d of d, 2H, CH-Ar Phenylalanine), 7.64-7.75 (t, 1H, CH-Ar Phenylalanine), 7.79-7.88 (d, 2H, CH-Ar Phenylalanine), 7.90-8 (t, 1H,NHCO), 8.03-8.16 (d, 1H, NHCO), 8.31-8.46 (s, br, 1H, NHCO), 8.47-8.55 (d, 1H, NHCO), 8.70-8.79 (d, 1H, NHCO), ^{13}C NMR (DMSO-d_6, 75 MHz,) δ = 12.39 (CH_3), 16.69, 18.03 (CH_2 Proline), 41.49 (CH_2C=O), 41.64 (CH_2-N Proline), 41.81 (CH Alanine), 53.57 (CH Tyrosine), 55.15 (CH Phenylalanine), 62.18 (CH-N Proline), 114.52, 115.77, 120.72, 127.14 (CH-Ar), 128.82 (C-Ar Tyrosine), 132.66 (CH-Ar), 133.16 (C-Ar Phenylalanine), 133.34 (C-OH Tyrosine), 134.66, 139.68, 141.71, 150.09, 151.10 (C=O) ; LC-MS (ESI) *m/z* Calcd for (3) 535.33, Found *m/z* = 536.00000(M+1).

Synthesis of [Cyclo-(Ala-Tyr-Pro-Phe-Gly)] **(4)**

Yield: 80%; White solid; IR (KBr): ν (cm^{-1}) 3209.33 (NH amide), 1674.10 (C=O amide), 1616.24 (C=C in amino acids Phenylalanine and Tyrosine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 7.55-7.66 (t, 1H, CH-Ar Phenylalanine), 7.74-7.85 (t, 2H, CH-Ar Phenylalanine), 7.90-8.09 (d, 2H, CH-Ar), 8.13-8.36 (d, 3H, NHCO), 8.42-8.64 (s, br, 1H, NHCO), ^{13}C NMR (DMSO-d_6, 75 MHz,) δ = 12.43 (CH_3), 16.64, 18.04 (CH_2 Proline), 23.60 (CH_2 Phenylalanine), 25.58 (CH_2 Tyrosine), 25.73 (CH_2-N Proline), 41.87 (CH_2 Glycine), 44.97 (CH_2-N Proline), 45.93 (CH Alanine), 47.72 (CH Tyrosine), 53.62 (CH-N Proline), 108.74, 120.36, 126.35, 127.48, (CH-Ar), 129.19 (C-Ar Tyrosine), 130.46 (CH-Ar), 134.11 (C-Ar Phenylalanine), 146.92 (C-OH Tyrosine), 150.11, 158.30, 164.65, 166.05, 168.36 (C=O) ; LC-MS (ESI) *m/z* Calcd for (4) 535.33, Found *m/z* = 536.00000(M+1).

Synthesis of [Cyclo-(Phe-Ser-Pro-Phe-Gly)] **(5)**

Yield: 80%; White solid; IR (KBr): ν (cm^{-1}) 3427.27 (NH amide), 1674.10 (C=O amide), 1641.32 (C=C in amino acids Phenylalanine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 4.04-4.10 (s, br, 2H, $NHCH_2CO$), 4.29-4.38 (s, br, 1H, CH-N Proline), 6.71-8.14 (m, CH-Ar), 8.13-8.45 (s, br, 4H, NHCO) ; LC-MS (ESI) *m/z* Calcd for (5) 535.33, Found *m/z* = 536.20000(M+1).

Synthesis of [Cyclo-(Val-Tyr-Pro-Ile-Ala)] **(6)**

Yield: 74%; White solid; IR (KBr): ν (cm^{-1}) 3209.33 (NH amide), 1676.03 (C=O amide), 1639.38 (C=C in amino acid Tyrosine), 600-800 (out of plane bending vibration C-H in amino acid Tyrosine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 1.60-2.01 (d, 3H, CH-CH_3), 8.12-8.87 (s, br, 4H, NHCO); LC-MS (ESI) *m/z* Calcd for (6) 543.39, Found *m/z* = 544.70000(M+1).

Synthesis of [Cyclo-(Pro-Tyr-Val-Phe-Ala)] **(7)**

Yield: 77%; White solid; IR (KBr): ν (cm^{-1}) 3205.47 (NH amide), 1676.03 (C=O amide), 1546.80 (C=C in amino acids Phenylalanine and Tyrosine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 4.05-4.09 (s, br, 1H, CH-N Proline), 4.31-4.37 (s, be, 1H, CH-N Proline), 6.70-6.80 (d, 2H, CH-Ar Tyrosine), 7.10-7.99 (m, CH-Ar), 8.15-8.57 (s, br, 4H, NHCO), 8.91-8.99 (s, br, 1H, OH Tyrosine) ; LC-MS (ESI) *m/z* Calcd for (7) 577.38, Found *m/z* = 578.10000(M+1).

Synthesis of [Cyclo-(Phe-Tyr-Pro-Val-Gly)] **(8)**

Yield: 79%; White solid; IR (KBr): ν (cm^{-1}) 3444.53 (NH amide), 1679.88 (C=O amide), 1554.52 (C=C in amino acids Phenylalanine and Tyrosine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); ^{1}HNMR (DMSO-d_6, 300MHz,): δ= 7.46-7.52 (d of d, 2H, CH-Ar Phenylalanine), 7.68-7.72 (d, 2H, CH-Ar), 7.93-7.98 (d, 2H, CH-Ar), 8.47-8.50 (d, 1H, NHCO), 8.50-8.53 (d, 1H, NHCO), 8.72-8.74 (d, 1H, NHCO), 8.74-8.75 (d, 1H, NHCO), ^{13}C NMR (DMSO-d_6, 75 MHz,) δ = 12.29 (CH_3), 16.66, 17.98 (CH_2 Proline), 18.68 (CH Valine), 26.81 (CH_2 Glycine), 40.65 (CH_2-N

Proline), 41.75 (CH Tyrosine), 49.09 (CH Phenylalanine), 53.49 (CH-NH Valine), 78.64 (CH-N Proline), 119.07, 120.67, 128.69, 132.15 (CH-Ar), 132.24 (C-Ar Tyrosine), 133.10 (CH-Ar), 134.09 (C- Phenylalanine), 134.65 (C-OH Tyrosine), 139.69, 151.03, 151.08, 151.13, 158.66 (C=O) ; LC-MS (ESI) *m/z* Calcd for (8) 563.36, Found *m/z* = 564.30000(M+1).

Synthesis of [Cyclo-(Phe-Tyr-Pro-Phe-Val)] **(9)**

Yield: 75%; White solid; IR (KBr): ν (cm^{-1}) 3444.57 (NH amide), 1674.10 (C=O amide), 1600.81 (C=C in amino acids Phenylalanine and Tyrosine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 4.39-4.41 (s, br, CH-N Proline), 6.73-6.76 (d, 2H, CH-Ar Tyrosine), 7.11-7.15 (d, 2H, CH-Ar, Tyrosine), 7.47-7.54 (d of d, 4H, CH-Ar, Phenylalanine), 7.66-7.74 (t, 1H, CH-Ar Phenylalanine), 7.80-7.86 (d, 2H, CH-Ar Phenylalanine), 7.91-7.99 (t, 1H, CH-Ar Phenylalanine), 8.05-8.15 (d, 2H, CH-Ar Phenylalanine), 8.49-8.52 (d, 1H, NHCO), 8.52-8.55 (d, 1H, NHCO), 8.73-8.75 (d, 1H, NHCO), 8.76-8.78 (d, 1H, NHCO), 13.61-13.98 (s, br, 1H, OH Tyrosine), ^{13}C NMR (DMSO-d6, 75 MHz,) δ = 10.86 (CH_3), 16.69, 18.06 (CH_2 Proline), 26.42 (CH Valine), 33.5 (CH_2 Phenylalanine), 38.27 (CH_2 Tyrosine), 41.48 (CH_2-N Proline), 41.76 (CH Tyrosine), 45.02 (CH Phenylalanine), 46.63 (CH Phenylalanine), 56.85 (CH Valine), 68.10 (CH Proline), 114.48, 120.73, 127.11, 128.86 (CH-Ar), 132.65 (C-Ar Tyrosine), 133.11 (CH-Ar), 134.63 (C-Phenylalanine), 139.62 (C-OH Tyrosine), 149.50, 150.02, 151.14 (C=O); LC-MS (ESI) *m/z* Calcd for (9) 653.41, Found *m/z* = 654.20000(M+1).

Synthesis of [Cyclo-(Phe-Tyr-Pro-Phe-Ala)] **(10)**

Yield: 75%; White solid; IR (KBr): ν (cm^{-1}) 3379.05 (NH amide), 1602.74 (C=O amide), 1558.38 (C=C in amino acids Phenylalanine and Tyrosine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 7.47-7.53 (m, CH-Ar), 8.50-8.52 (d, 1H, NHCO), 8.52-8.55 (d, 1H, NHCO), 8.73-8.75 (d, 1H, NHCO), 8.75-8.77 (d, 1H, NHCO), 13.72-13.89 (s, br, 1H, OH Tyrosine) ; LC-MS (ESI) *m/z* Calcd for (10) 625.38, Found *m/z* = 626.10000(M+1).

Synthesis of [Cyclo-(Thr-Val-Pro-Phe-Ala)] **(11)**

Yield: 76%; White solid; IR (KBr): ν (cm^{-1}) 3211.26 (NH amide), 1676.03 (C=O amide), 1598.88 (C=C in amino acid Phenylalanine), 600-800 (out of plane bending vibration C-H in amino acid Phenylalanine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 3.50-3.70 (s, br, 1H, OH), 7.42-7.55 (d of d, CH-Ar), 7.67-7.73 (d, CH-Ar), 7.93-7.99 (d, CH-Ar), 8.26-8.43 (d, 2H, NHCO), 8.45-8.56 (d, 1H, NHCO), 8.67-8.82 (d, 1H, NHCO), ^{13}C NMR (DMSO-d_6,75 MHz,) δ = 12.44 (CH_3), 16.70, 18.05 (CH_2-N Proline), 41.88 (CH Valine), 53.61 (CH Alanine), 53.65 (CH Phenylalanine), 120.76, 128.87, 134.71 (CH-Ar), 139.71 (C-Ar Phenylalanine), 151.04, 151.16, 151.27 (C=O) ; LC-MS (ESI) *m/z* Calcd for (11) 515.36, Found *m/z* = 516.20000(M+1).

Synthesis of [Cyclo-(Phe-Tyr-Pro-Phe-Ile)] **(12)**

Yield: 78%; White solid; IR (KBr): ν (cm^{-1}) 3396.41 (NH amide), 1672.17 (C=O amide), 1600.81 (C=C in amino acids Phenylalanine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 3-3.04 (m, 6H, CH_2-Ph), 6.69-6.78 (m, 4H, CH-Ar Tyrosine), 7.10- 7.17 (t, 2H, CH-Ar Phenylalanine), 7.47-7.50 (d, 1H, NHCO), 7.50-7.53 (d, 1H, NHCO), 7.69- 7.73 (d, 2H, CH-Ar), 7.81-7.85 (d, 2H, CH-Ar), 7.94-7.99 (d, 2H, CH-Ar), 8.08-8.12 (d, 2H, CH-Ar), 8.49-8.51 (d, 1H, NHCO), 8.52-8.54 (d, 1H, NHCO), ^{13}C NMR (DMSO-d_6, 75 MHz,) δ = 12.41, 16.70 (CH_3), 18.05 (CH_2 Isoleucine), 38.22 (CH_2 Phenylalanine), 41.50 (CH_2 Tyrosine), 41.81 (CH_2-N Proline), 53.53 (CH Tyrosine), 53.62 (CH Phenylalanine), 118.78, 120.70, 128.86, 129.35 (CH-Ar), 132.65 (C-Ar Tyrosine), 133.31 (CH-Ar), 134.63 (C-Ar Phenylalanine), 139.64 (C-OH Tyrosine), 151.06, 151.16, 157.31, 158.09, 158.57 (C=O) ; LC-MS (ESI) *m/z* Calcd for (12) 667.42, Found *m/z* = 668.60000(M+1).

Synthesis of [Cyclo-(Val-Thr-Pro-Phe-Leu)] **(13)**

Yield: 80%; White solid; IR (KBr): ν (cm^{-1}) 3375.20 (NH amide), 1674.10 (C=O amide), 1598.88 (C=C in amino acid Phenylalanine), 600-800 (out of plane bending vibration C-H in amino acid Phenylalanine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 3.98-4.05 (s, 1H, CH_2NHCO), 4.07-4.11 (d, 1H, CH_2NHCO), 4.31-4.43 (m, 1H, CH_2-N Proline), 7.09-7.18 (t, 2H, CH-CH_2-Ph), 7.46-7.53 (d of d, CH-Ar), 7.65-7.73 (t, CH-Ar), 7.80-7.86 (d, CH-Ar), 8.06-8.12 (d, 1H, NHCO), 8.24-8.37 (s, br, 1H, NHCO), 8.47- 8.55 (d, 1H, NHCO), 8.71-8.78 (d, 1H, NHCO), ^{13}C NMR (DMSO-d_6, 75 MHz,) δ = 12.47 (CH_3), 16.70, 18.07 (CH_2 Proline), 41.85 (CH_2 Leucine), 53.63 (CH Leucine), 120.74, 128.86, 134.70 (CH-Ar), 139.68 (C-Ar), 151.11 (C=O); LC-MS (ESI) *m/z* Calcd for (13) 577.41, Found *m/z* = 578.20000(M+1).

Synthesis of [Cyclo-(Pro-Tyr-Phe-Phe-Leu)] **(14)**

Yield: 72%; White solid; IR (KBr): ν (cm^{-1}) 3207.40 (NH amide), 1666.38 (C=O amide), 1598.88 (C=C in amino acids Phenylalanine and Tyrosine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 3.99-4.10 (m, 1H, CH-N Proline), 4.32-4.42 (m, 1H, CH-N Proline), 4.65-4.71 (s, br, 1H, OH Tyrosine), 7.06-7.29 (m, CH-Ar), 7.44-7.57 (d of d, 2H, CH-Ar), 8.22-8.43 (d, 2H, NHCO), 8.46-8.58 (d, 1H, NHCO), 8.70-8.80 (d, 1H, NHCO), ^{13}C NMR (DMSO-d_6, 75 MHz,) δ = 12.44 (CH_3), 16.70, 18.04 (CH_2 Proline), 41.84 (CH_2 Leucine), 53.60 (CH Leucine), 120.75, 128.86, 134.66 (CH-Ar), 139.66 (C-Ar), 151.14 (C=O); LC-MS (ESI) *m/z* Calcd for (14) 667.42, Found *m/z* = 668.40000(M+1).

Synthesis of [Cyclo-(Pro-Tyr-Gly-Phe-Val)] **(15)**

Yield: 73%; White solid; IR (KBr): ν (cm^{-1}) 3420 (NH amide), 1676.03 (C=O amide), 1537.16 (C=C in amino acids Phenylalanine and Tyrosine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 3.99-4.05 (s, 1H, CH_2NHCO), 4.06-4.11 (d, 1H, CH_2NHCO), 4.32-4.42 (m, 1H, CH_2-N Proline), 6.70-6.79 (d, 2H, CH-Ar Tyrosine), 7.09-7.19

(t, 2H, CH-CH_2-Ph), 7.45-7.55 (d of d, CH-Ar), 7.63-7.75 (t, CH-Ar), 7.77-7.88 (d, CH-Ar), 7.90-8 (t, CH-Ar), 8.05-8.14 (d, 1H, NHCO), 8.26-8.46 (s, br, 1H, OH Tyrosine), 8.47- 8.54 (d, 1H, NHCO), 8.56-8.63 (d, 1H, NHCO), 8.71-8.78 (d, 1H, NHCO), 8.86-8.93 (d, 1H, NHCO), ^{13}C NMR (DMSO-d_6, 75 MHz,) δ = 12.39 (CH_3), 16.69, 18.09 (CH_2 Proline), 38.25 (CH_2 Phenylalanine or CH_2 Tyrosine), 41.54 (CH_2 Glycine), 41.88 (CH_2-N Proline), 53.63 (CH Phenylalanine or CH Tyrosine), 115.80, 118.85, 127.21, 129.44 (CH-Ar), 132.68 (C-Ar Tyrosine), 133.21 (CH-Ar), 133.40 (C-Ar Phenylalanine), 134.71 (C-OH Tyrosine), 150.12, 151.19, 157.42, 158.34, 158.83 (C=O) ; LC-MS (ESI) *m/z* Calcd for (15) 563.36, Found *m/z* = 564.50000(M+1).

Synthesis of [Cyclo-(Phe-Thr-Pro-Phe-Val)] **(16)**

Yield: 76%; White solid; IR (KBr): ν (cm^{-1}) 3439.34 (NH amide), 1674.10 (C=O amide), 1600.81 (C=C in amino acids Phenylalanine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 6.70-6.78 (m, 2H, CH-Ar), 7.10-7.26 (m, 4H, CH-Ar), 7.26-7.56 (d of d, 4H, CH-Ar), 7.64-7.76 (t, 1H, CH-Ar), 7.78-7.89 (d, 4H, CH-Ar), 7.90-8.01 (t, 1H, CH-Ar), 8.05-8.16 (d, 1H, NHCO), 8.21-8.34 (s, br, 1H, OH Threonine), 8.38-8.44 (d, 1H, CH_2-NHCO), 8.46-8.56 (d, 1H, NHCO), 8.71-8.80 (d, 1H, NHCO), 8.82-8.87 (d, 1H, CH_2NHCO), ^{13}C NMR (DMSO-d_6, 75 MHz,) δ = 12.46, 16.71 (CH_2 Proline), 18.06 (CH_2 Phenylalanine), 41.50 (CH_2 Glycine), 41.76 (CH_2-N Proline), 41.86 (CH Threonine), 53.63 (CH Phenylalanine), 114.53, 127.17, 132.67 (CH-Ar), 133.19 (C-Ar Phenylalanine), 134.67, 139.66, 150.09, 151.16 (C=O) ; LC-MS (ESI) *m/z* Calcd for (16) 591.39, Found *m/z* = 592.40000(M+1).

Synthesis of [Cyclo-(Phe-Ser-Pro-Phe-Ala)] **(17)**

Yield: 77%; White solid; IR (KBr): ν (cm^{-1}) 3203.54 (NH amide), 1676.03 (C=O amide), 1575.73 (C=C in amino acids Phenylalanine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 7.06-7.32 (m, 2H, CH-Ar), 7.40-7.44 (d, 2H, CH-Ar), 7.35-7.57 (d, 2H, CH-Ar), 7.69-7.73 (d, 2H, CH-Ar), 8.13-8.42 (s, br, 4H, NHCO) ; LC-MS (ESI) *m/z* Calcd for (17) 549.34, Found *m/z* = 550.30000(M+1).

Synthesis of [Cyclo-(Phe-Ser-Pro-Ala-Gly)] **(18)**

Yield: 76%; White solid; IR (KBr): ν (cm^{-1}) 3454.34 (NH amide), 1676.03 (C=O amide), 1633.59 (C=C in amino acid Phenylalanine), 600-800 (out of plane bending vibration C-H in amino acid Phenylalanine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 7.11-7.28 (d, CH-Ar), 8.36-8.74 (s, br, 4H, NHCO) ; LC-MS (ESI) *m/z* Calcd for (18) 459.3, Found *m/z* = 460.20001(M+1).

Synthesis of [Cyclo-(Phe-Tyr-Pro-Phe-Gly)] (Cyclic Longicalycinin A) **(19)**

Yield: 78%; White solid; IR (KBr): ν (cm^{-1}) 3420.12 (NH amide), 1679.88 (C=O amide), 1610.95 (C=C in amino acids Phenylalanine and Tyrosine), 600-800 (out of plane bending vibration C-H in amino acids Phenylalanine and Tyrosine); ^{1}HNMR (DMSO-d_6, 300 MHz,): δ= 3.09-3.14 (d of d, 2H, CH_2-Ph), 8.22-8.31 (s, br, 4H, NHCO), ^{13}C NMR (DMSO-d_6, 75 MHz,) δ = 25.86 (CH_3), 25.97, 26.98 (CH_2 Proline), 27.15 (CH_2 Phenylalanine), 41.80 (CH_2-N Proline), 44.75, 45.91, 45.96, 47.74 (CH_2), 53.52 (CH-N Proline), 124.42, 128.05, 129.14 (CH-Ar), 130.5 (C-Ar Phenylalanine), 158.39, 158.53, 158.81, 168.67, 169.03 (C=O); LC-MS (ESI) *m/z* Calcd for (19) 611.36, Found *m/z* = 612.50000(M+1).

MTT assay results

The deprotected linear and cyclic peptides of Longicalycinin A analogues have shown cytotoxicity to HepG2 and HT-29 cancer cell lines in varying degree (ranging IC_{50} values from 8.76 to 17.2µg/mL and 9.06 to 17.34 µg/mL, respectively). Tables 1 and 2 show the IC_{50} results for all deprotected linear and cyclic analogues of Longicalycinin A peptide, respectively, along with IC_{50} results for 5-flurouracil, chosen as a standard toxic drug. Tables 3 and 4 contain viability percentage data for linear and cyclic analogues of Longicalycinin A peptide, respectively, on three cell lines; skin Fibroblast, HepG2 and HT-29 cancer cells.

Table 1. IC_{50} values (µg/mL) for anti-proliferative activity of peptides on HepG2 and HT-29 cells in MTT assay. Values are presented as mean ± SD of three independent experiments (n= 3). The stars show the values are significantly different from the corresponding control (**P* <0.05, ***P* <0.01, *** *P* <0.001).

Linear	IC_{50} cell line HepG2	IC_{50} cell line HT-29
1	9.66±0.0007***	10.45±0.0042***
2	8.76±0.0035***	12.33±0***
3	9.77±0.0007***	11.4±0***
4	9.53±0.0014***	10.78±0.0021**
5	8.99±0.0014***	10.97±0.0049***
6	10.98±0.0014***	12.31±0.0063
7	9.6±0.0007***	9.78±0.0212***
8	10.35±0.0014***	12.88±0.0014***
9	9.33±0.0007***	10.44±0.5536
10	10.5±0.0042***	10.5±0.0007***
11	9.56±0.0021***	11.67±0.0014***
12	11.32±0.0014***	14.2±0.0014**
13	9.26±0.0028***	17.2±0.0014**
14	9.67±0.0007***	9.12±0.0042***
15	9.44±0***	10.55±0.0989
16	11.1±0.0007***	12.11±0.0056***
17	9.33±0.0007***	11.33±0.0035***
18	10.33±0.0007***	10.2±0.0014**
19 (Linear Longicalycinin A)	9.18±0.0014***	9.61±0.0007*
5-Fluorouracil (standard drug)	3.16	6.08

Table 2. IC_{50} values (μg/mL) for toxicity activity of peptides on HepG2 and HT-29 cells in MTT assay. Values are presented as mean ± SD of three independent experiments (n= 3). The stars show the values are significantly different from the corresponding control (*** P <0.001).

Cyclic	IC_{50} cell line HepG2	IC_{50} cell line HT-29
1	10.2±0.0007***	11.4±0.0028***
2	10.4±0.0014***	11.09±0.0014***
3	9.5±0.0056***	11.28±0.0063
4	9.77±0.0007***	11.33±0.0035***
5	9.45±0.0056***	9.98±0.4256***
6	17.34±0.0014***	10.2±0.0021
7	9.21±0.0056***	12.11±0.0035
8	9.12±0.0007***	12.67±0.0028
9	10.67±0***	10.7±0.0056***
10	10.28±0***	11.56±0.0028***
11	10.36±0***	10.61±0.0007***
12	12.37±0.0014***	13.2±0***
13	9.44±0.0014***	9.08±0.0007
14	10.46±0.0028***	10.92±0.0021***
15	10.33±0***	11.23±0.0007
16	12.1±0.0007***	10.4±0.0028***
17	9.4±0.0014***	11.2±0.0007
18	9.68±0.0014***	9.06±0.0014***
19 (Cyclic Longicalycinin A)	10.45±0.0042***	11.87±0.0021
5-Fluorouracil (standard drug)	3.16	6.08

LDH analysis results

The results of viability percentage peptides-treated HepG2 and HT-29 cells calculated by measuring optical density in the LDH assay are shown in Tables 5 and 6.

Table 5. LDH assay of linear Longicalycinin A analogues for cell lines HepG2 and HT-29

Linear	Viability percentage (HepG2)	Viability percentage (HT-29)
1	44.52	54.02
2	34.56	47.8
3	63.65	51.31
4	76.62	78.36
5	46.45	60.51
6	67.05	21.26
7	80.94	64.87
8	45.29	61.56
9	66.04	51.31
10	65.97	41.29
11	41.58	64.87
12	61.18	52.65
13	71.91	40.92
14	73.53	64.63
15	42.97	74.41
16	53.31	40.92
17	74.76	77.58
18	48.45	52.65
19	60.03	31.29
5-FU	27.46	7.6
Control	100	100

Table 6. LDH assay of cyclic Longicalycinin A analogues for cell lines HepG2 and HT-29.

Cyclic	Viability percentage (HepG2)	Viability percentage (HT-29)
1	52.16	15.85
2	60.49	21.56
3	43.67	33.75
4	73.91	15.95
5	48.14	30.53
6	60.18	63.12
7	47.53	93.21
8	45.21	22.63
9	43.51	17.26
10	73.45	53.12
11	45.06	35.85
12	43.36	13.51
13	79.16	35.6
14	50	11.26
15	59.72	20.53
16	72.53	21.12
17	44.13	16.97
18	68.13	30.78
19	44.29	14.04
5-Fu	27.46	7.6
Control	100	100

Flow cytometry analysis results

Tables 7 and 8 show flow cytometery results of linear and cyclic analogues of Longicalycinin A. Figures 1 and 2 show the histograms 1 and 5 as example. All the other related histograms are included in the supplementary file. The left dot plots present the forward scatter (FSC) parameter in the horizontal axis and side scatter (SSC) parameter in the vertical axis, which can be correlated with the relative size and granularity of the cells, respectively. The histograms show the intensity of fluorescence of the samples in the FL-2 channel, which corresponds to PI emission wavelength.[26]

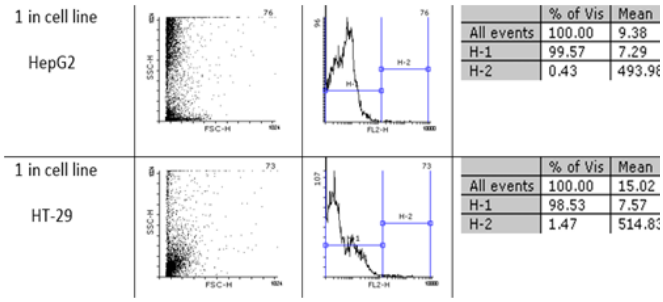
Table 7. Apoptosis percentageof linear Longicalycinin A analogues for cell lines HepG2 and HT-29

Linear	Apoptosis percentage (HepG2)	Apoptosis percentage (HT-29)	Viability percentage (HepG2)	Viability percentage (HT-29)
1	99.57	98.53	0.43	1.47
2	0.62	2.65	99.18	97.57
3	99.78	99.66	0.22	0.34
4	99.56	67.12	0.44	32.76
5	99.76	99.43	0.24	0.59
6	99.41	99.57	0.6	0.43
7	99.66	99.6	0.33	0.4
8	99.74	99.63	0.25	0.37
9	99.69	99.78	0.31	0.22
10	99.68	99.63	0.32	0.37
11	1.57	2.45	98.18	97.05
12	0.04	1.84	99.76	97.84
13	1.78	2.63	97.77	96.55
14	99.6	99.68	0.41	0.32
15	99.59	99.79	0.41	0.21
16	99.05	99.53	0.96	0.47
17	99.55	99.65	0.45	0.35
18	99.57	99.38	0.43	0.62
19	99.76	99.34	0.24	0.66

Table 8. Apoptosis percentageof cyclic Longicalycinin A analogues for cell lines HepG2 and HT-29

Cyclic	Apoptosis percentage (HepG2)	Apoptosis percentage (HT-29)	Viability percentage (HepG2)	Viability percentage (HT-29)
1	73.15	2.86	3.17	95.18
2	4.24	91.67	81.3	7.87
3	6.33	0.18	93.09	99.79
4	4.26	91.32	90.57	0.44
5	56.79	12.29	0.3	87.61
6	4.35	10.91	94.02	89.32
7	1.64	97.91	97.86	2.09
8	1.48	6	98.18	94.03
9	99.24	96.21	0.42	0.48
10	1.4	98.65	97.83	1.01
11	2.48	9.95	97.48	88.75
12	99.78	99.6	0.22	0.4
13	98.81	99.46	0.87	0.47
14	11.25	4.9	88.78	86.81
15	99.58	6.5	0.4	90.17
16	98.06	4.93	0.65	93.2
17	2.29	0.96	97.77	98.68
18	99.76	97.66	0.24	0.48
19	90.85	95.24	0.85	4.34

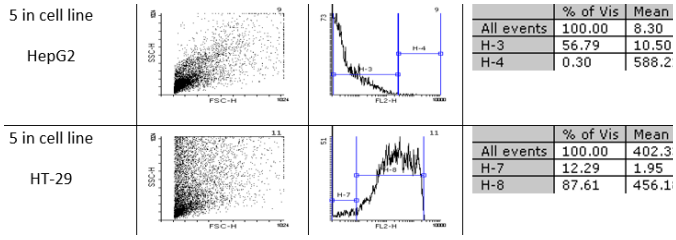
Figure 1. Flow cytometery results obtained from deprotected linear peptide-treated HepG2 and HT-29 cells



	% of Vis	Mean
All events	100.00	9.38
H-1	99.57	7.29
H-2	0.43	493.98

	% of Vis	Mean
All events	100.00	15.02
H-1	98.53	7.57
H-2	1.47	514.83

Figure 2. Flow cytometery results obtained from deprotected cyclic peptide-treated HepG2 and HT-29 cells



	% of Vis	Mean
All events	100.00	8.30
H-3	56.79	10.50
H-4	0.30	588.2

	% of Vis	Mean
All events	100.00	402.3
H-7	12.29	1.95
H-8	87.61	456.1

Determination of Lysosomal membrane integrity results

Lysosome damage using acridine orange as a probe was assayed in isolated lysosome obtained from the treated HepG2 and HT-29. Values are presented as mean ± SD of three independent experiments performed for determination of Lysosomal membrane integrity of HepG2 and HT-29 cells (Figs 3-6). As shown in Figs 3 and 5, linear peptides with numbers 18 and 17 caused the highest absorption of acridine orange dye in HepG2 and HT-29 cell lines, respectively and in Figs 4 and 6, cyclic peptides with numbers 14 and 11 caused the highest absorption of acridine orange dye in HepG2 and HT-29 cell lines, respectively.

Figure 3. Determination of Lysosomal membrane integrity assay on HepG2. The values show acridine orange absorbance of the damaged lysosomes of peptide-treated HepG2 cells and are presented as mean ± SD of three independent experiments (n= 3). The stars show the values are significantly different from the corresponding control (***P* <0.01, *** *P* <0.001).

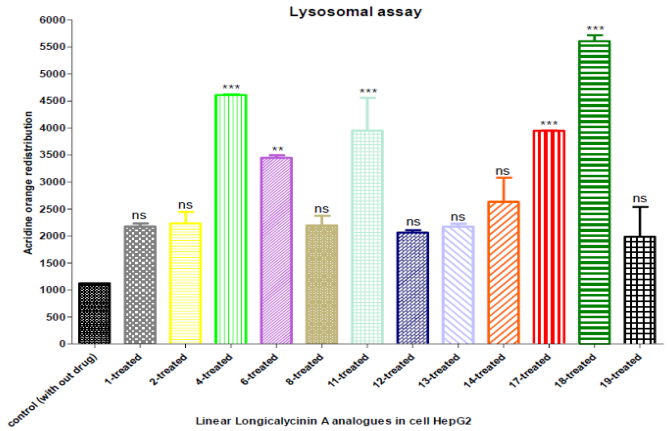


Figure 4. Determination of Lysosomal membrane integrity assay on HepG2. The values show acridine orange absorbance of the damaged lysosomes of peptide-treated HepG2 cells and are presented as mean ± SD of three independent experiments (n= 3). The stars show the values are significantly different from the corresponding control (**P* <0.05, ***P* <0.01, *** *P* <0.001).

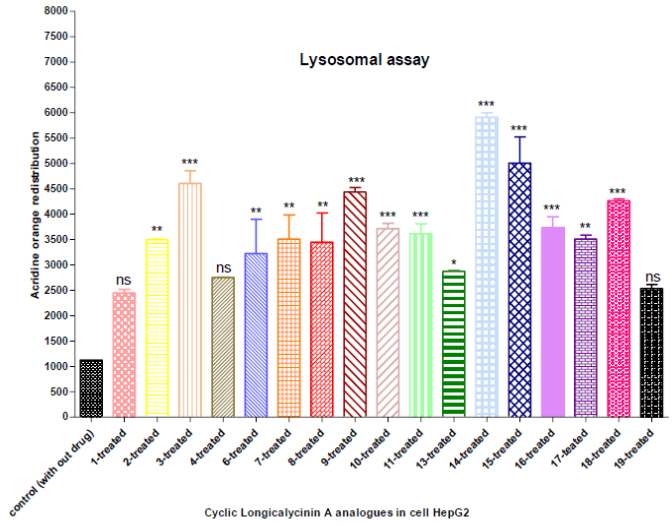


Figure 5. Determination of Lysosomal membrane integrity assay on HT-29. The values show acridine orange absorbance of the damaged lysosomes of peptide-treated HT-29 cells and are presented as mean ± SD of three independent experiments (n= 3). The stars show the values are significantly different from the corresponding control (**P* <0.05, ***P* <0. 01, *** *P* <0.001).

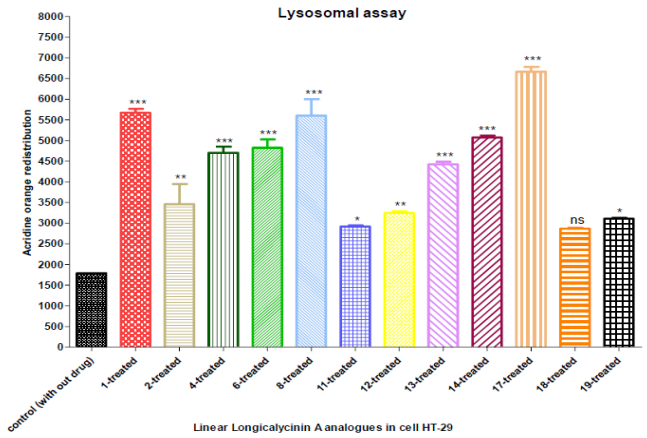
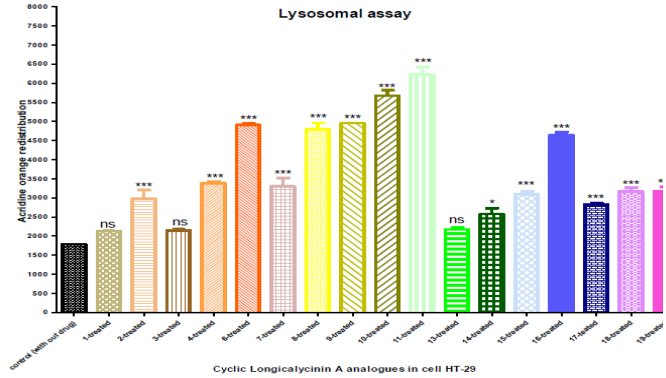


Figure 6. Determination of Lysosomal membrane integrity assay on HT-29. The values show acridine orange absorbance of the damaged lysosomes of peptide-treated HT-29 cells and are presented as mean ± SD of three independent experiments (n= 3). The stars show the values are significantly different from the corresponding control (**P* <0.05 and *** *P* <0.001).



Discussion:

Two-step synthesis of peptides on 2-CTC resin was chosen as the main strategy in this study due to several advantages achievable from this kind of resin. At the end of synthesis to obtain protected linear peptides, the trityl linker could be readily cleaved under a mild acidic condition by treatment with TFA 1% in dichloromethane owing to the high stability of trityl cations attached to the resin.[28] Meanwhile desired Longicalycinin A analogues with protected side chains could be obtained in a good yield and high purity without producing byproducts due to side reactions of the protecting groups released in the solution. In the second step, deprotection of the side chain (*O-t-Bu*) linear Longicalycinin A analogues were achieved with TFA 95% containing scavengers. For the synthesis of cyclic peptides, cyclization of linear *(Ot-Bu)* Longicalycinin A analogues was achieved using coupling reagents and then the final deprotection was applied on the cyclic *(Ot-Bu)* Longicalycinin A analogues by treatment with TFA 95% containing scavengers to obtain cyclic Longicalycinin A analogues**.**

Synthesized linear and cyclic Longicalycinin A analogues exhibited considerable cytotoxic activity against cell lines of HepG2 (human liver cancer Cell Line) and HT-29 (Human Colorectal Adenocarcinoma Cell Line) with mean IC_{50} values ranging from 8.76 to 17.2 µg/mL and 9.06 to 17.34 µg/mL, respectively, in comparison with standard drug 5-fluorouracil (5-FU). The results of MTT assay are shown in Table 1. According to these results, the synthesized compounds 2, 3, 5, 7, 9, 10, 11, 15 and 19 with IC_{50} values (8.76-10.5 µg/mL) on HepG2 cell lines showed relatively high toxic activity against cell viability. All of the compounds had high toxic effects on colon cancer cell line with the exception of compounds 9 and 15 (see Table 3). Compounds 1, 8, 12, 13, 14, 16 and 18 with IC_{50} values (9.26-11.32 µg/mL) on HepG2, compounds 9 and 15 with IC_{50} values (10.44-10.55 µg/mL) on HT-29 cell lines showed low potency on cell death and were classified as fewer toxic agents on cell lines, assuming at least containing less than one third potential activity as that of 5-FU. Skin Fibroblast cells as the normal cells with a relatively high-rate division could provide a safety profile for the synthesized peptide compounds. The higher IC_{50} should give the better safety profile. Among the compounds thus prepared, compounds 1, 6, 8, 12, 13, 14 and 18 showed a high safety result on normal cell line with a low toxicity activity on HepG2 cell line. Moreover, comparing with linear Longicalycinin A, compounds 1, 8, 12, 13 and 14 give higher toxicity on cancerous HT-29 cell line. Structurally, compound 1 is different from Longicalycinin A linear peptide by two amino acids Gly and Phe being replaced by Val and Gly, respectively. Compound 8 is different from Longicalycinin A linear peptide by one amino acid Phe being replaced by Val. Compound 12 is different from Longicalycinin A linear peptide by one amino acid Gly being replaced by Ile. Compound 13 is different from Longicalycinin A linear peptide by three amino acids Gly, Tyr and Phe being replaced by Leu, Thr and Val, respectively. Compound 14 is different from Longicalycinin A linear peptide by three amino acids Gly, Pro and Phe being replaced by Leu, Phe and Pro, respectively. Comparing substitution of amino acids as above can direct this notion that the lipophilicity of the peptide compounds is in favour of high toxicity towards cancerous colon cells (HT-29) along with low toxicity on Fibroblast cells.

The results of MTT assay for Longicalycinin A cyclic analogues are shown in Table 4. According to these results, the synthesized compounds 1, 2, 4, 9, 10, 11, 12, 14, 17 and 19 with IC_{50} values (9.4-12.37 µg/mL) on HepG2 and compounds 1, 2, 5, 9, 10, 11 and 17 with IC_{50} values (9.98-11.56 µg/mL) on HT-29 cell lines showed relatively high toxic activity against cell viability. Cyclic analogues in HepG2 cell line than HT-29 cell line have high toxic effects. Compound 8 with IC_{50} value (9.12 µg/mL) on HepG2, compounds 3, 6, 7, 8, 13, 15, 16 and 18 with IC_{50} values (9.06-12.67 µg/mL) on HT-29 cell lines showed low potency on cell death and were classified as fewer toxic agents on cell lines, assuming at least containing more than one third potential activity as that of 5-FU. Skin fibroblast cell line as the

normal cells with a relatively high-rate division could provide a safety profile for the synthesized peptide compounds. Among the compounds thus prepared, compounds 3, 5, 6, 8, 13, 15 and 16 showed a high safety result on normal cell line. Moreover, comparing with Longicalycinin A cyclic peptide, compounds 3, 5, 6, 8, 13, 15 and 16 showed a high safety result on normal cell line, compounds 1, 2, 4, 9, 10, 11, 14 and 17 showed higher toxicity on cancerous HepG2 cell line and compounds 1, 2, 5, 9, 10, 11 and 17 showed higher toxicity on cancerous HT-29 cell line. In comparing with Longicalycinin A cyclic peptide, cyclic peptides 1, 2, 9, 10, 11, 17 have higher toxicity on cancerous HepG2 and HT-29 cell lines. Among the peptides, compound 5 showed a considerable toxicity activity on cancer cell line HT-29 than HepG2 along with a high safety on normal cells. Structurally, compound 5 is different from Longicalycinin A cyclic peptide by one amino acid Tyr being replaced by Ser.

Table 3. Viability percentage of linear Longicalycinin A analogues for cell lines skin Fibroblast, HepG2 and HT-29 in MTT assay

Linear	Viability percentage (Fibroblast)	Viability percentage (HepG2)	Viability percentage (HT)
1	75.86	86.71	16.96
2	25.8	28.63	11.53
3	27.82	17.67	12.44
4	51.72	72.87	24.09
5	27.94	23.28	8.48
6	82.28	61.36	50.67
7	40.66	17.67	15.83
8	86.8	84.65	11.99
9	26.99	27.26	98.41
10	42.09	23.28	7.91
11	27.46	29.72	11.53
12	76.21	87.94	18.66
13	76.45	88.21	18.89
14	75.86	86.98	17.64
15	26.51	26.02	84.84
16	52.08	84.24	11.76
17	50.89	72.46	24.32
18	74.68	84.45	43.49
19	42.21	23.28	9.16
5-FU	28.53	14.79	6.9
Control	100	100	100

Table 4. Viability percentage of cyclic Longicalycinin A analogues for cell lines skin fibroblast, HepG2 and HT-29 in MTT assay.

Cyclic	Viability percentage (Fibroblast)	Viability percentage (HepG2)	Viability percentage (HT-29)
1	34.24	30.27	28.5
2	33.41	27.94	24.88
3	72.05	43.28	71.49
4	39.12	28.9	47.51
5	83.7	58.63	2.26
6	71.93	48.21	72.39
7	56.48	52.32	77.14
8	69.08	63.15	70.13
9	37.45	26.02	23.75
10	34.6	27.94	28.73
11	46.84	27.94	25.11
12	38.76	36.16	47.28
13	70.74	44.65	71.38
14	39.47	29.31	47.62
15	68.01	47.67	72.51
16	82.28	57.67	70.13
17	45.65	25.47	23.86
18	55.61	53.42	77.2
19	38.76	36.71	47.62
5-Fu	28.53	14.79	6.9
Control	100	100	100

The LDH assay of the samples was obtained by measuring optical density. Increase in optical density indicates greater LDH activity of cancerous cells lines HepG2 and HT-29 while decrease in optical density represents lower LDH activity of cancerous cells lines HepG2 and HT-29. As shown in Table 5, compound 6 results in a low viability percentage of the cancerous cell HT-29 while the compound 2 gave a low viability of HepG2. Considering Tables 3 and 5, it can be concluded that compounds 1, 8, 12, 13 and 14 are favorite compounds from the point of toxicity on cancerous cells HT-29 with a safety profile on normal cells. As shows in Table 6, for Longicalycinin A cyclic analogues, compound 14 result in a low viability percentage of the cancerous cell HT-29 while the compound 12 gave a low viability of HepG2. Figs 1 , 2 and supplementary file show the histograms obtained from flow cytometry analysis of the peptides-treated cancerous cell lines. The signals obtained in H-1 area mean that PI was able to enter the cells and bind to DNA and so apoptosis has occurred, whereas, the signals appeared in H-2 area indicate that the cancerous cells treated with peptides remained intact and so no apoptosis has happened. [26]

In this work, MTT results for some linear analogues of Longicalycinin A were not associated with the results of apoptosis, whereas for some other analogues, the MTT results did confirm the apoptosis data (compounds 3, 5, 7, 9, 10, 15, 16). MTT results for all analogues of cyclic Longicalycinin A with the exception of compounds 5 and 12 were not associated with the results of apoptosis.

In order to find the mechanism of apoptotic action of the linear and cyclic peptides analogues, lysosomal membrane integrity experiment was employed for measuring lysosomal damage of the cancerous cells affected by these peptides. As shown in Fig. 3, HepG2 cells gave more acridine orange absorption after contacting with linear peptide 18 than linear other peptides compared with untreated cells (control group). This means that HepG2 cell lysosomes were more damaged and thus more release of cathepsin occurred which by binding with acridine orange gave higher absorption detected by the instrument. The more release of cathepsin means more apoptosis has occurred.[29] For HT-29 cells, the above phenomena were happened for linear peptide 17 rather than linear other peptides comparing with control, Fig.5. As shown in Fig. 4, HepG2 cells gave more acridine orange absorption after contacting with cyclic peptide 14 than cyclic other peptides compared with untreated cells (control group). This means that HepG2 cell lysosomes were more damaged and thus more release of cathepsin occurred which by binding with acridine orange gave higher absorption detected by the instrument. For HT-29 cells, the above phenomena were happened for cyclic peptide 11 rather than cyclic other peptides comparing with control, Fig.6.

Conclusion:

The synthesized linear analogues of Longicalycinin A showed toxic effects on chosen cancerous cells of liver and colon with varying degrees. Meanwhile, the peptides 2, 3, 5, 9, 11 and 15 were toxic on the skin Fibroblast cells, whereas the other linear peptides were not (see Table 3). The compounds 1, 8, 12, 13, and 14 had higher toxicity on the cell line colon and lower toxicity on the cell line liver in MTT assay. It can be

suggested that for these peptides, toxicity on the colon cell line occurs through mitochondrial pathway and lipophilicity plays a major role in this phenomenon. On the other hand, MTT results for some analogues of linear Longicalycinin A were not associated with the results of apoptosis. In the Lysosomal pathway, compound 18 showed higher toxicity towards the liver cells while compound 17 demonstrated more toxicity on the colon cells. The results of determination of Lysosomal membrane integrity experiment showed that the mechanism of apoptotic action of the linear peptides 17 and 18 might be through lysosomal damage not by the mitochondria pathway. The synthesized cyclic analogues of Longicalycinin A showed toxic effects on chosen cancerous cells of liver and colon with varying degrees. Meanwhile, the peptides 1, 2, 4, 9, 10, 12, 14 and 19 were toxic on the skin fibroblast cells, whereas the other cyclic peptides were not. The compounds 3, 5, 6, 8, 13, 15 and 16 showed a high safety result on normal cell line, compounds 1, 2, 9, 10, 11 and 17 showed higher toxicity both on cancerous HepG2 and HT-29 cell line and compound 5 showed higher toxicity on cancerous HT-29 cell line. In comparing with Longicalycinin A cyclic peptide, cyclic peptides 1, 2, 9, 10, 11, 12, 17 have higher toxicity on cancerous HepG2 and HT-29 cell lines. Among the peptides, compound 5 showed a considerable toxicity activity on cancer cell line HT-29 than HepG2 along with a high safety on normal cells. On the other hand, MTT results with the exception of compounds 5 and 12 were not associated with the results of apoptosis. In the lysosomal membrane integrity pathway, compound 14 showed higher toxicity towards the liver cells while compound 11 demonstrated more toxicity on the colon cells. The results of membrane integrity experiment showed that the mechanism of apoptotic action of the cyclic peptides might be through lysosomal damage not by the mitochondria pathway.

In conclusion, linear compounds 1, 8, 12, 13 and 14, considering the property of toxicity action good enough on cancerous cell lines HT-29 along with high safety profile on normal skin cells and cyclic compound 5 with the sequence *[Cyclo-(Phe-Ser-Pro-Phe-Gly)]*, considering the property of toxicity action good enough on cancerous cell lines along with high safety profile on normal skin cells led us to choose these peptides as good candidates for further works to make more relevant derivatives in order to gain structure-activity relationship data and also more closer inside understanding the mechanism of action for such peptide compounds on cancerous cells.

Compliance with ethical standards

Conflict of interest the authors declare that there is no conflict of interest on this research work.

Ethical approval this work did not involve any studies on human or animal experiment undertaken by any of these authors.

Informed consent Informed consent was obtained from all individual participants included in this study.

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References

1. Anand, P.; Kunnumakara, A.B.; Sundaram, C.; Harikumar, K.B.; Tharakan, S.T.; Lai, OS. Cancer is a preventable disease that requires major lifestyle changes. *Pharmaceutical research*. **2008**, *25*, 2097-2116.
2. Sandhu, I.K.; Nair, M.; Aharwal, R.P; Sandhu, S.S. Diagnosis of Cancer Using Artificial Neural Network and Cloud Computing Approach. *World Journal of Pharmacy and Pharmaceutical Sciences*. **2014**, *3*, 1533-1548.
3. Tawfiq, L.; Abdul-Jabbar, S. Diagnosis of cancer using artificial neural network. *International Journal of Advances in Applied Mathematics and Mechanics*. **2015**, 3, 45-49.
4. Xiao, Y.F.; Jie, M.M.; Li, B.S.; Hu, C.J.; Xie, R.; Tang, B. Peptide-Based Treatment: A Promising Cancer Therapy. *Journal of immunology research.* **2015**, 1-13, 2015.
5. X. Maa.; C. Wu, W.; Wang, X. Li. Peptides from Plants: A New Source for Antitumor Drug Research. *Asian J Tradi Med.* **2006**, 2, 85-90.
6. Y. Sakai.; T. Yoshida.; T. Tsujita.; K. Ochiai.; T. Agatsuma.; Y. Saitoh.; GE3, a novel hexadepsipeptide antitumor antibiotic, produced by Streptomyces sp. I. Taxonomy, production, isolation, physico-chemical properties, and biological activities. *The Journal of antibiotics*. **1997**, 50, 659-664.
7. W. Inman.; P. Crews, Novel marine sponge-derived amino acids. 8. Conformational analysis of jasplakinolide. *Journal of the American Chemical Society.* **1989,** 111, 2822-2829.
8. N. Shinde.; A. Dhake.; K. Haval. Biological activities of cyclic peptides: an overview. Res *J Pharm Biol Chem Sci*. **2013,** 4, 142-158.
9. Y.F. Xiao.; M.M. Jie.; B.S. Li.; C.J. Hu.; R. Xie.; B. Tang. Peptide-Based Treatment: A Promising Cancer Therapy. *Journal of immunology research*. **2015**, 13.
10. Y.Z. Shu. Recent natural products based drug development: a pharmaceutical industry perspective. *Journal of natural products.* **1998**, 61, 1053-1071.
11. C.J. White.; A.K. Yudin. Contemporary strategies for peptide macrocyclization. *Nature chemistry*. **2011**, 3, 509-524.
12. L. Yang.; G. Morriello. Solid phase synthesis of 'head-to-tail'cyclic peptides using a sulfonamide 'safety-catch'linker: the cleavage by cyclization approach. *Tetrahedron letters.* **1999**, 40, 8197-8200.
13. Park JG.; Langenwalter KJ.; Weinbaum CA.; Casey PJ.; Pang YP. Improved loading and cleavage methods for solid-phase synthesis using chlorotrityl resins: synthesis and testing of a library of 144 discrete chemicals as potential farnesyltransferase inhibitors. *Journal of combinatorial chemistry.* **2004**, 6, 407-413.
14. Olsen CA.; Witt M.; Jaroszewski JW.; Franzyk H. Solid-phase synthesis of rigid acylpolyamines using temporary N-4,4'-dimethoxytrityl protection in the presence of trityl

linkers. *The Journal of organic chemistry.* **2004,** 69, 6149-6152.
15. Lundquist IV JT.; Satterfield AD.; Pelletier JC. Mild and adaptable silver triflate-assisted method for trityl protection of alcohols in solution with solid-phase loading applications. *Organic letters.* **2006**, 8, 3915-3918.
16. Crestey F.; Ottesen LK.; Jaroszewski JW.; Franzyk H. Efficient loading of primary alcohols onto a solid phase using a trityl bromide linker. *Tetrahedron Letters.* **2008,** 49, 5890-5893.
17. Ahmad W.; Shaheen F.; Zhou Y.; Zhang L. Solid-phase total synthesis of cyclic pentapeptide Longicalycinin A, by using 2-chlorotrityl chloride resin. *Journal of Cancer Research and Experimental Oncology.* **2013**, 5, 8-19.
18. Oshima Y.; Ohsawa T.; Oikawa K.; Konno C.; Hikino H. Structures of dianosides A and B, analgesic principles of Dianthus superbus var. longicalycinus herbs1. *Planta medica.* **1984,** 50, 40-43.
19. Oshima Y.; Ohsawa T.; Hikino H. Structure of Dianosides C, D, E and F, Triterpenoid Saponins of Dianthus superbus var. longicalycinus Herb1. *Planta medica.* **1984,** 50, 43-47.
20. Oshima Y.; Ohsawa T.; Hikino H. Structures of dianosides G, H and I, triterpenoid saponins of Dianthus superbus var. longicalycinus herbs. *Planta medica.* **1984**, 50, 254-258.
21. Hikino H.; Ohsawa T.; Kiso Y.; Oshima Y. Analgesic and Antihepatotoxic Actions of Dianosides, Triterpenoid Saponins of Dianthus superbus var. longicalycinus Herb. *Planta medica.* **1984**, 5, 353-355.
22. T. Mosmann. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods.* **1983**, 65, 55-63.
23. E. Amador.; L.E. Dorfman.; W.E. Wacker. Serum lactic dehydrogenase activity: an analytical assessment of current assays. *Clinical Chemistry*. **1963**, 9, 391-399.
24. T. Decker.; M.L Lohmann-Matthes. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *Journal of immunological methods.* 1988, 115, 61-69.
25. C. Legrand.; J. Bour.; C. Jacob.; J. Capiaumont.; A. Martial.; A. Marc. Lactate dehydrogenase (LDH) activity of the number of dead cells in the medium of cultured eukaryotic cells as marker. *Journal of biotechnology.* **1992**, 25, 231-243.
26. C. Riccardi.; I. Nicoletti. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nature protocols.* **2006**, 1, 1458-1461.
27. J. Pourahmad.; M. Amirmostofian.; F. Kobarfard.; J. Shahraki. Biological reactive intermediates that mediate dacarbazine cytotoxicity. *Cancer chemotherapy and pharmacology*. **2009**, 65, 89-96.