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Synthesis, cytotoxicity of Carnosine peptide analogues on mitochondria obtained from cancerous rats' liver

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ABSTRACT

In this study, HCC *(Hepatocellular carcinoma)* was induced by diethylnitrosamine (DEN), as an initiator, and 2-acetylaminofluorene (2-AAF), as a promoter. Mitochondria from cancerous rats liver for evaluation of the cytotoxic effect of dipeptide Carnosineanalogues was isolated and cellular parameters related to apoptosis signaling were then determined. Our results showed that high toxicity synthesized linear and cyclic Carnosine analogues with concentration 10μg/mL in MTT assay, a raise in mitochondrial reactive oxygen species (ROS) level, swelling in mitochondria, ATP generation, mitochondrial membrane potential (Δψm) collapse, release of cytochrome c and caspase-3 activation after exposure of mitochondria isolated from the *Hepatocellular carcinoma (*HCC group). Based on the overall results, cyclic Carnosine analogues rather than linear Carnosine analogues would be encouraging to develop new anticancer agents and may be considered as a promising complementary therapeutic agents for the treatment of HCC. The synthesized Carnosine analogues were characterized by using different methods such as, LC-MS, FT-IR.

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Introduction

Cancer is a complex disease that is characterized by cell proliferation, cell transformation and disruption apoptotic process.[1]Cancer is a group of diseases marked by uncontrolled growth and spread of abnormal cells. If the tumor spread is not controlled, it can result in mortality. Cancer is caused by both external different factors (tobacco, chemicals, radiation, and infectious organisms) and internal different factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism).[2]Cancer can start in the breast, lungs, colon, or even in the blood cells. Cancers are similar in some ways, but they are different in growth ways and sprea.[3] Normal cells divide in a regular route. They die when they are damaged, and new cells take their place. Cancer is formed when the cells begin to grow out of control. The cancer cells keep on growing and new ones will replace the damaged cells.[4]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 improve cancer, chemotherapy based on peptides has attracted a great interest and considerable attention due to the unique advantages of peptides, such as a low molecular weight, the ability to specifically target tumor cells, and low toxicity in normal tissues. During the past decade, peptides have gained a wide range of applications in medicine, drug delivery and biotechnology.[5]Liver cancer represents one of the most common malignancy global. Hepatocellular carcinoma represents a major form of primary liver cancer in adults. The most important risk factors are hepatitis B and C infections. The ideal anticancer candidates would have a tendency to kill cancer cells without affecting normal cells. Despite these efforts, anticancer drugs also have a side effects on normal cells.[6] Possible functions of the dipeptide of Carnosine include buffer, anti-oxidant, antiglycator, aldehyde and carbonyl scavenger, chelator of metal ion, immuno-stimulant, wound healing agent and neurotransmitter. In 1986, it was reported that Carnosine can inhibit growth of tumor cells.[7] In 2008, Carnosine was shown to inhibit growth of cultured glioblastoma cells,[8] most probably via effects on glycolysis.[9,10] Other results indicated that Carnosine can suppress tumor growth in animals.[11,12] The number of biochemical markers have been identified in the induction of apoptotic cell death, including an increase of reactive oxygen species (ROS), collapse of mitochondrial membrane potential (MMP). In the intrinsic apoptotic cell death pathway, ROS are

potent inducers of oxidative damage and have been suggested as main regulators of apoptotic cell death. Remarkably, intracellular ROS increase prior to cytochrome c release from mitochondria during the activation of apoptotic process.[13] Cytochrome c release is an endpoint of destruction to mitochondria and a starting point of cell death signaling resulting in either apoptosis or necrosis in the exposed tissue depending on cellular ATP levels.[14,15] In this study, cyclic Carnosine analogues than linear Carnosine analogues leads to increased ROS formation, reduction of collapse of MMP, reduction of ATP generation and mitochondria swelling that finally produce cytochrome c release. The functions and the cytotoxicity mechanisms of linear and cyclic Carnosine analogues on hepatocytes and mitochondria isolated from the HCC rat model by the DEN and 2-AAF were not completely reported till now. This study focused on the apoptotic effects of Carnosine analogueson hepatocytes and mitochondria obtained from the liver of HCC rats and the detailed mechanism. It suggests that therapeutic methods to inhibit anti-apoptotic signals in HCC cells might have the potential to provide powerful tools in the future to treat in the patients with HCC.[16,17]

2. Materials and methods

All other commercially obtained reagents and solvents were used without further purification. Trifluoroacetic acid (TFA), Triisopropylsilane (TIS), Fmoc amino acids and coupling reagents O-(7-Azabenzotriazol-1-yl)-*N,N,N,N*-tetramethyluroniumhexafluorophosphate (HATU), (benzotriazol-1-yloxy)tripyrrolidinophosphonium-hexafluorophosphate(PyBop), were supplied by Merk. Solvents like acetonitrile (CH_3CN), Piperazine, *N,N*-diisopropylethylamine (DIPEA), Diethylether, Dichloromethane (DCM), *N,N*-dimethylformamide (DMF), and methanol (MeOH) were purchased from Merk. 2-chlorotritylchloride resin (1% DVB, 200-400 mesh, 1mmol/g) was purchased from Aldrich. Commercially available chemicals were used as received unless otherwise stated. Flash column chromatography were carried out using silica Gel 60 (particle size0.04–0.06 mm / 230–400 mesh). The mass spectral measurements were performed on a 6410 Agilent LCMS triple quadrupole mass spectrometer (LCMS) with an electrospray ionization (ESI) interface.

3. Experimental

3.1. General procedure for the synthesis of protected linear Carnosine analogues (*N-Trt*)

Synthesis was carried out using 2-chlorotritylchloride resin (1mmol/g) following the standard Fmoc strategy. Fmoc-His(Trt)-OH (680mg, 2mmol) was attached to the 2-CTC resin with DIPEA (1mL) in anhydrous DCM:DMF (30 mL, 1:1) at room temperature for 2h. After filtration, the remaining tritylchloride groups were capped by a solution of DCM / MeOH / DIPEA (2:2:1.5) for 30 min. Then, it was filtered and washed thoroughly with DCM (1 × 10 mL), 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). Then, a solution of Fmoc-β-alanine-OH (780mg, 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 coupling, resin was washed with DMF (2 × 10 mL). The resin-peptide was treated with Piperazine 10% in DMF (100 mL) for 30 min and the resin was washed with DMF (4 × 20 mL). The produced peptide of Carnosine was cleaved from resin by treatment of TFA 1% in DCM and neutralization with pyridine 4% in MeOH. The solvent was removed under reduced pressure and precipitated in water. Other analogues of Carnosine peptide were synthesized in the same way.

3.2. General procedure for the synthesis of deprotected linear Carnosine analogues

A mixture of trifluoroacetic acid / dichloromethane / triisopropylsilane (TFA / DCM / TIPS) (10:10:1) were added to the protected linear Carnosine analogues and stirred for one hour. Under such strong acidic condition, His(Trt) side chains deprotection (*N-Trt*) were carried out in one step. Then this mixture was filtered and the excess TFA / DCM was removed under reduced pressure. The desired peptide was precipitated in cold diethylether and deprotected linear Carnosine analogues were obtained.

3.3. General procedure for the synthesis of cyclic Carnosine analogues

The precipitates of protectedlinear Carnosine analogueswere 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 (1:4, chloroform:methanol). Final deprotection on cyclic (*N-Trt*) Carnosine analogues were done by treatment of TFA 95%, phenol, anisole, distilled water and triisopropylsilane 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.

3.4. Animals

The rats (Male Sprague Dawley rats) fed a standard chow diet and given water ad libitum were used in all experiments. The animals for this work were purchased from Institute Pasteur (Tehran, Iran). All animals were maintained in a controlled room temperature of 20–25°C and a humidity of 50–60% and were exposed to 12-hr of daylight. The protocols approved for the study were conducted according to the ethical standards and the Committee of Animal Experimentation of ShahidBeheshti University of Medical Sciences, Tehran, Iran. All efforts were made to minimize the number of animals used and their suffering.

3.5. Experimental design

The rats were divided into two groups (5 rats in each): group A, normal rats fed with standard diet and group B (HCC group), rats were injected intraperitoneally (i.p.) with a single dose of DEN 200 mg/kg body-weight dissolved in corn oil. Two weeks after DEN administration, cancer development was promoted

with dietary 2-AAF (0.02%, w/w) for 2 weeks.[18] At the end of the HCC induction period (week 15), the body-weight of each rat was recorded and then cardiac puncture blood samples were collected (5 rats) in chilled non heparinized tubes and left at room temperature for 25 min. and then centrifuged at 1000 × g for 10 min. at 4°C and the serum kept at -80°C until biochemical analysis. According to published studies, HCC was confirmed through the histopathological evaluations, determinations of blood amounts of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and finally alfa-fetoprotein (AFP) as a specific HCC marker were assayed in the mentioned serum.[19,20]

3.6. Biochemical assessments

Alkaline phosphatase (ALP), Serum alanine transaminase (ALT) and aspartate transaminase (AST) determinations were carried out spectrophotometrically using the Hitachi-912 Chemistry Analyzer and standard diagnostic kits (18).

3.7. Serum alpha-fetoprotein (AFP)

AFP concentrations in serum were estimated using the ADVIA Centaur AFP bioassay. This assay is a two-site sandwich immunoassay using direct chemiluminometric technique (18).

3.8. Isolation of Mitochondria from Rat Hepatocytes

At week 15, post-HCC induction, the rats were anaesthetized with ketamine (80 mg/kg, ip) and xylazine (5 mg/kg, ip).[22] Differential centrifugation (5 min at 760 × g for the first stage and 20 min at 8000 × g for the second stage) was used for isolation of mitochondria from hepatocytes.[23] In this study, for the determination of mitochondrial ROS generation, mitochondrial swelling and MMP collapse, the mitochondria were suspended in corresponding buffers, respectively. All tests were carried out three times. The concentration used for peptides (10 µg/mL) were selected based on MTT assay.

3.9. Determination of Cytotoxicity

Hepatocytes from normal and HCC cells (1×10^7/well) were plated in 96-well plates and treated with 10 µg/mL concentration of Carnosine analogues for 6h (Cells were maintained in RPMI 1640, supplemented with 10% FBS) and antibiotics (50 U/mL of penicillin and 50µg/mL streptomycin). After treatment, MTT (5 mg/mL in RPMI 1640) reagent was added to each well. After 4 h, the reaction was stopped by addition of 50 µL of DMSO. The absorbance at 570 nm of the solubilized MTT products was measured with an ELISA reader. The process was repeated in triplicate to confirm accuracy.

3.10. ROS formation assay

The fluorescent dye DCFH was used for the mitochondrial ROS measurement. Mitochondria isolated from both normal and HCC hepatocytes were suspended in respiration buffer (0.32 mM of sucrose, 10 mM of Tris, 20 mM of Mops, 50 µM of EGTA, 0.5 Mm of $MgCl_2$, 0.1 mM of KH_2PO_4, and 5 mM of sodium succinate). Then, DCFH (final concentration, 10 µM) was added to mitochondria of both groups and incubated at 37°C for 15 min. The fluorescence intensity of DCF which is an indicator of ROS concentration was then assayed by a Shimadzu RF-5000U fluorescence spectrophotometer at EXλ = 488 nm and EMλ = 527 nm.[19,21]

3.11. Mitochondria membrane potential (MMP) assay

Mitochondrial accumulation and also redistribution of the cationic fluorescent dye, rhodamine 123 (Rh 123), from mitochondria into the cytosol has been used for the determination of the collapse of MMP. Rh 123 (10 µM) was added to the mitochondrial suspensions (1000 µg mitochondrial protein/mL) in MMP assay buffer (220 mM sucrose, 68 Mm D-mannitol, 10 mMKCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 50 µM EGTA, 5 mM sodium succinate, 10 mM HEPES, 2 µM rotenone). The cytosolic Rh 123 fluorescence intensity which represents the redistribution of the dye from mitochondria into the cytoplasm was determined using Shimadzu RF-5000U fluorescence spectrophotometer at the EXλ = 490 nm and EMλ = 535 nm.[21]

3.12. Mitochondrial swelling assay

For evaluation mitochondrial swelling, mitochondria from HCC and normal groups were suspended in corresponding assay buffer (70 mM of sucrose, 230 mM of mannitol, 3 mM of HEPES, 2 mM of Tris-phosphate, 5 mM of succinate, and 1 µM of rotenone) and incubated at 37°C with 10µg/mL of peptides. The absorbance was measured at 540 nm at 15-min. Intervals using an ELISA reader (Tecan, Rainbow Thermo). A decrease in the absorbance indicates an increase in mitochondrial swelling.[19]

3.13. Determination of ATP Production

The ATP assay was performed according to the manufacturer's instruction. BCA Protein Assay Kit and ATP Assay Kit were bought from Beyotime Institute of Biotechnology (Nanjing, China). Harvested cultured cells were lysed with a lysis buffer, followed by centrifugation at 10.000×g for 2 min, at 4°C. Finally, in 6-well plates, the level of ATP was assayed by mixing 20 mL of the supernatant with 95 mL of luciferase reagent, which catalyzed the light production from ATP and luciferin. Luminance was measured by a monochromator microplate reader. Standard curve was generated and the protein concentration of each group was determined using the BCA protein assay kit. Total ATP levels were expressed as nmol/mg protein.[24]

3.14. Cytochrome c release

The release of cytochrome c by peptides was assayed by the Quantikine Rat/Mouse Cytochrome c Immunoassay kit provided by R & D Systems, Inc. (Minneapolis, MN, USA).

3.15. Caspase-3 activity assay

The effect of Carnosine peptide analogues on the activation of caspase-3 on the mitochondria from HCC and normal groups were assayed using Sigma's caspase-3 colorimetric assay kit (Sigma-Aldrich, Taufkirchen, Germany). Finally, the concentration of the p-nitroaniline released from the substrate at 405 nm used for evaluate caspase- 3 activity.

3.16. Statistical analysis

Results are presented as mean ± SD. All statistical analyses were performed using Graph Pad Prism software, version 5. Statistical significance was assayed using the one-way ANOVA test, followed by the post hoc Tukey test. The one-way ANOVA test was used as a specific statistical analysis for the determination of effect of DEN/2-AAF on body weight and liver weight (the average body weight and average liver weight considered as single variable respectively between normal and HCC groups), for determinations of cytotoxicity (the concentration was the single variable) and the cytochrome c release (the concentration was the single variable). The two-way ANOVA test, followed by the post hoc Bonferroni test, was used for determinations of mitochondrial ROS level, MMP and mitochondrial swelling (time and concentration considered as two variables). Statistical significance was set at $P<0.05$.

4. Results

4.1. The protected and deprotected Carnosine analogues

S*ynthesis ofβ-alanine-His-OH (1)*

a) Synthesis of protected peptide

Yield: 80%; White oily liquid; IR (KBr): ν (cm^{-1}) 3438.64 (NH), 2165.88 and 2556.05 (C=N in amino acid Histidine), 1664.42 (C=O amide), 1546.45 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (1a) 468.22, Found *m/z* = 469.5$[M+H]^+$.

b) *Synthesis of deprotected peptide*

Yield: 80%; Yellow solid; IR (KBr): ν (cm^{-1}) 3438.64 (NH), 2164.07 and 2561.28 (C=N in amino acid Histidine), 1666.62 (C=O amide), 1547.02 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (1b) 226.11, Found *m/z* = 227.2$[M+H]^+$.

S*ynthesisofβ-alanine-His-β-alanine-His-OH (2)*

a) Synthesis of protected peptide

Yield: 78%; White oily liquid; IR (KBr): ν (cm^{-1}) 3434.17 (NH), 2166.47 and 2555.28 (C=N in amino acid Histidine), 1664.92 (C=O amide), 1546.34 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (2a) 918.42, Found *m/z* = 919.6 $[M+H]^+$.

b) *Synthesis of deprotected peptide*

Yield: 78%; Yellow solid; IR (KBr): ν (cm^{-1}) 3440.64 (NH), 2163.68 and 2565.33 (C=N in amino acid Histidine), 1666.61 (C=O amide), 1546.72 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (2b) 434.2, Found *m/z* = 435.5$[M+H]^+$.

S*ynthesisofβ-alanine-His-Pro-β-alanine-His-OH (3)*

a) Synthesis of protected peptide

Yield: 75%; White oily liquid; IR (KBr): IR (KBr): ν (cm^{-1}) 3428.45 (NH), 2167.10 and 2559.85 (C=N in amino acid Histidine), 1666.66 (C=O amide), 1546.62 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (3a) 1015.48, Found *m/z* = 1016.6$[M+H]^+$.

b) *Synthesis of deprotected peptide*

Yield: 75%; Yellow solid; IR (KBr): ν (cm^{-1}) 3439.64 (NH), 2164.39 and 2556.76 (C=N in amino acid Histidine), 1665.21 (C=O amide), 1546.14 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (3b) 531.26, Found *m/z* = 532.3$[M+H]^+$.

SynthesisofPro-β-alanine-His-β-alanine-His-OH (4)

a) Synthesis of protected peptide

Yield: 75%; White oily liquid; IR (KBr): ν (cm^{-1}) 3451.30 (NH), 2164.07 and 2551.33 (C=N in amino acid Histidine), 1664.07 (C=O amide), 1545.98 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (4a) 1015.48, Found *m/z* = 1016.6$[M+H]^+$.

b) *Synthesis of deprotected peptide*

Yield: 75`%; Yellow solid; IR (KBr): ν (cm^{-1}) 3438.64 (NH), 2165.77 and 2569.91 (C=N in amino acid Histidine), 1666.21 (C=O amide), 1546.57 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (4b) 531.26, Found *m/z* = 532.3$[M+H]^+$.

SynthesisofHis-β-alanine-β-alanine-His-OH (5)

a) Synthesis of protected peptide

Yield: 75%; White oily liquid; IR (KBr): ν (cm^{-1}) 3477.52 (NH), 2161.95 and 2576.03 (C=N in amino acid Histidine), 1667.99 (C=O amide), 1546.81 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine);

LC-MS (ESI) *m/z*Calcd for (5a) 918.42, Found *m/z* = 919.6$[M+H]^+$.

b) *Synthesis of deprotected peptide*

Yield: 75`%; Yellow solid; IR (KBr): ν (cm^{-1}) 3438.64 (NH), 2165.21 and 2558.13 (C=N in amino acid Histidine), 1663.92 (C=O amide), 1545.83 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (5b) 434.2, Found *m/z* = 435.5$[M+H]^+$.

SynthesisofHis-β-alanine-Pro-β-alanine-His-OH (6)

a) Synthesis of protected peptide

Yield: 75%; White oily liquid; IR (KBr): ν (cm^{-1}) 3437.16 (NH), 2165.15 and 2559.93 (C=N in amino acid Histidine), 1665.85 (C=O amide), 1547.00 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (6a) 1015.48, Found *m/z* = 1016.6$[M+H]^+$.

b) *Synthesis of deprotected peptide*

Yield: 75`%; Yellow solid; IR (KBr): ν (cm^{-1}) 3441.96 (NH), 2165.88 and 2569.91 (C=N in amino acid Histidine), 1679.60 (C=O amide), 1548.23 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (6b) 531.26, Found *m/z* = 532.3$[M+H]^+$.

SynthesisofPro-His-β-alanine-β-alanine-His-OH (7)

a) Synthesis of protected peptide

Yield: 75%; White oily liquid; IR (KBr): ν (cm^{-1}) 3429.71 (NH), 2166.65 and 2557.15 (C=N in amino acid Histidine), 1665.90 (C=O amide), 1546.67 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (7a) 1015.48, Found *m/z* = 1016.6$[M+H]^+$.

b) *Synthesis of deprotected peptide*

Yield: 75`%; Yellow solid; IR (KBr): ν (cm^{-1}) 3438.64 (NH), 2162.11 and 2587.56 (C=N in amino acid Histidine), 1670.61 (C=O amide), 1547.35 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (7b) 531.26, Found *m/z* = 532.3$[M+H]^+$.

SynthesisofPro-β-alanine-His-His-β-alanine-OH (8)

a) Synthesis of protected peptide

Yield: 75%; White oily liquid; IR (KBr): ν (cm^{-1}) 3458.84 (NH), 2161.39 and 2629.39 (C=N in amino acid Histidine), 1677.66 (C=O amide), 1548.05 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (8a) 1015.48, Found *m/z* = 1016.6$[M+H]^+$.

b) *Synthesis of deprotected peptide*

Yield: 75`%; Yellow solid; IR (KBr): ν (cm^{-1}) 3440.13 (NH), 2163.05 and 2573.18 (C=N in amino acid Histidine), 1669.65 (C=O amide), 1546.74 (C=C in amino acid Histidine), 600-800 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (8b) 531.26, Found *m/z* = 532.3$[M+H]^+$.

4.2. The deprotected cyclic Carnosine analogues

Synthesisof [Cyclo-(β-alanine-His-Pro-β-alanine-His-OH)] (1c)

Yield: 75%;Yellow solid; IR (KBr): IR (KBr): ν (cm^{-1}) 3438.45 (NH), 1673.24 (C=O amide corresponding to cyclization), 1600.18 (C=O amide), 1537.69 (C=C in amino acid Histidine), 558.35-836.76 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (1c) 513.26, Found *m/z* = 514.3$[M+H]^+$.

Synthesisof [Cyclo-(Pro-β-alanine-His-β-alanine-His-OH)] (2c)

Yield: 75%; Yellow solid; IR (KBr): ν (cm^{-1}) 3415.11 (NH), 1742.54 (C=O amide corresponding to cyclization), 1673.93 (C=O amide), 1544.00 (C=C in amino acid Histidine), 558.03-836.30 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (2c) 513.26, Found *m/z* = 514.3$[M+H]^+$.

Synthesisof [Cyclo-(His-β-alanine-Pro-β-alanine-His-OH)] (3c)

Yield: 75%; Yellow solid; IR (KBr): ν (cm^{-1}) 3447.80 (NH), 1743.20 (C=O amide corresponding to cyclization), 1673.09 (C=O amide), 1544.00 (C=C in amino acid Histidine), 557.98-835.26 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (3c) 513.26, Found *m/z* = 514.3$[M+H]^+$.

Synthesisof [Cyclo-(Pro-His-β-alanine-β-alanine-His-OH)] (4c)

Yield: 75%; Yellow solid; IR (KBr): ν (cm^{-1}) 3440.56 (NH), 1743.35 (C=O amide corresponding to cyclization), 1674.14 (C=O amide), 1544.00 (C=C in amino acid Histidine), 558.06-835.26 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (4c) 513.26, Found *m/z* = 514.3$[M+H]^+$.

Synthesisof [Cyclo-(β-alanine-His-Pro-His-β-alanine-OH)] (5c)

Yield: 75%; Yellow solid; IR (KBr): ν (cm^{-1}) 3413.90 (NH), 1747.05 (C=O amide corresponding cyclization), 1673.38 (C=O amide), 1544.00 (C=C in amino acid Histidine), 558.41-841.62 (out of plane bending vibration C-H in amino acid Histidine); -

LC-MS (ESI) *m/z*Calcd for (5c) 513.26, Found *m/z* = 514.3-$[M+H]^+$.

Synthesisof [Cyclo-(Pro-β-alanine-His-His-β-alanine-OH)] (6c)

Yield: 75%; Yellow solid; IR (KBr): ν (cm^{-1}) 3443.41 (NH), 1742.42 (C=O amide corresponding to cyclization), 1671.86 (C=O amide), 1629.02 (C=C in amino acid Histidine), 558.25-837.62 (out of plane bending vibration C-H in amino acid Histidine); LC-MS (ESI) *m/z*Calcd for (6c) 513.26, Found *m/z* = 514.3$[M+H]^+$.

4.3. Effect of DEN/2-AAF on body weight and liver weight

Table 1 shows the body (final) and liver weights of control (1) and HCC group (2) of rats. According to this table, the average body weight of the normal group (1) of rats (284.41± 10.11g) was higher than that of the HCC group (2) rats (230.23 ± 5.63 g), significantly ($P < 0.001$). Moreover, in the HCC group, the average liver weight of rats (12.20 ± 0.76 g) after 15 weeks was significantly ($P < 0.05$) higher than that of the normal group rats (9.11 ± 0.88 g).

Table 1. Effect of DEN/2-AAF regimen on final body and liver weights (g). *: $P < 0.05$, ***: $P < 0.001$ compared with the untreated normal group.

Group	Final body weight(g)	Liver weight
Untreated control group (1)	284.41±10.11	9.11±0.88
HCC group (2)	230.23±5.63***	12.20±0.76*

4.4. Effect of DEN/2-AAF on AFP, AST, ALT and ALP

The results suggested that the levels of AST, ALT, ALP and AFP concentrations in the serum of cancerous group was considerably higher than of the normal group (Table 2).

Table2. Effects of DEN/AAF regimen on the ALP, ALT, AST and AFP in serum. Three rats were placed in each group**.** All results were reported as mean ± SD. *: $P < 0.05$, ***: $P < 0.001$ compared with the untreated normal group.

Group	ALT(IU/L)	AST(IU/L)	ALP(IU/L)	AFP(IU/L)
Untreated control group (1)	85±12	91±9	598±14	0.18±0.02
HCC group (2)	730±63***	608±39***	794±22***	3.03±0.23*

4.5. Effect of Carnosine analogues in cytotoxicity

As shown in Figure 1; the HCC hepatocytes viability were decreased by Carnosine analogues after 6 hour treatment. For the measurement of cell viability, we assessed cytotoxicity using theMTT test after 6h incubation of cells with Carnosine analogues (1.25, 2.5, 5, 10, 20, 50 and 100 µg/mL). Our results with MTT assay showed that even at the 1.25, 2.5, 5 µg/mL concentrations have not toxic effects toward HCC group and these concentrations no significant difference with the control group. In addition to, 20, 50, 100 µg/mL concentrations have toxic effects toward HCCand at these concentrations all cells were killed. Then we chosed effective concentration on HCC.-The IC_{50} value obtained for the mean of the three independent experiments for Carnosine analogues was 10 µg/mL. Also, significant at viability was observed in the compared with the corresponding HCC group.

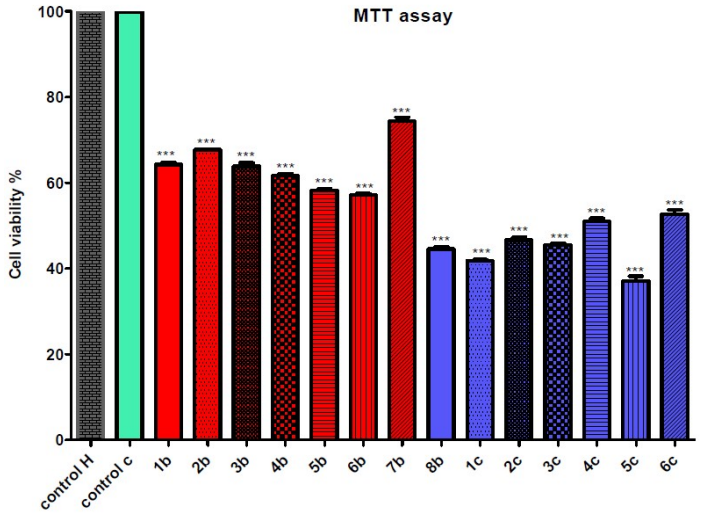

Figure 1. Cytotoxicity assay. Cytotoxic effects of linear and cyclic Carnosine analogues on hepatocytes from normal group and comparative effects of linear and cyclic Carnosine analogues at 10 µg/mL on the cytotoxic of HCC hepatocytes. The cells was treated with peptides for 6 h, and cytotoxic effects were determined by MTT assay. Data represent the mean ± SD of three replicates in three independent experiments (n=3). The stars show that values were significantly different from the corresponding control (***$p < 0.001$).

4.6. Effect of Carnosine analogues on ROS

Our data indicated that the ROS generation in the mitochondria isolated from the HCC rat group after 6h of incubation with concentration 10 µg/mL of linear and cyclic peptides was significantly ($P < 0.05$) raised compared to the corresponding control (Figs 2 and 3). On the other hand, ROS generation of linear Carnosine analogues 1b, 4b, 5b, 7b and 8b at 30 min and 7b at 60 min no have significant difference in comparison with HCC group and linear Carnosine analogues 2b, 3b and 6b at 30 min and 1b, 2b, 3b, 4b, 5b, 6b and 8b at 60 min have significant difference in comparison with HCC group. In addition to significant difference in linear Carnosine analogues, all cyclic Carnosine analogues have significant difference in comparison with HCC group with the exception of 7b.

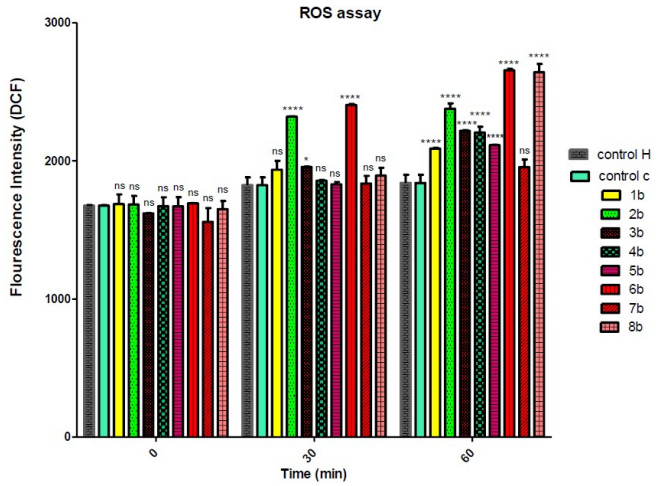

Figure 2. ROS formation assay. The effect of linear Carnosine analogues at 10 µg/mL on ROS generation at different times (0, 30, and 60min) in the liver mitochondria obtained from hepatocytes of the normal and HCC groups. Data represent the mean ± SD of three replicates in three independent experiments (n=3). The two-way ANOVA test was performed. * and **** significantly different from the corresponding control ($p<0.05$ and $p<0.0001$, respectively).

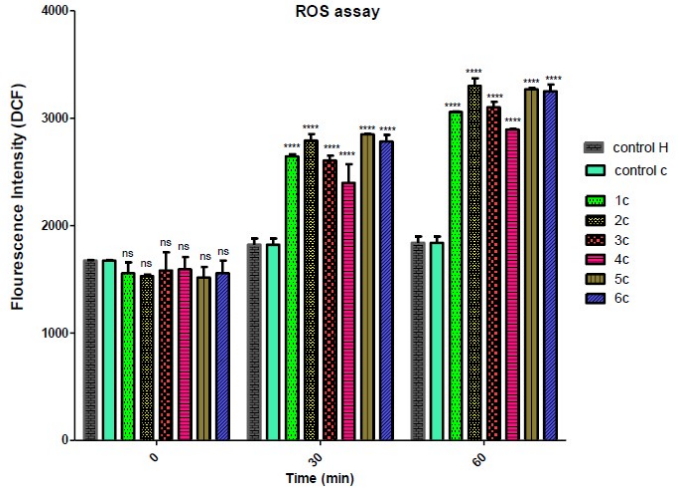

Figure 3. ROS formation assay. The effect of cyclic Carnosine analogues at 10 µg/mL on ROS generation at different times (0, 30, and 60min) in the liver mitochondria obtained from hepatocytes of the normal and HCC groups. Data represent the mean ± SD of three replicates in three independent experiments (n=3). The two-way ANOVA test was performed. **** Significantly different from the corresponding control ($p<0.0001$).

4.7. Effects of Carnosine analogues on MMP

Results in Figure 4 showed that all Carnosine analogues hadsignificant difference in comparison with HCC group ($p<0.01$ and $p<0.0001$).

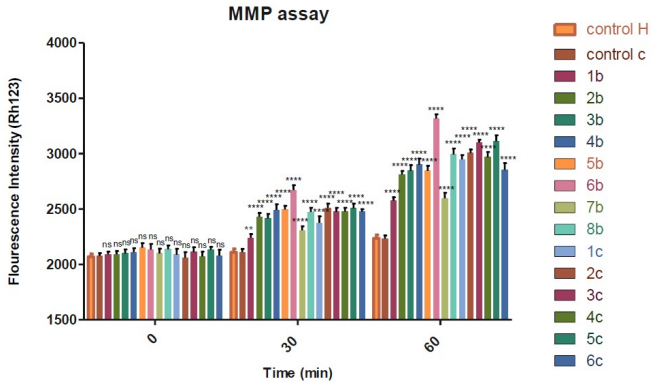

Figure 4. Mitochondrial membrane potential (MMP) assay. The effect of 10 µg/mL of linear and cyclic Carnosine analogues on collapse MMP at different times (0, 30 and 60 min) in the liver mitochondria obtained from hepatocytes of the normal and HCC groups. Data represent the mean ± SD of three replicates in three independent experiments (n=3).The two-way ANOVA test was performed. ** And **** significantly different from the corresponding control ($p<0.01$ and $p<0.0001$, respectively).

4.8. Effect of Carnosine analogues on mitochondrial swelling

Addition of linear and cyclic Carnosine analogues to mitochondrial suspensions obtained from the HCC group led to significant ($P<0.05$) mitochondrial swelling (Figure 5) within 30 and 60 min of incubation. Compounds 2b and 4c, compounds 6b, 1c, 2c, 3c, 5c and 6c at 30 min have significant difference in comparison with HCC group ($p<0.01$ and $p<0.0001$, respectively). At 60 min, compounds 1b, 2b, 3b, 4b, 5b and 8b, compounds 6b, 1c, 2c, 3c, 5c and 6c, compound 4c have significant difference in comparison with HCC group ($p<0.01$, $p<0.0001$ and $p<0.001$, respectively). All peptides show with the exception of 7b in Figure 5 (significantly decrease), significantly increase mitochondrial swelling at 60 min.

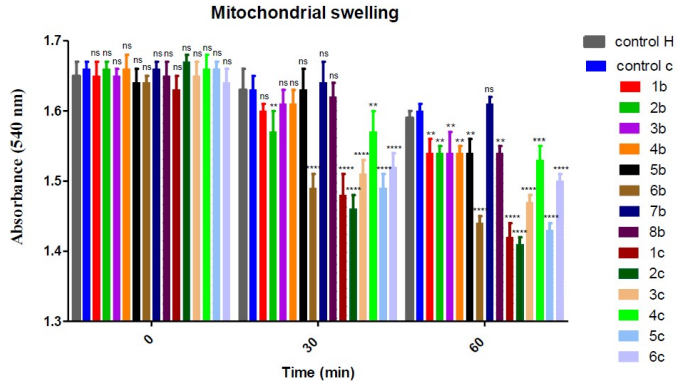

Figure 5. Mitochondrial swelling assay. The effect of 10 µg/mL of linear and cyclic Carnosine analogues on mitochondria swelling at different times (0, 30 and 60 min) in the liver mitochondria obtained from hepatocytes of the normal and HCCgroups. Data represent the mean ± SD of three replicates in three independent experiments (n=3). The two-way ANOVA test was performed **, *** and **** show a significant difference in

comparison with the corresponding control (P<0.01, P<0.001 and P<0.0001, respectively).

4.9. Effect of Carnosine analogues on ATP

Results in Figure 6 showed that,linear and cyclic Carnosine analogues to mitochondrial suspensions obtained from the HCC group led to ATP generation. Compounds 3b, 5b, 6b and 7b no have significant difference in comparison with HCC group and compound 1b and compounds 2b, 4b, 8b, 1c, 2c, 3c, 4c, 5c and 6c have significant difference in comparison with HCC group (p<0.05 and p<0.001, respectively).

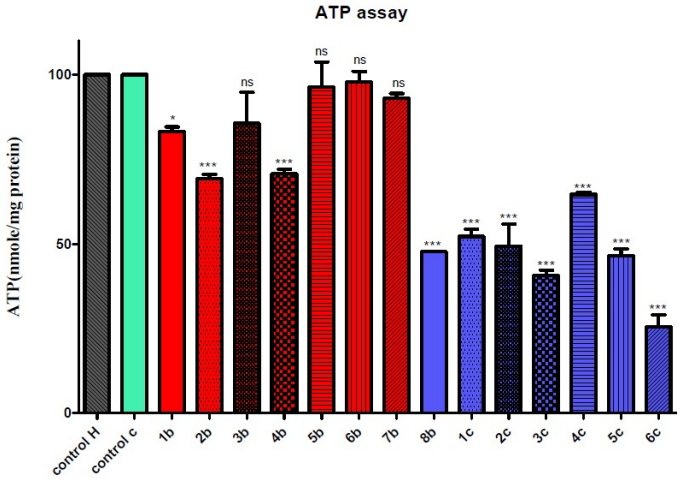

Figure 6. ATP assay. The effect of linear and cyclic Carnosine analogues at 10 µg/mL on ATP generation in the liver mitochondria obtained from hepatocytes of the normal and HCC groups. Data represent the mean ± SD of three replicates in three independent experiments (n=3).The one-way ANOVA test was performed. * And *** significantly different from the corresponding control (p<0.05 and p<0.001, respectively).

4.10. Effect of Carnosine analogues on cytochrome c release

As shown in Figure 7, Carnosine analogues induced a significant (*P* <0.05) release of cytochrome c in comparison with mitochondria isolated from the HCC group. Also, the results showed that compound 1b no have significant difference in comparison with HCC group and all another peptides with the exception of 3b (*P* <0.05)have significant difference in comparison with HCC group (*P* < 0.001).

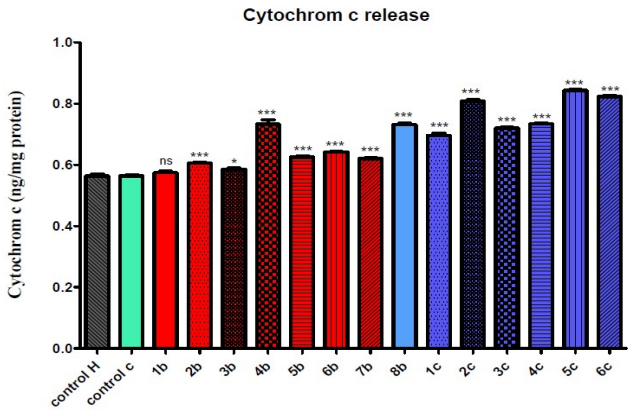

Figure 7.Cytochrome c release assay. The amount of expelled cytochrome c from the mitochondrial fraction into the suspension buffer was determined using a rat/mouse cytochrome c ELISA kit. Data represent the mean ± SD of three replicates in three independent experiments (n=3).The one-way ANOVA test was performed. * And *** show significant difference in comparison with the corresponding control (p<0.05 and p<0.001, respectively).

4.11. Effect of Carnosine analogues on caspase-3 activation

Our results showed that peptides at concentration of 10 µg/mL induced a significant (P<0.001) increase at activity of caspase-3 only in the hepatocytes isolated from the HCC but not normal group (see **Figure** 8).

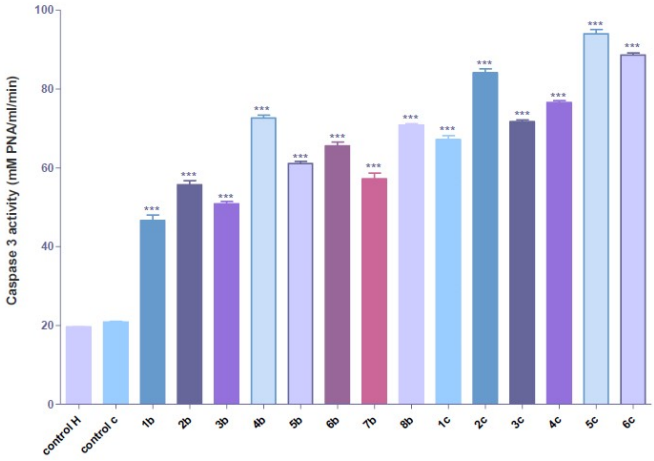

Figure 8. Evaluation of caspase 3 activity. The caspase-3 activity was measured by using Sigma-Aldrich kit. Caspase-3 activation in the both HCC and untreated control rat hepatocytes following the exposure to peptides (10 µg/mL). Data represent the mean ± SD of three replicates in three independent experiments (n=3). The one-way ANOVA test was performed *** Significant difference in comparison with corresponding control HCC group (p < 0.001).

5. Discussion

At the moment in worldwide, cancer is one of the causes leading to death and therefore there is a very urgent need for

discovering a new therapy. Current techniques for cancer treatment included chemotherapy, surgery and radiation therapy.[25] Currently, many therapeutic techniques used for the treatment of liver cancer, but these therapeutic techniques have not been successful (26). Therefore, the designation of choice therapy with high potency and efficacy has led to the raised use of anticancer agent developed from natural resources.[25] The major aim of this research was to evaluate the apoptotic effect of Carnosine analogues via ROS mediated mitochondrial targeting on hepatocytes and mitochondria from HCC rat model. Recently, many studies of functional and structural differences between normal and cancerous cells (For instance, there are various changes in the size, shape, and number of the mitochondria in liver tumor cells in comparison with their corresponding normal cells) have been used to design new anticancer drugs.[18,27] Our results in MTT assay indicated that Carnosine analogues at applied 10 µg/mL concentration caused a significant increase in the cell viability only in the mitochondria isolated from the HCC rats. Cell viability percentage of synthesized cyclic peptides was low in the compared with synthesized linear peptides and this means that cyclic peptides were toxic than linear peptides in comparison with HCC group. In this study to justify that the Carnosine analogues induce apoptosis signaling via increasing ROS generation, the level of ROS was evaluated. The results of the study show that all synthesized linear and cyclic peptides significantly increased level of ROS in comparison with mitochondria isolated from the HCC group. A significantly increased level of ROS in linear and cyclic Carnosine analogues (compounds 6b and 2c, respectively) than other linear and cyclic peptides, in comparison with mitochondria isolated from the HCC group (Figs 2 and 3). It has been documented which ROS act a vital role in the regulation of intracellular signaling pathways at physiological low levels (as "redox messengers"), but at higher levels cause the oxidation of cellular macromolecules and promote apoptotic cell death through the mitochondrial oxidative stress (OS) pathways.[28] It was shown that the during apoptosis the MMP is disrupted via the formation of permeability transition pores due to the effect of various agents, which produce intracellular signals that induce the collapse of MMP. Rh 123 staining showed that the structure of the mitochondria had changed, indicating that disruption of the mitochondrial membrane is actually main mechanism in the induction of apoptosis by peptides. In addition to, our results showed that all linear and cyclic peptides could induce the collapse of MMP in comparison with mitochondria isolated from the HCC group. Compound 6b significantly increased the collapse of MMP in Carnosine analogues in comparison with mitochondria isolated from the HCC group (see **Figure** 4). The induction of MPT (Mitochondrial permeability Transition), also leads to mitochondrial swelling and release of pro-apoptotic proteins such as cytochrome c from mitochondria into cytosol. Compound 2c significantly increased mitochondrial swelling in comparison with mitochondria isolated from the HCC group. We also assayed level of swelling in mitochondria as an indicator of MPT pore opening in this study. All cyclic peptides than linear peptides significantly increased mitochondrial swelling in comparison with mitochondria isolated from the HCC group whereas linear peptide 7b significantly decreased mitochondrial swelling in comparison with mitochondria isolated from the HCC group (see **Figure** 5). All linear and cyclic peptides decreased ATP generation in comparison with mitochondria isolated from the HCC group. On the other hand, cyclic peptides significantly decreased than linear peptides in comparison with mitochondria isolated from the HCC group (see **Figure** 6). The release of cytochrome c from mitochondria to media buffer as subsequent events after mitochondrial swelling and collapse of MMP was also determined. The most important result was that all linear and cyclic peptides significantly increased release of cytochrome c in comparison with mitochondria isolated from the HCC group (see **Figure** 7). Compound 5c than other peptides significantly increased release of cytochrome c in comparison with HCC mitochondria.

The caspase-3 plays a terminable role of the apoptosis signaling as an important executioner. When activated, it cleave a series of substrates, orchestrating apoptosis (29). To investigate the contention of caspases cascade in apoptosis pathway, we detected the activity of caspase-3 in analogues of Carnosine-treated hepatocytes. Our results showed that peptides could activate caspase-3. Cyclic peptides in the compared with linear peptidescaused considerable caspase-3 activation only in hepatocytes from HCC group (see **Figure** 8). Apoptosis is the best-characterized form of programmed cell death and is main importance in tissue homeostasis. In mammalian systems, there are two major pathways and cellular apoptosis signaling could usually be initiated either through the cell death receptor-mediated extrinsic pathway or the mitochondrial-mediated intrinsic pathway.[28]However these pathways act independently to initiate the death machinery in some cellular systems, in many cell types, including numerous tumor cells. Following the each of synthesized Carnosine analogues treated with hepatocytes from HCC rat, to assess whether we could cause apoptosis. Apoptosis process through the mitochondrial pathway induces MMP loss, cytochrome c release. A number of researches have shown that the activation of the apoptotic pathway in malignant cells is a main protective mechanism against the development and progression of cancer.[28]The following, results of our study showed that peptidescaused a significant decline in the level of MMP only in mitochondria from the HCC rat group. It is well known that $\Delta\psi m$ is a vital variable toward the adjustment of mitochondrial function, also its decline is the critical point of death signaling (especially apoptosis).[30,31] The results implies that the increasing of ROS generation level and also the changing of the MPT pore play vital roles in the induction of apoptosis by peptides.Eventually, the results of our study showed that Carnosine analoguesraise the mitochondrial ROS level via the disruption of mitochondrial respiratory chain in comparison with mitochondria isolated from the liver of HCC rats. This process resulted in a decline of the MMP, alteration of mitochondrial swelling and release of cytochrome c, which can induce apoptosis signaling in liver hepatocytes of HCC rats. Also, the results of our study show that raise of the ROS level proposed as important regulators of mitochondria-mediated apoptosis.

6. Conclusion

In summary, the present study showed the selective apoptosis effects of synthesized linear and cyclic Carnosine analogues against HCC in a typical animal model. The results provide evidence for the hypothesis that synthesized peptides may exert an apoptotic effect on HCC liver hepatocytes through increasing ROS production which finally ends in cytochrome C release. Based on a raise in mitochondrial reactive oxygen species (ROS) level,swelling in mitochondria, decreasing of ATP generation, mitochondrial membrane potential (Δψm) collapse, release of cytochrome c and caspase-3 activation after exposure of mitochondria isolated from the *Hepatocellular carcinoma,* cyclic Carnosine analogues than linear Carnosineanalogues would be encouraging to develop new anticancer agents and may be considered as a promising complementary therapeutic agents for the treatment of HCC.

Acknowledgements

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