In Vivo Study of a Newly Synthesized Chromen-4-One Derivative as an Antitumor Agent Against HCC

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Abstract

Background: Chromenes are a wide group of natural compounds that can be synthesized chemically. The chromen-4-one nucleus acts as a skeleton for varieties of additional active groups that makes the chromenes activity varies between antioxidant and anti-inflammatory agents. In the present study, a newly synthesized chromene compound exhibits different behavior other than anti-inflammatory and antioxidant activities that it is the first time that a member of chromen-4-one compound can control the cancer progress. Inflammation is the first step in tumor development where the severity grade can potentiate tumor growth and progression. In many tumors pro-inflammatory genes record high expression level such as tumor necrosis factor (TNF-α) and vascular endothelial growth factors (VEGF). These pro-inflammatory factors act as rate limiting steps in tumor initiation and controlling its expression acts as an early therapeutic way to control the tumor proliferation. The chromone derivatives have biological activities such as anti-inflammatory and antitumor activity.

Methods: In the present study a new chromene derivative (Ch) was studied against HCC induced by diethylnitrosamine (DEN) in rats.

Results: The treatment strategy Ch compound is to down regulate pro-inflammatory gene expression of early genes as TNF-α as well as VEGF and subsequently controls other factors such as p53, Cyt C and MMP-9. Also, retrieve the balance between Bcl2 and Bax proteins in DEN induced HCC in rats.

Conclusion: The ability to control the primary initiators of HCC offers the new Ch derivative an antitumor activity and encourages further researches to follow and monitor its effect on the molecular level.

Summary

- **Chromones are effective compounds as anti-inflammatory agents and some researches pointed out the antitumor activity of chromones in vitro.**
- **The Ch compound is one of chromone derivatives and we studied its antitumor activity invivo.**
- **HCC was induced in rats DEN and treated with Ch compound.**
- **The treatment with Ch compound rebalances the biochemical markers to shift the body status away from HCC disturbance to the normal balance through blocking of TNF-α.**
- **Ch compound could be an effective agent for controlling the tumor status.**

Background:

Chromones (benzopyran-4-one) are known as bioactive agents due to their anti-inflammatory properties. Moreover , some of chromones are natural such as UMB , 7-hydroxy coumarin in golden apples with its antioxidant property and can be used as modulator to protect against gamma radiation .[1]

Also, one of 5,7-dihydroxy chromen-4-one (DCO-6) inhibits (NO) produced by lipopolysaccharide and considered as an anti-inflammatory agent .[1]

palifermine, and amifostine are only two compounds used as radiopprotectors in spite of many compounds of several classes were studied as radioprotectors.[2] A novel Chromone derivative ((2E) 2- (4-oxo – 4H—chromen—3–yl) methylene amino-4- nitrobenzoic acid)shows antitumor activity towards EAC (Ehrlish Ascites carcinoma) cell line [3] and was tested as a promising radioprotector agent.[4]

The aim of this work is to study the previous chromone derivative to treat the HCC and study its mode of action.

The majority problems of health facing the world recently are tumor and infectious diseases.[5] In developing countries, Cancer is a main cause of death and new therapeutic strategies take great attention in biomedical researchs.[6] Hepatocellular cancer (HCC) is a third fatal cancer leading to death . [7]

HCC is related to many risk factors such as HCV [8] which leads to a multistep cascade related to cytokines initiation of inflammation and activation of oxidative stress leading to chronic hepatitis, cirrhosis, fibrosis, regeneration hepatic tissues.[9] In addition, carcinogenesis in hepatic tissues may be related to HCV gene over production causing liver proliferation .[10]

Diethylnitrosamine **(DEN)** is known as powerful hepatocarcinogen causing malignant transformation present in tobacco, water, some fried foods, cheddar cheese, chemical feltilizers of plants, cosmetics as well as some pharmaceutical products.[11]

DEN induces damage of DNA repair hepatic enzymes, such damage leads to liver tumors of modeling in experimental animal [12] through hepatocellular accumulation of reactive oxygen species (ROS).[13] The overload of DEN causes many dramatic changes such as induction of lipid peroxidation; which can be detected by the levels of MDA (malonaldehyde); as well as elevation of circulating AFP levels [14] and disturbance of p53 as tumor suppressor and pro-apoptotic protein, which subsequently shifts the balance between the antiapoptotic Bcl-2 and apoptotic Bax proteins to tumorigenesis.[15]In addition, the MMP (metalloproteinase) which involved in invasion and metastasis via disturbance of the mitochondrial permeability. Also, signals of mitochondrial Cyt c release to the cytosol. C (Cyt c) leads to activation of caspase-9. This event activates caspase-3 cleavage.[16] Treatment of tumors is related to several mechanisms of action of chemotherapeutic agents should be take place in treatment of cancer.[17]

Methods

The present study has been done at the Biology of Radiation Department, National Center of Radiation Research and Technology (NCRRT), Cairo, Egypt.

Chemical compound

Diethylnitrisamine (DEN) as well as chemicals were bought from (Sigma Aldrich Company), Street Louis-MO. The molecular formula of DEN is $(C_2H_5)_2$ NNO and the molecular weight is 102.1

Chromene

The Chromene Derivative was previously prepared, designed and characterized.[3] Its chemical structure was illustrated in Figure 1.

In the present study the antitumor activity was determined against hepatocytocarcinoma cell line Hep G2 by MTT method by colorimetric change of 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) to blue product of formazan in mitochondria of viable cells. The color is measured by spectrophotometer at 595 nm by 990-win 6 software of ELISA microplate reader (DV990BV4) Roma, Italy .[18]

Experimental Design and Animal Grouping

Male Wister rats (Adult) weighting 120-150 g were purchased from the El- Nile Pharmaceutical and Chemical Industries Company, Cairo, Egypt. The dealing conditions and treatment were approval of the Institution of Animal Care and Uses Committee (IACUC).[19]

Forty male rats were selected randomly into four groups, with (10) animals in group as mentioned in Table 1.

Table 1: Groups of rats.

Group 1 (control):	Animal receive interpertonial injection of saline
Group 2 (Ch):	Four weeks, animals were received (ip) injection with Ch in saline suspension (5 times per week) the dose is 20 mg./.kg b.wt.[4]
Group 3 (DEN):	DEN dissolved in 0.9 % saline, with a dose of 20 mg per kg b.wt per day, 5 times per week for six weeks.[20]
Group 4 (DEN + Ch):	Rats were received DEN like group 3 after that treated with chromene for 4 weeks like group 2 after DEN gavage.

Blood Samples

After completion of experiment rats were incubated for 24 hrs. and sacrificed under diethyl ether anesthesia. Collection of blood were done by heart puncture, the samples were centrifuged for 10 minutes at rate of 3000 rpm to obtain plasma for biochemical analysis.

Liver tissue

Liver tissue of experimental animals were dissected out and divided into 2 parts, one of them was embeded in 10% formalin for histopathology and the second part was homogenized (10% weight/volume) in phosphate—buffered–saline (0.02 Mole sodium phosphate buffered with 0.15 M of NaCl, pH=7.4) in a glass tissue homogenizer with a teflon pestle .[21]

Experimental parameters

Activities of L-alanine-aminotransferase (ALT) and Aspartate-amino-transferase (AST) were estimated in plasma using colorimetric assays as described by Reitman and Frankel .[22]Serum samples were tested for alpha-fetoprotein (AFP) using (ELISA) kit of AFP from Cloud,Clone Corp. in USA while The Rat C-Reactive Protein ELISA from Abcam, Canada.

Homogenates of hepatic tissue samples were used to assay for malondialdehyde (MDA) was spectrophotometrically determined according to Tsikas.[23] The TNF-α, VEGF, cytochrome C and MMP-9 concentrations were assayed by Sandwich ELISA kit for rats [Novus Biological, USA]

Apoptotic markers

Evaluation of Bcl2 and Bax gene expression in HCC tissue samples. The anti-apoptotic (Bcl2) and Bax apoptotic genes expression were quantified upon RNA extraction and synthesis of cDNA.

50mg of EC tissues were used to isolate total RNA by TRIzol reagent .[24] Then the enzyme reverse transcriptase is used to synthesize standard cDNA from one mg of RNA as a template. The mentioned primers are in Table 2.

Quantitative real–time of polymerase chain reaction [qPCR] RT-PCRs is assayed according to Pfaffl. [25] Expression mRNA of Bcl2 and Bax relative and the normalization average by reference b-actin.

A of Bcl2 and Bax relative and the normalization average by reference b-actin.

Table 2: Primer sequence of all studied genes.

Gene symbol	Primers sequence
Bax	F:. ’5 AGGGTGGCTGGGAAGGC’ -3'
	R:. ’5 TGAGCGAGGCGGTGAGG’-3'
Bcl2	F:5'ATCGCTCTGTGGATGACTGAGTAC3'
	R: 5' AGAGACAGCCAGGAGAAATCAAAC 3'
β-Actin	F; 5’-CGCAGTCAACGCATTTCGTCCTAT-3’
(rat)	R; 5’-ACGCTTGTCGATCCTCTGAGAC-3’

Histopathological examination

At the time of sacrifice, the liver tissues were excised from the animals. Samples from each liver tissue were fixed in 10% formalin and embedded in paraffin. Section of 5 microns thickness were cut and stained with haematoxylin and eosin [21]and examined by light microscope for histopathological investigation.

Statistical analysis

Statistical analysis between differences of means was figured out using one-way analysis variance (ANOVA). In case of a significant [F-ratio], post hoc the least significant difference (LSD) test of multiple comparisons for evaluation of the statistical significance between treated groups at $P<0.05$ significance level. Cross-tabulation analysis was carried out and the significance (χ^2) was calculated at $P<0.01$ to evaluate percentage of survival of rats. All of the statistical analysis was calculated using Statistical Package of Social Science [SPSS] version 20.0 [SPSS Inc.- Chicago- IL- USA].

Results

In vitro study

The antitumor activity of Ch was estimated by using [HepG2] cell lines. The Ch derivative was tested at different concentrations and results were plotted in Figure 2.

Effect of Ch derivative treatment on liver function enzymes

Induction of HCC by DEN uptake significantly raise the concentrations of ALT and AST enzymes with respect with control group but the levels of such enzymes decrease upon treatment with Ch derivativenear the normal ranges Figure 3.

Effect of Ch derivative on MDA of the induced HCC in rats

A significant induction of MDA in the HCC induced group in the concentration with respect to control group and Ch group. While CH treatment lowers the concentration of MDA in a significant manner in treated group in comparison with HCC group (Figure 4).

Effect of Ch derivative on TNF-α and VEGF of the induced HCC in rats The gavage of DEN induces the over expression of TNF-α and VEGF as a pro-inflammatory initiator but the ip injection of Ch derivative down regulate the TNF-α and VEGF significantly with respect to HCC induced group by (48.58% and 55.46%) respectively (Figure 5)

Chromene and DEN groups, respectively).

Effect of Ch derivativetreatment on cytochrome C

The exposure to DEN leads to lowering in the level of cytochrome c in comparison with normal control group. The Ch treatment with derivative elevates the level of cytochrome c significantly in comparison with DEN group (Figure 6).

Effect of Ch derivativetreatment on MMP-9

The gavage of DEN increases the level of MMP-9 in DEN group with respect to control group while the level of MMP-9 decreased significantly upon Ch treatment in group DEN + Ch group with respect to DEN group (Figure 7).

Effect of Ch derivativetreatment on p53

Figure 8 mentions the increase of p53 in DEN group and significantly exceeds the control group and the Ch treatment compound leads to inhibition in the level of p53 in comparison with DEN group.

Effect of Ch derivativetreatment on AFP and CRP

The treatment of rats with DEN elevates the AFP and CRP respectively significant with to control group while the treated HCC rats in (HCC+ Ch) group with Ch derivative leads to decrease the levels of AFP and CRP (13% and 28%) respectively in comparison with HCC group Figure 9.

Effect of Ch derivative on apoptosis

The balance between the Bcl2 and Bax proteins are very important in normal cases but a balance shift appears upon DEN gavage by increasing Bcl2 protein and decreasing Bax to keep the viability of tumor cells while this balance is returned again near the normal by treatment with Ch derivative Figure 10.

Histopathological examination

Studying the histological section of male rat liver under light microscope showed that, there was no histological alteration observed and the normal controls histological structure. Liver tissue section of male rats treated with Chromene under light microscope showing spotty necrosis. Treatment with diethylnitrosamine under light microscope showed that, showing hepatocellular carcinoma formation (note the trabecular and pseudoacinar pattern. The histological section of HCC induced male rat liver treated with Chromene showed that, markedly dilated congested blood vessels and areas of hemorrhage as well as tumor tissue degenerative changes and apoptosis as recorded in (Figure 11).

Discussion

Hepatocarcinogenesis is a complicated process including several steps, with a plenty of signals, leading to a diversified molecular profile.[26]

DEN generates ROS which result in DNA damage, mutations and cancer.[27] Appling of DEN is accompanied by over expression of inflammatory cytokines such as TNF which is the rate limiting step of carcinogenesis cascade. The oxidative stress produced upon DEN application can activate NOX1 (NADPH oxidase) axis which subsequently activate Kupffer cells and newly recruited macrophages to express TNF in abnormal concentration to start the inflammation and carcinogenesis.[28]

In the present study the Ch compound exerts an ability to inhibit TNF-α molecules. Two docking studies were carried out to deduce that chromen-4-one nucleus has special behavior to inhibit TNF-α. They mentioned that the TNF-α molecule has pharmacophoric features can act as hydrogen bond acceptors as the chromen-4-one has aromatic rings and (C = O) in aromatic ring which can donate electrons to the residues Leu 120 and Gly 121 in the TNF-α in addition the two aromatic rings can donate electrons to leu 57 and Gly 122 and Ile 58.[29–30]

The data of the present study revealed that the level of TNF- α was increased in the DEN induced HCC but decreased upon Chromene treatment our finding was supported by Afzal *et al.* [31] who mentioned that, once the expression of TNF- α increased, the cascade of inflammation starts to activate tumor promotion, angiogenesis, proliferation and survival via activation of NF-kB.

The angiogenesis is initiated by up regulation of VEGF as a step next to NF-kB activation [31] that, VEGF is known as an angiogenic agent which has a motivated facility for angiogenesis and growth of tumors and metastasis in the conditions like HCC.[32]

Saleem *et al.*[33] observed that levels of VEGF in the DEN group were significantly elevated implying advanced pace of angiogenesis which supports our results of increasing VEGF level in the group treated with DEN.

Also, the activation of NF-kB initiates over expression of other tumor promotors such as MMP-9 and COX-2 .[34]

In the present study MMP-9 level is increased in the group treated with DEN while the treatment with Chromene derivative decrease the MMP-9 level.

Among them, MMP-2 (72 kDa) and MMP-9 (92 kDa) are found to regulate tumor invasion and metastasis. Researches in malignant tumors reported the overexpression and enhanced activity of both of these MMPs .[35] Enhanced metastasis is evidently linked with enhanced MMP-2 and MMP-9 expressions as a tumor progressor. On the other hand, COX-2 enzyme enhances tumor through over production of MDA as a biproduct of COX-2 catalyzed breaking downof PGH 2 (prostaglandin H2).[36]

Malondialdehyde is considered as a mutagen in mammalian cells and carcinogen for rats [37] due to its affinity toward DNA to form adducts with deoxyguanosine to yield pyrimidopurinone – deoxyguanosine both MDA carbonyls react with nitrogen, forming the pyrimido [1,2-α]purine-10(3*H*)-one-2'-deoxyribose, or malondialdehyde-2'-deoxyguanosine adduct (M1dG) [38] and *N*6-(3-oxoprenyl) deoxyadenosine (M1dA)

and *N*4-(3-oxoprenyl) deoxycytidine (M1dC).[39] Although, formation of DNA adducts is a fateful step in cancer development, that it can be exerted by another pathway in addition to the effect of MDA. That, DEN itself is not a carcinogen but it exerts its carcinogenic effect after metabolism in hepatic cytochrome P450 (CYP) enzymes to yield alkylated guanine [40] while, Kang *et al.*[41] revealed that, the CYP deficient mice show less tumor incidence in comparison with the wild type. Also, as the CYP enzymes decrease, the bioactivation of DEN is declined leading to less availability of formation of DNA adduct and less tumorigenesis. [42] As a normal defense response, the hepatic cells start to express the protein p53 to obligate the mutated cells for entrance the apoptotic pathway .[15] The mode of action of p53 involves stimulation of reactive oxygen species so that the over production of p53 leads to more inflammatory response, accumulation of ROS and initiation of tumorigenesis in the surrounding environment as mentioned by[43] who recorded the accumulation of p53 in wild type rates treated with DEN with histopathological evidence of malignant hepatic tumors while less p53 filtration in hepatic p53+/− rats with lower risk of tumor formation.

Chiu *et al.* [44] studied two groups of humans with HCC, the first group is negative immune staining of p53 and the second group is positive immune staining p53. They found an increase in the level of Bax in both groups but the expression of Bcl2 decreased in the first group and increased in the second group with more advanced histopathological of tumor grade. They explain their results by finding out the ratio of anti-apoptotic and pro apoptotic proteins Bcl2 and Bax where, this ratio decreased in the first group immune negative p53 leading to apoptotic fate of the tumor cells but increased in the second group immune positive p53 to exceeds the percentage 88% with respect to controls that means that the tumor cells have more tendency to an anti-apoptotic behavior in group 2.

It has been demonstrated extensively that translocation of Bax from cytosol to mitochondria facilitates the release of the cytochrome c[45] because Bax can form channels, which allow direct cytochrome c release.[46]

The Bcl2 itself binds to pro apoptotic members such as Bax, preventing pore formation and cytochrome c release .[47] In contrast, increase in expression of Bax, induces cell death eliminating tumor cells.[48]

It has been suggested that a high ratio of Bax to Bcl2 can lead to collapse of mitochondrial membrane potential, resulting in release of cytochrome c and consequently causes cell apoptosis .[49] Our data also confirm that decreased Bcl2 protein expression, its inhibitory effect on Bax and caspase-9 was removed and leads to over expression of Bax and finally activation of caspase-9. Although, activation of caspase-9 also leads to loss of mitochondrial membrane potential by cleaving anti apoptotic members of Bcl2 family including Bcl-xL and Bcl2.[50]

However, it has been recently reported that ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-Chromene-3-carboxylate (HA14-1) binds to Bcl2 protein and blocks its anti-apoptotic function in HL-60 cells.[51]

Our results revealed that increase in the Bcl2 in the DEN treated group accompanied by drop in the level of the Bax leading to anti-apoptotic behavior appears in pathological observation while the treated group with DEN + Ch shows rebalanced Bcl2 and Bax levels near the control group.

The previous events are concerned with the cell proliferation which appeared as elevation in alpha fetoprotein (AFP) to develop the proliferation in HCC .[52] This finding supports our data where the treatment with DEN leads to elevation of AFP parallel to over expression of p53. In addition, after the inflammatory event and DNA disturbance affected by DEN administration, the inflammatory cascade reaching HCC, the hepatic function is disturbed as shown in elevation of liver function enzymes ALT and AST [43] as well as, increase in the expression of CRP in the HCC induced group because, hepatocyte is the main origin of the CRP and its synthesis is increased due to inflammatory stimulation .[53–54]

The administration of Ch derivative treats with the inflammatory event by deactivation of NF-κB which in turn controls the cascade of inflammation this finding is supported by .[4]

Conclusion

Tumor is one of the inflammatory response draw backs but the mentioned Ch derivative has the ability to down regulate TNF-α and VEGF which; subsequently; cut off the cross talk between other inflammatory, proliferation and angiogenesis mediators to decrease the severity of tumor development and the antiapoptotic behavior of the tumor cells can be rebalanced again upon Ch administration. Other studies should be done to detect more about the chromene based compounds behavior and their ability to sustain the normal status.

Abbreviations

TNF-α: tumor necrosis factor alfa; **VEGF**:vascular endothelial growth factors; **HCC:** Hepatocellular cancer; **DCO-6:** (E)-5,7-dihydroxy-3- (3-oxo-3-phenylprop-1-en-1-yl) -4H-chromen-4-one; **NO**: nitric oxide; **EAC:** Ehrlish Ascites carcinoma; **HCV**: Hepatitis C Virus; **MDA:** malonaldehyde; **ROS:** reactive oxygen species; **MMP:** Metalloproteinase; **Cyt c**: Cytochrome C; **NCRRT:** National Center for Radiation Research & Technology; **DEN:** Diethylnitrisamine; **Ch:** [(2E)-2- (4-oxo- 4H-chromen-3-yl) methylene amino-4-nitrobenzoic acid; **IACUC:** Institutional Animal Care and Use Committee; **ALT:** L-alanine aminotransferase; **AST:** Aspartate amino transferase; **ELISA:** Enzyme-Linked Immunosorbent Assay; **AFP:** alpha-fetoprotein; **RT-PCR:** real time polymerease chain reaction; **cDNA:** cloned Deoxyribonucleic acid; **RNA:** Ribonucleic acid; **qPCR:** Quantitative real-time polymerase chain reaction; **LSD:** least significant difference; **SPSS:** Statistical Package for Social Science.

Declarations

We declare no conflict of interest.

Ethical approval and consent to participate:

Animal dealing conditions and treatment were guided as per the National Institute of Health Guide for Animal and approved by the Institutional Animal Care and Use Committee (IACUC).

Reference: National Research Council (US) Committee for the Update of the Guide. Guide for the care and use of laboratory animals. 8th ed. Washington, DC: National Academies Press, 2011.

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Authors Contribution

all authors have read and approved the manuscript

1. **A. I. Nabeel** Donated the chromen-4-one new derivative and wrote the manuscript
2. **S. Z. Mansour** Shared the laboratory and animal house for work
3. **E. M. E. Mahdy** contributed the idea and plan of work
4. **H. A. El-Mezayen** Revision role
5. **S. A. Mohamed** apply the practical work and share the writing of manuscript

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Figures

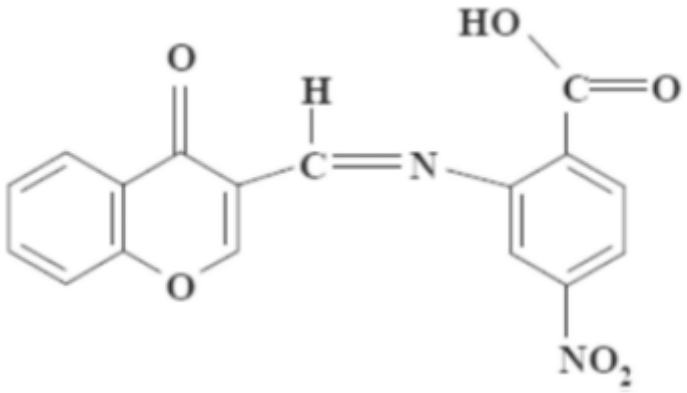

Figure 1

Chemical structure of Ch compound [.(2E).-2- (4-oxo– 4H—chromen—3–yl). methylene amino – 4 – nitrobenzoic. acid].

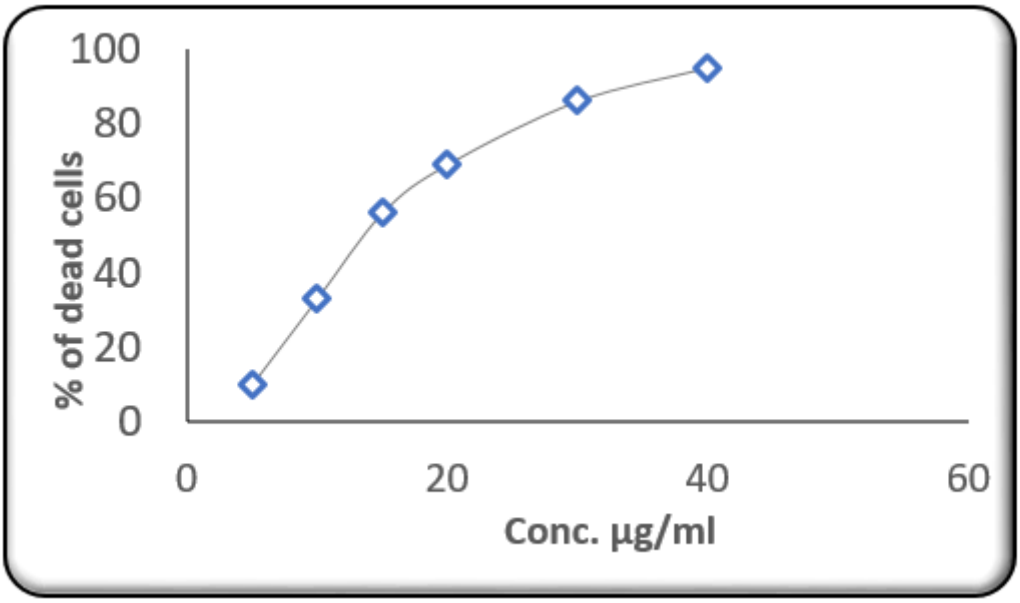

Figure 2

Different concentrations of Ch derivative and activity against HCC cell line. In vivo studies

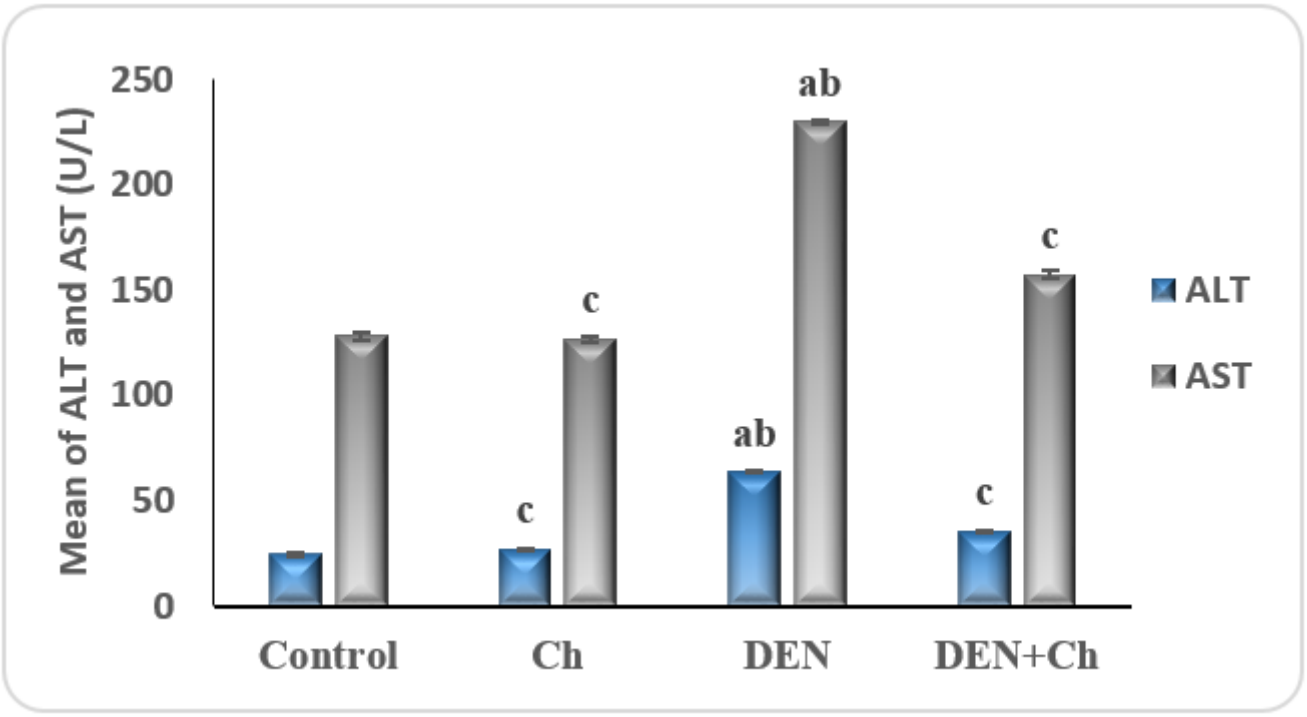

Figure 3

the changes of the liver function enzymes due to treatment with the Ch derivative (a, b and c denote significant change at p< 0.05 versus control, Chromene and DEN groups, respectively).

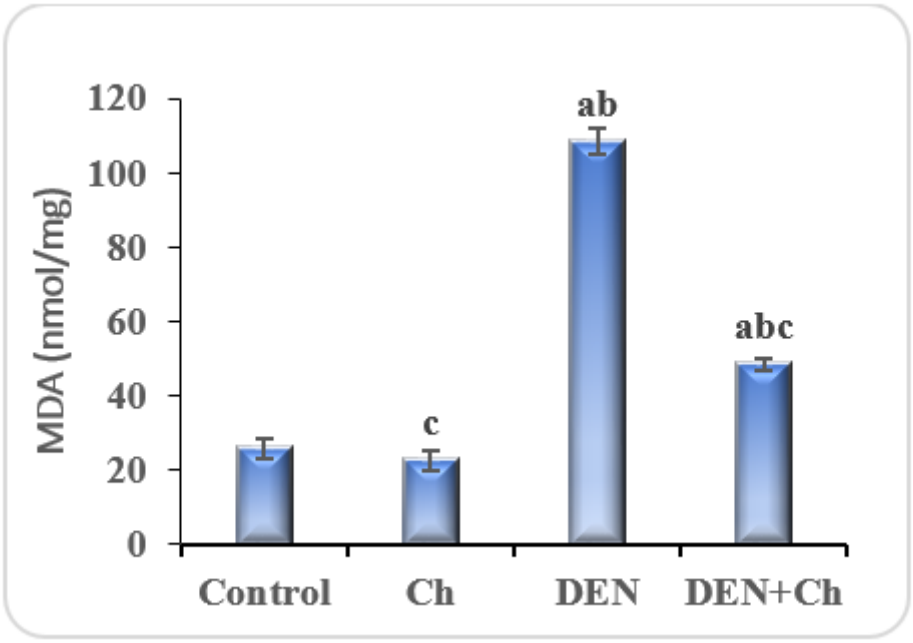

Figure 4

Mean of values of MDA concentration in different treated groups compared with controls (a, b and c denote significant change at p<0.05 versus control, Chromene and DEN groups, respectively).

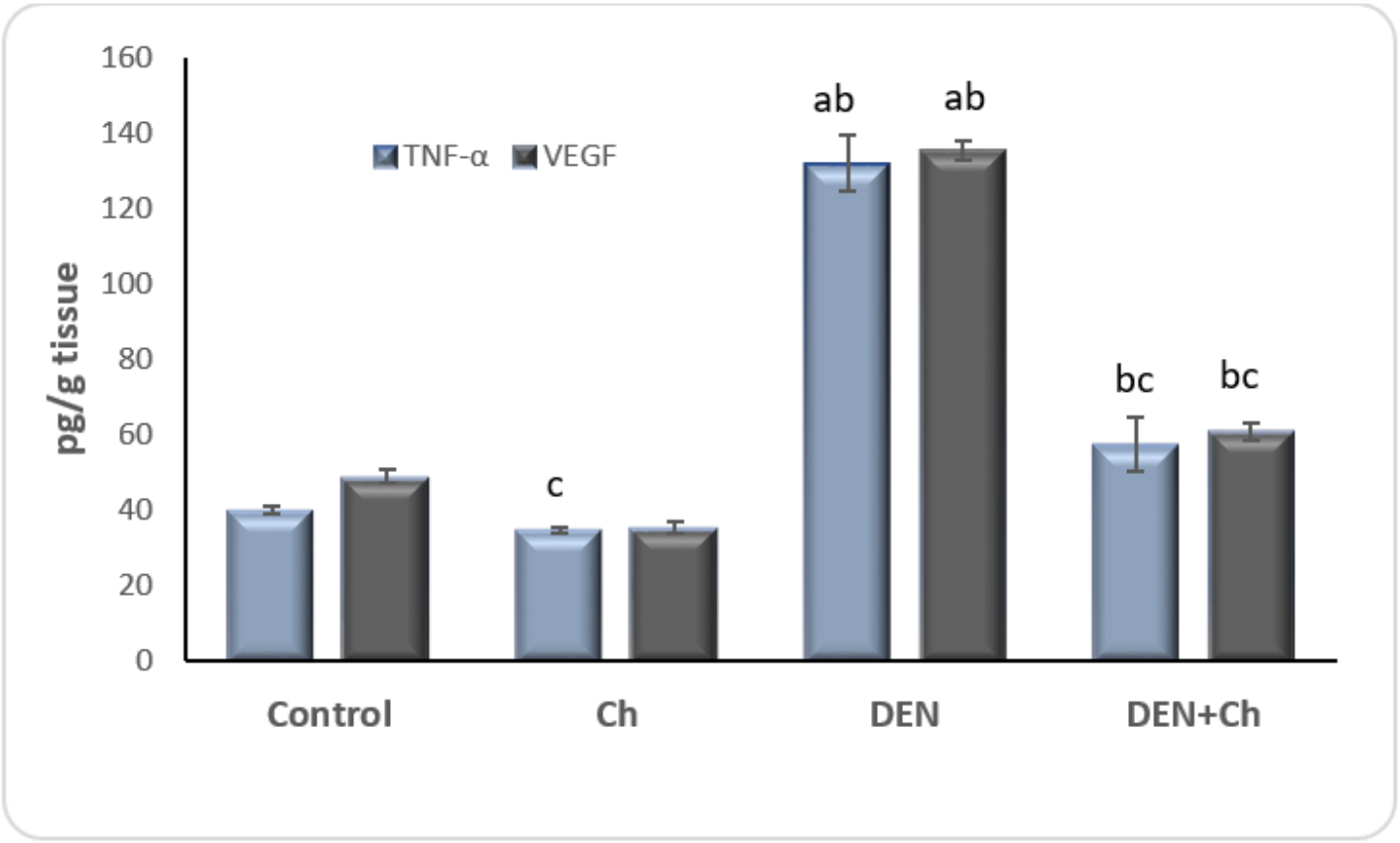

Figure 5

Mean values of TNF-α and VEGF concentration in different treated groups compared with controls (a, b and c denote significant change at p< 0.05 versus control, Chromene and DEN groups, respectively).

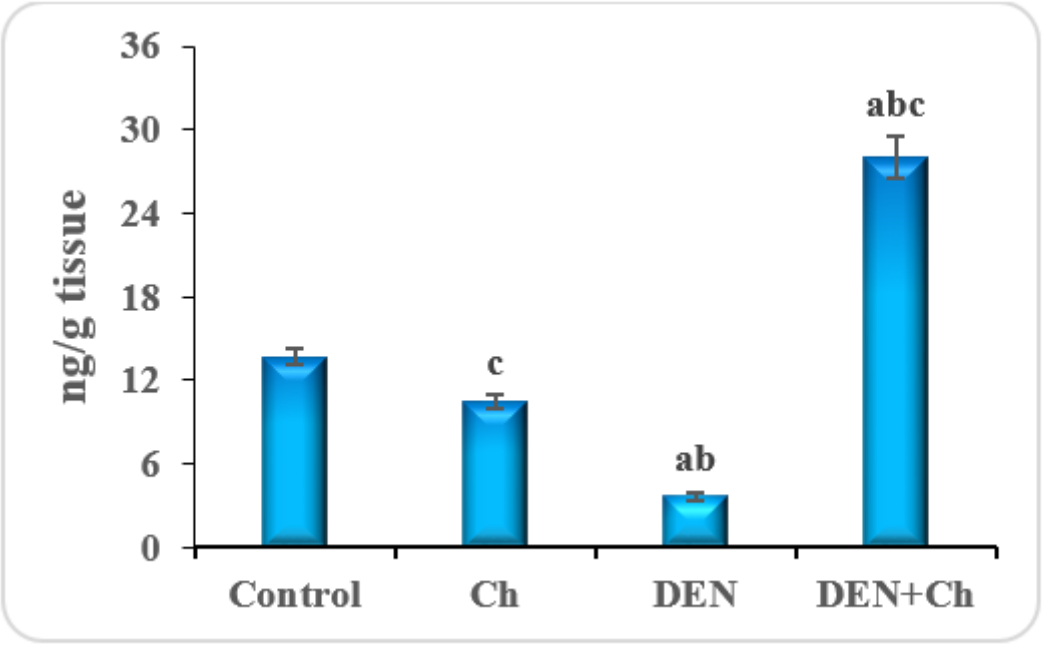

Figure 6

Mean values of cytochrome C concentration in different treated groups compared with controls (a, b and c denote significant change at p<0.05 versus control, Chromene and DEN groups, respectively).

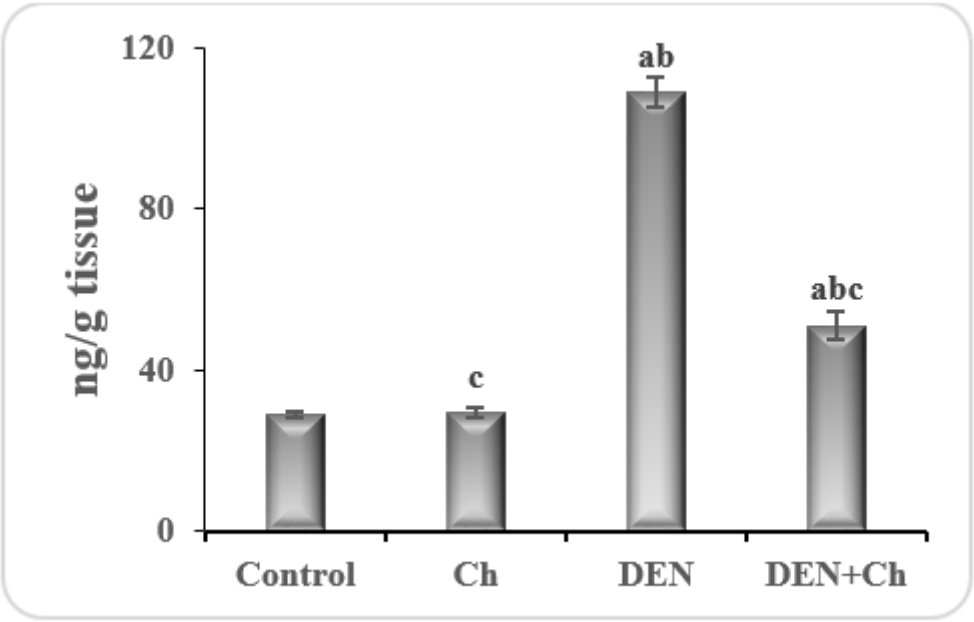

Figure 7

Mean values ofMMP-9 concentration in different treated groups compared with controls (a, b and c denote significant change at p< 0.05 versus control, Chromene and DEN groups, respectively).

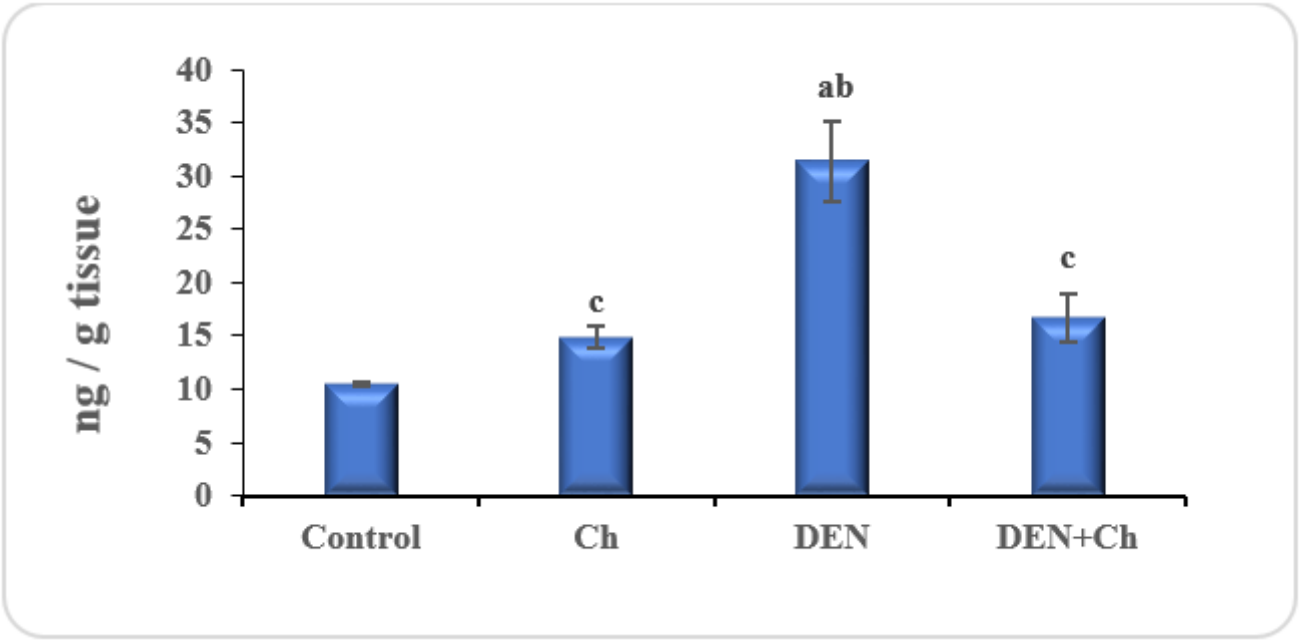

Figure 8

Mean values of p53 concentration in different treated groups compared with controls (a, b and c denote significant change at p<0.05 versus control, Chromene and DEN groups, respectively).

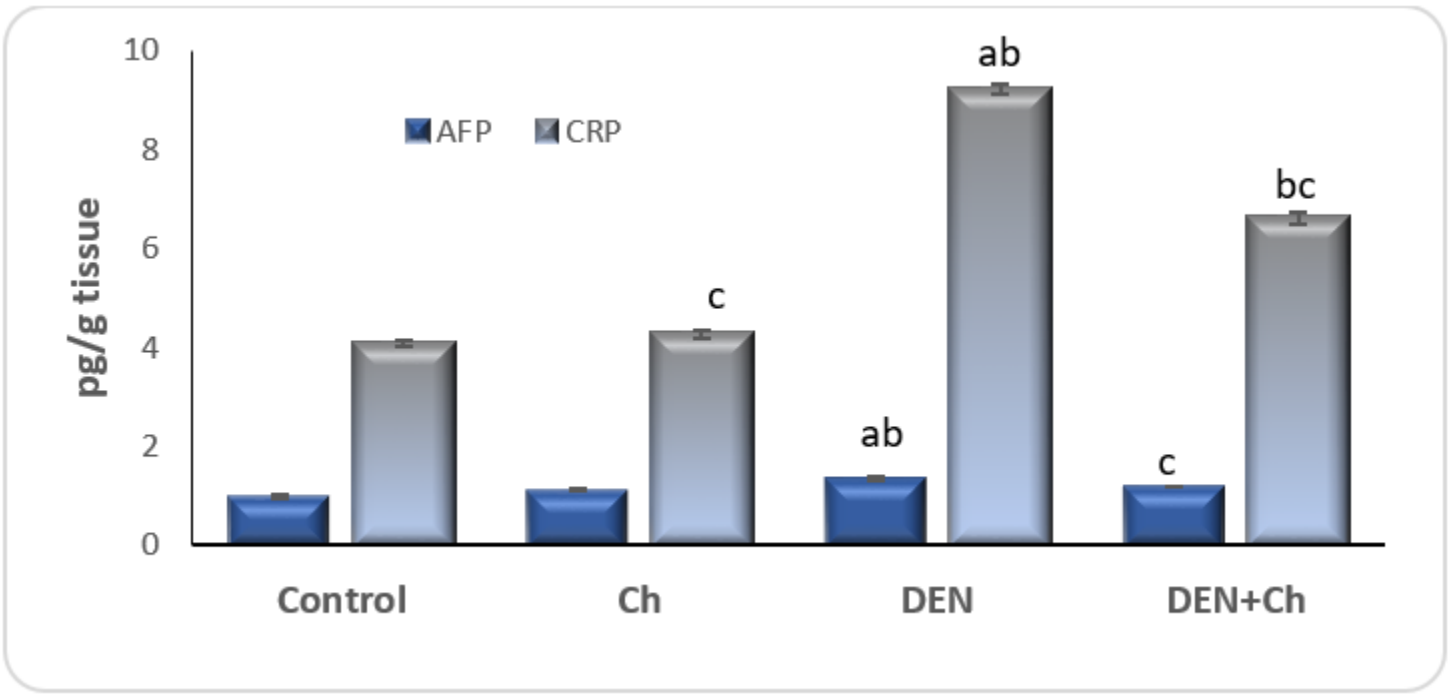

Figure 9

Mean values of AFP and CRP concentration in different treated groups compared with controls (a, b and c denote significant change at p< 0.05 versus control, Chromene and DEN groups, respectively).

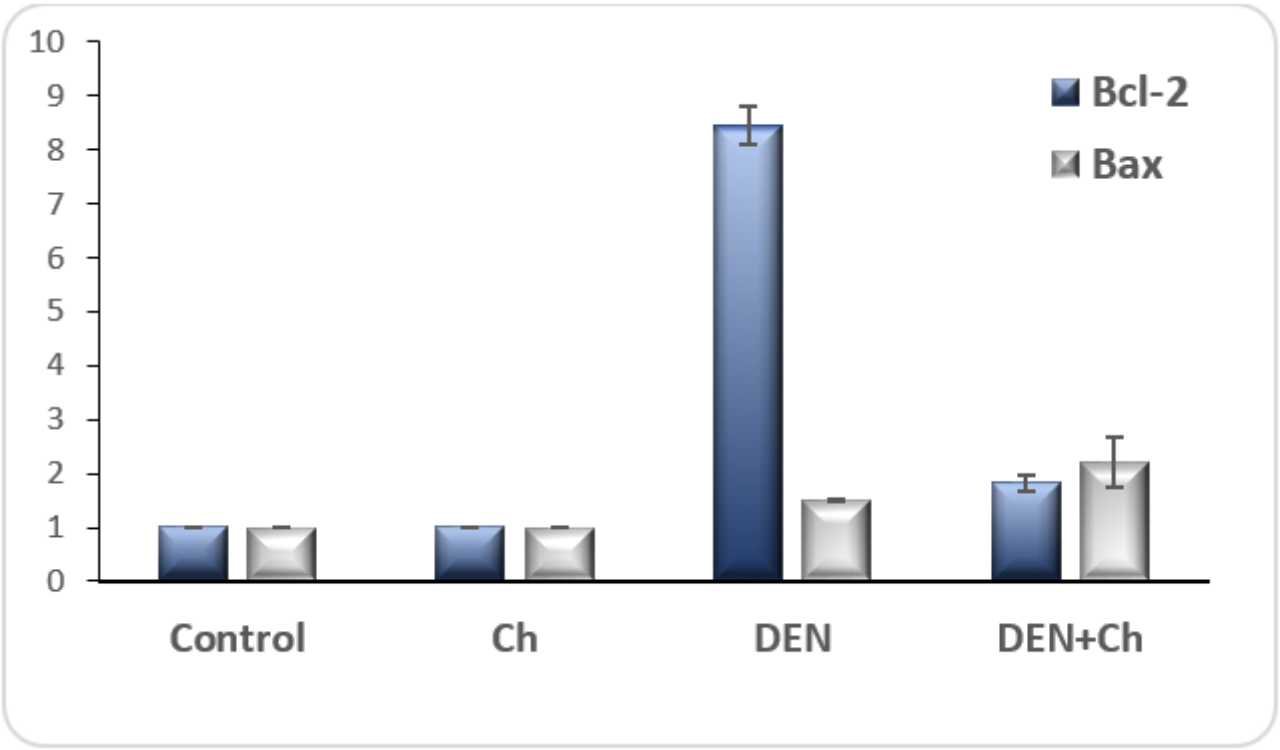

Figure 10

Bcl2 and Bax in different treated groups compared with controls (a, b and c denote significant change at p< 0.05 versus control, Chromene and DEN groups, respectively).

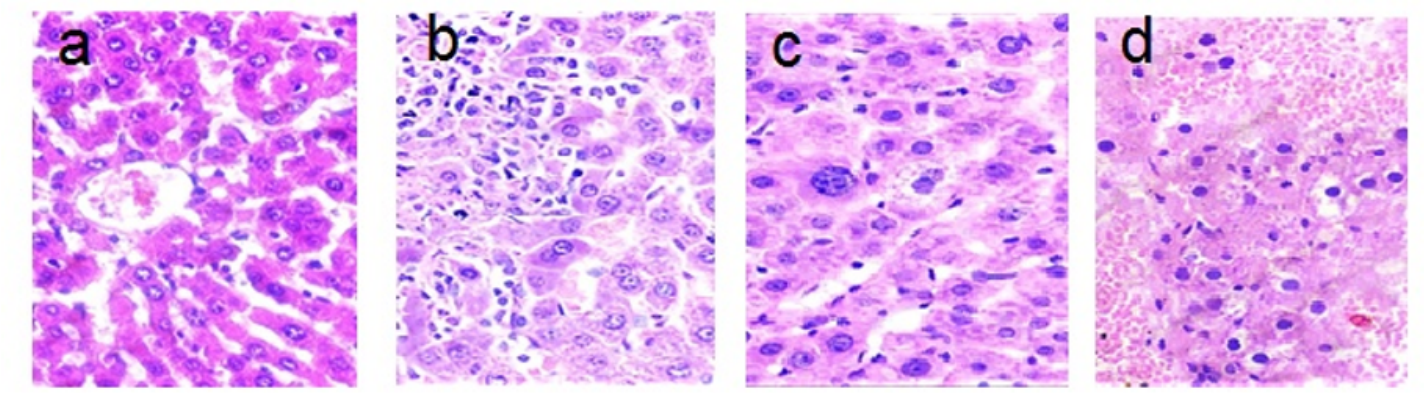

Figure 11

Histopathological examination while (a) represents control group, (b) represents Ch group, (c) represents DEN group and (d) represents DEN + Ch group.

Supplementary Files

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