

Anticancer activity of chicken cathelicidin peptides against different types of cancer

Maged Mostafa Mahmoud

King Abdulaziz University (KAU)

Ahmed M. Al-Hejin

King Abdulaziz University (KAU)

Turki S. Abujamel

King Abdulaziz University (KAU)

Modhi Alenezi

King Abdulaziz University (KAU)

Fadwa Aljoud

King Abdulaziz University (KAU)

Abdulwahab Noorwali

King Abdulaziz University (KAU)

Ibrahim A. Awad

King Abdulaziz University (KAU)

Mohammed. Alkhaled

University of Jeddah

Haitham. A. Yacoub (✉ Haithamyacoub46@gmail.com)

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Abstract

This study served as the pioneer in studying the anti-cancer role of chicken cathelicidin peptides. Chicken cathelicidins were used as anticancer agent against the breast cancer cell line (MCF-7) and human colon cancer cell line (HCT116). An *in vivo* investigation was also achieved to evaluate the role of chicken cathelicidin in Ehrlich ascites cell (EAC) suppression as a tumor model after subcutaneous implantation in mice. In addition, the mechanism of action of the interaction of cationic peptides with breast cancer cell line MCF-7 was also investigated. It was found during the study that exposure of cell lines to higher concentration of chicken cathelicidin for 72 h reduced cell lines growth rate by 90%-95%. These peptides demonstrated down-regulation of (cyclin A1 and cyclin D genes) which are essential for G1/S phase transient and S/G2 phase and consequently causes “prometaphase arrest” ultimately leading to death of MCF-7 cells. The study showed two- and three-times higher expression of the caspase-3, and -7 genes respectively in MCF-7 cells treated with chicken peptides (especially cathelicidin-2 and -3) relative to untreated cells which encouraged pro-apoptotic pathway, autophagy, and augmentation of the anti-proliferative activity. Our data showed that chicken (CATH-1) enhance releasing of TNF α , INF- γ and upregulation of granzyme K in treated mice groups, in parallel, the tumor size and volume was reduced in the treated EAC-bearing groups after cathelicidin administration compared to untreated EAC-bearing group. Additionally, animals received high dose of cathelicidin-1 (40 μ g/ml) displayed an apical survival rate compared to untreated carcinoma control and animals which received low dose of cathelicidin (10 and 20 μ g/ml). Tumor of mice groups treated with chicken cathelicidin displayed high area of necrosis compared to untreated EAC-bearing mice. Based on histological analysis and immunohistochemical staining revealed that the tumor section in Ehrlich solid tumor exhibited a strong Bcl2 expression in untreated control compared to mice treated with 10 & 20 μ g/ml of cathelicidin. Interestingly, low expression of Bcl2 were observed in mice taken 40 μ g/ml of CATH-1. This study drive intention in treatment of cancer through the efficacy of anticancer efficacy of chicken cathelicidin peptides.

Introduction

There is an in-built immune and defense system in every organism that protects its body from disease-causing microbes or pathogens. The immune system generates small microbe-killing molecules in order to prevent and counter the pathogen attack; such molecules are known as antimicrobial peptides. Multifunctional properties of AMPs have been lately recognized; they are known to perform multiple roles of immune regulation, wound healing, angiogenesis, and anticancer functions. They show different anticancer properties according to cancer types (1). Since the antimicrobial peptides are positively charged particles, they easily undergo reaction with the microbial cell surface that is negatively charged (2). As a result, the cell surfaces are disrupted. In healthy animals, the negative charge is not visible to the AMPs due to absence of such negatively charged particles on the cell surface. The growth of cancer cells placed in a dish was found to be attacked by the AMPs according to various studies but AMPs attack on cancer cells within a living body have not been established yet (2).

It may be an extremely beneficial cancer treatment strategy to determine the peptides that are effective from therapeutic point-of-view and those having anticancer potential and to consequently work for their development. As per the Antimicrobial Peptide Database (APD), anticancer properties are found in over 170 peptides (3). Cell death may be caused by AMPs when apoptotic pathways or other pathways are affected due to their interaction with cancer cells (4). Many efforts are being invested to explore the anticancer properties of AMPs keeping in view their multifunctional character.

One of the peptides essential for the natural immune system is Cathelicidins that is one of the two types of Host Defense Peptides (HDP). These peptides are characterized with multiple antimicrobial activities for prevention of attack by Gram-negative bacteria, Gram-positive bacteria, fungi and parasites. The in vitro examinations revealed that cathelicidins-1,2 and -3 depicted potent broad spectrum antibacterial activities; on the other hand, only some of the bacteria were subjected to CATH-B1. Considering other peptides, gene duplication leads to the formation of mature peptides CATH-1 and CATH-3 having about 70% similar sequence (5-7). However, only their antibacterial properties have been extensively studied in majority of research (8-12). Several studies have been investigated the antimicrobial and immunomodulatory potentialities of chicken cathelicidin-2 through activating toll like receptor-2 and neutralization of LPS (13-18).

The significance and processes of LL-37 in case of human cancer was only investigated at molecular and cellular levels. It was found that LL-37 may act as pro-tumorigenic or as an anti-cancer agent. LL-37 effects at molecular level in various cellular settings have not been comprehended completely; but its function as a ligand for different membrane receptors has been recognized which indicates the tissue-specific activity of LL-37 in various types of cancers (19). Various combinations of processes like aberrant cell cycle regulation, decline in rate of apoptosis and increased stimulation of growth factor pathway affect the malignant transformation and onset and progression of the tumor (20). The progression of the cell cycle is controlled through the coordination of serine/threonine kinases namely the Cyclin-dependent kinases (CDKs).

When the CDK enzyme complex binds to the appropriate cyclin, it is activated. When the phosphoryl group is added to this CDK-cyclin complex at specific activating residues, then planned activation and breakdown of CDK complexes is essential for controlling cell cycle progression (21,22). The CDK activity is greater in transformed cells than in normal ones; hence, the former show quicker cell proliferation. This implies the therapeutic significance of inhibiting CDK/cyclin complexes in protection from cancer. Cells are blocked during the G phase of cell cycle by compounds inhibiting CDK4/6 activity, while cell arrest during G/S and G/M phases of cell cycle is caused by compounds inhibiting CDK1/2 activity (Nurse,2002). Moreover, apoptosis may be stimulated in cancer cells by a few inhibitors of CDK2 activity. With respect to structure, CDK inhibitors (CDKIs) are associated with adenosine-5'-triphosphate (ATP) (22).

The mechanism of action involved is as follows: the helical cathelicidin peptides cause depolarization and alteration in cell membrane and its associated aspects. The helical structure may be due to higher

concentration of peptides, anions, pH, detergent, and lipids (23,24). This peptide undergoes interaction with eukaryotic cancer cell membranes owing to its hydrophobicity and amphiphilicity (25).

Earlier research conducted on designed peptides indicated that the interactions of peptides with target cytoplasm membranes and the consequent anti-cancer mechanism is highly dependent on hydrophobicity (26-28). In addition, high specificity was observed for anticancer peptides that had been developed through de novo approach. This high specificity helped distinguishing cancerous cells from non-cancerous cells. Moreover, it was found that the peptides' membrane partitioning property is highly affected by Amphiphilicity (1).

The same observations were recorded in a few other reports which indicated that apoptosis may be initiated by human cathelicidin through mitochondrial depolarization and DNA fragmentation; however, it may not be initiated through caspase activation. In addition, apoptotic cell death was highly stimulated by analogous peptides as compared to original peptide (10–40 µg/mL). The reaction of peptides on the membrane of cancer-cell showed that designing of analogous peptides is done in such a way as to enhance antimicrobial effects (29). Since cancerous cells have membranes with greater negative charge as compared to non-cancerous cells, apoptotic cell death may be induced in cancer cells by the action of peptides, LL-37 fragment, and its derivatives with amino acid replacement.

The established model pertaining to the course of interaction between AMP and cancer cell membranes suggests that AMPs play an essential part in the intracellular site utilization and takes part in bilayer interaction, membranolytic mechanism and membrane translocation. The unique characteristics that distinguish cancer cells from other cells were identified in some studies as cholesterol as well as different anionic components (25,27,30-31).

Cholesterol is one of the main sterols found in eukaryotic cell membranes (32) and its excess can prevent various α -helical peptides from performing lysis on non-cancerous cell membranes and the corresponding lipid mimics. Hence, cholesterol is known to inhibit anticancer activity on membranes of cells (25,33-35). It is important to note that cancer cells sometimes depict more cholesterol–lipid rafts (36), and the toxicity of such cancer cells was found to be lower due to interaction of these rafts with AMPs (31). The cholesterol-depleted membranes are characterized with greater fluidity and loosely-held lipids and hence they are more vulnerable to be attacked by peptides; however, development of lipids raft with more cholesterol content can decrease these chances of attack, hence protecting phosphatidylserine (30). The current study intended to evaluate the regulatory effects on cell viability and apoptosis of breast cancer cell line (MCF-7) and human colon cancer cell line (HCT116) under the action of chicken cathelicidin peptides. This was followed by investigation of prometaphase arrest of cancer cells and other essential molecular events to come up with molecular basis for use of such peptides as an anticancer agent for breast cancer cells. Additionally, an *in vivo* investigation was also achieved to evaluate the role of chicken cathelicidin in Ehrlich ascites cell (EAC) suppression as a tumor model after subcutaneous implantation in mice.

Results

The main objective of this study was to examine the *in-vitro* inhibition of breast and colon cancer cells under the effect of chicken antimicrobial peptides (chicken cathelicidins-1, -2, and -3) besides examining the mechanism of action behind the stimulation of program cell death and processes preventing cancer-cell from undergoing cell division. An *in vivo* investigation was achieved to evaluate the role of chicken cathelicidin in Ehrlich ascites cell (EAC) suppression as a tumor model after subcutaneous implantation in mice. Several parameters in blood lymphocytes and tissues were investigated to study the possible therapeutic antitumor immunity effects of chicken cathelicidin on carcinoma animal model.

In vitro experiments

Breast and colon cancer cell lines cytotoxicity

As per our study, the rate of inhibition of cancer cell growth at lower concentration (10 µg/ml) of chicken cathelicidins was between 66% and 85%. As the concentration of chicken cathelicidins peptides was changed to moderate level (10 µg/ml), there was a significant decline in the rate of cell survival. Figure 1 shows that as the concentration was increased to high level (40 µg/ml) and observations were made at 24 h of treatment, dramatic decrease in growth was seen in the cancer cell line.

When the cells were observed after 48 h of low concentration (10 µg/ml) peptide treatment, they depicted 25% survival rate for chicken cathelicidins-1, 64% for chicken cathelicidins-2 and 58% for chicken cathelicidins-3. When treated for 48h with moderate concentration (10 µg/ml) of peptides, the treated cells showed survival rates of 12% for cathelicidins-1, 64% for cathelicidins-2 and 31% for cathelicidins-3. Considering the treatment with 40 µg/ml of cathelicidin, there was a sharp decline of about 7% in the cell growth of both cell lines for cathelicidin-1 and cathelicidin-3 while 10 % decline was observed in cell growth after 48h long treatment with cathelicidin-2.

It became evident from the study outcomes that at the time interval of 72h of treatment, cancer cells depicted a development rate of 43% and 48% for cathelcidin-1, 61.3% and 72% for cathelcidin-2 and 65% and 73% for cathelcidin-3 for both HCT116 and MCF-7 cell lines respectively, while the concentration of each peptide was low. On the other hand, as the peptide concentration was increased from low to moderate (20 µg/ml), we observed a decline in cancer cell growth at the time interval of 72 h of treatment with 23.8 % and 22% % development rate for chicken cathelicidin-1, 24% and 44% for chicken cathelicidin-2, and 43.5% and 73% for chicken cathelicidin-3 treatment for both HCT116 and MCF-7 cell lines, respectively. This shows that cancer cell growth was significantly inhibited (around 85-95%) when treated with high concentration of chicken cathelicidins (40 µg/ml) at 72 h of treatment for both HCT116 and MCF-7 cell lines, respectively ,which proves to be a strong anti-cancer agent.

Expression Levels of Cyclin A1 and Cyclin D1

Figures 2 and 4 depict the change in cyclin A1 gene and cyclin D1 gene expression after subjecting the breast cancer cell line to chicken cathelicidins. Cyclin A1 and Cyclin D1 are the genes associated with cell

cycle division. It was found that the untreated cell (MCF-7 control) show expression of both the genes while cells treated with chicken cathelicidin peptides do not depict clear expression of these genes. The cells treated in this way may sometimes even depict cell cycle division arrest in the cell cycle phases of G1/S and G2/S (prometaphase arrest) consequently inhibiting the growth of breast cancer cell line and ultimately causing death of cancer cell. All the chicken cathelicidin peptides showed similar outcomes in terms of reducing the cancer cell survival.

Expression Levels of Caspase-3 and -7

The MCF-7 cells treated with chicken cathelicidin peptides were then subjected to RT-PCR testing in order to determine the patterns of expression of the Caspase-3 gene and Caspase-7 gene associated with cysteine protease family. It is recognized that these genes are responsible for mediation of apoptotic pathways through the execution of intracellular proteins associated with cytoskeleton protein fibres. In this context, figures (3 and 4) shows that cathelicidin-1 induced a detectable level of caspase-3 gene expression which was 3 times greater than the expression of this gene in untreated cells whereas, treatment with cathelicidin-2 and -3 induced two times greater gene expression. The treatment with cathelicidin-2 and -3 resulted in two-fold caspase-7 gene expression than the expression of this gene in untreated MCF-7 cells. However, treatment of cancer cells with cathelicidin-1 did not show any change in caspase-7 gene expression.

Cell Membrane Morphology

The changes caused by chicken cathelicidin peptides in the morphology and survival of breast cancer MCF-7 cells were determined with the help of DAPI and PI dyes. These dyes are used to distinguish between living and dead cells on the basis that they have the ability to stains dead cells while they cannot cause any staining in live cells due to impermeability in such cells. Fluorescent microscopy was used to determine the extent of damage caused by cathelicidin peptides to the membranes of MCF-7 cell.

The outcomes of our study indicated that before the treatment of MCF7 cells with peptides, the membranes of these cells were not disrupted; however, when the breast cancer cells were subjected to chicken peptide, bright staining was evident in most of the cells indicating greater stain permeability post-treatment in comparison to untreated MCF-7 cells (Figure 5). Moreover, the shape of the mitochondria in treated cells is also elongated due to the effect of peptides on mitochondrial membrane. The nucleus of the treated cells undergoes fragmentation and is also smaller in size than in untreated cells.

***In vivo* experiments**

The level of INF- γ and TNF α population after chicken cathelicidin administration

Our results indicated that the level of INF- γ was significantly increased in group treated with 20 & 40 $\mu\text{g/ml}$ of CATH-1 (0.67 ± 0.11) (0.73 ± 0.18) respectively when compared to that of normal mice (0.25 ± 0.02 , $P=0.0017$, $P= 0.0064$) respectively and carcinoma group (0.16 ± 0.03 , $P= 0.0128$, $P= 0.0397$) respectively as shown in figure (6 A). Meanwhile, the INF- γ level was significantly decreased in group

treated with 10 µg/ml of CATH-1 (0.1 ± 0) in comparison with normal mice (0.25 ± 0.02 , $P= 0.0007$). On the other hand, the level of INF-γ was non significantly different in mice treated with 10 µg/ml of CATH-1 (0.1 ± 0) as compared to carcinoma control mice (0.16 ± 0.03 , $P=0.0624$). A similar trend was found in case of the level of level of TNFα showed a significant increase in mice treated with 20 & 40 µg/ml of CATH (0.3 ± 0.05) (0.4 ± 0.1) respectively as compared with that of the normal mice (0.12 ± 0.01 , $p=0.0027$, $P= 0.003$) respectively and untreated carcinoma mice (0.05 ± 0.02 , $P= 0.0031$, $p=0.0057$) respectively. Moreover, TNFα showed a significant increase in mice treated with 10 µg/ml of CATH (0.1 ± 0) in comparison with that of the untreated carcinoma group (0.05 ± 0.02) ($P=0.0438$). While the TNFα showed a non-significant different in mice taken 10 µg/ml of CATH-1 (0.1 ± 0) compared to normal group (0.12 ± 0.01 , $P=0.0630$) as noted in (figure 6).

Changes in tumor volume after chicken cathelicidin administration

The figure 7 represents the effect of treatment with CATH-1 on tumor growth through 21 days. The figure showed the changes of tumor volume of mice measuring by calliper in each group during the in vivo experiment. The tumor volume of mice in each group was significantly different, and the tumor volume of mice treated with CATH-1 (10, 20, & 40 µg/ml) was significantly smaller than of carcinoma control ($p \leq 0.0001$).

The high -frequency ultrasound technique was used to calculate the tumor volume from ultrasonography measurements (three diameters) at second and third week after tumor transplantation and doses administration. We did scan for five mice in each group via ultrasound and some follow up mice were died, so we got equal number of mice in each group (three mice per group). As shown in (figure 7), the size of tumor in untreated EAC-bearing was elevated at third week by 85.19, 79.13&115.97 mm³ respectively. On the other hand, the tumor volume of treated EAC-bearing mice with different concentration of CATH-1 (10, 20 & 40 µg/ml) showed decreased in tumor volume at third week. The group treated with 10 µg/ml of CATH-1 exhibit decrease in tumor size by 147.12, 144.53& 194.51 mm³ respectively. In addition, group treated with 20 µg/ml of CATH revealed decrease in tumor size by 158.78, 72.89 & 230.15 mm³ respectively. Similarly, mice treated with 40 µg/ml of CATH-1 showed decreased in tumor size by 53.92, 43.92 & 57.98 mm³ respectively.

The results revealed that no significant difference in tumor size in mice group treated with 10 µg/ml of CATH-1 at second week (502.77 ± 47.02) as compared with carcinoma control (558.37 ± 55.005 , $P= 0.485$). While the tumor size of mice given 10 µg/ml of CATH-1 showed a significant decrease at third week (340 ± 30.97) in comparison to untreated carcinoma control (651.79 ± 64.53 , $P=0.012$) as depicted in (Figure 8). The mice treated with 20 µg/ml of CATH-1 showed a significant decrease in tumor size at second week (342.42 ± 51.33) and third week (255.08 ± 79.68) as compared to untreated tumor mice at second week (558.37 ± 55 , $P= 0.045$) and third week (651.8 ± 64.53 , $P= 0.018$). Similarly, tumor size in mice taken 40 µg/ml of CATH-1 showed a significant decrease at second week (107.55 ± 16.43) and third week (55.61 ± 12.79) as compared to carcinoma mice at second week (558.37 ± 55 , $P = 0.0014$) and third week (651.8 ± 64.53 , $P = 0.0008$). additionally, the untreated EAC-bearing mice and mice received dose

of 10 of CATH-1 showed high rate of death, whereas the mice treated with 40 µg/ml of CATH showed high survival rate as indicated in (figure 9).

The level of expression of granzyme B and K after 4 chicken cathelicidin administration

We noticed in (figure 10), that exposure to cathelicidin with concentration of 40 µg/ml significantly increased granzymes K expression by (39.3 ± 12.4) (47.6 ± 15) fold in comparison to normal mice and carcinoma mice respectively. However, there was a significantly decreased in granzyme K expression in EAC-bearing groups treated with 10 µg/ml of cathelicidin by (0.36 ± 0.1) (0.293 ± 0.1) fold as compared to normal mice and carcinoma control, respectively. In addition, cathelicidin did not affect expression of granzyme B in mice groups.

Histological examination

Subcutaneous implantation of Ehrlich tumor cells resulted in the development of Ehrlich solid tumor. The hematoxylin & eosin staining sections prepared from solid tumors of the untreated control group showing high-grade malignant growth formed with minimal foci of necrosis. Ehrlich solid tumor showed large, round, and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei, and binucleation. In addition, several degrees of cellular, nuclear mitotic figures, and scattered giant cells with multiple nuclei were also observed. Some areas showed multiple malformed, widely dilated blood vessels (angiogenesis) were seen in the surrounding tissue with leukocyte infiltration (Figure 11).

Based upon histopathology results, cotreatment of Ehrlich solid tumor with 10 µg/ml of CATH-1 revealed mild wide zones of necrosis and cells with mitotic figures, and few giant cells in between inflammatory cell infiltrations. The 20 µg/ml of CATH-1 treated groups showed a moderate focal area of necrosis. Interestingly, the histopathological pictures showed improvement in mice given 40 µg/ml of CATH-1 as evidenced by increasing necrosis degrees and most of the cells showing apoptosis progressively and most cells appeared with chromatin dust and fragmented nuclei as illustrated in (Figures 11).

Necrosis area percentage % and Histological Scoring for solid tumors

Histopathological analysis has been carried out of tumor sections with an emphasis on necrotic proliferation, characteristic mitotic feature, and neoplastic gigantic cell presence. Blindly examined and their frequency and strength were measured: (0) absent, (1) weak to mild, (2) mildly to moderately and (3) strong or regular histopathologic inspection. In the present study, there was a significant increase in the necrosis area percentage in group treated with 10 µg/ml of CATH-1 (33.20 ± 1.31), group treated with 20 µg/ml of CATH (44.80 ± 1.31), and group treated with 40 µg/ml of CATH (59.00 ± 2.91) as compared to Ehrlich solid tumor control ($P \leq 0.0001$) (Figure 12). In addition to, group treated with 10 µg/ml of CATH-1 showed a non-significant difference in the score of the necrosis area (0.60 ± 0.24), Giant cell (2.60 ± 0.24), Mitotic figures (2.60 ± 0.24) as compared to solid tumor control ($P \leq 0.05$). Interestingly, group treated with 20 µg/ml of CATH exhibited significant increase of score of the necrosis area (1.80 ± 0.20) as

compared to tumor control group ($P < 0.001$) and showed a significant decrease in the score of giant cells (1.40 ± 0.40) and mitotic figures (1.60 ± 0.24) as compared to carcinoma control group ($P \leq 0.01$).

Moreover, group treated with 40 $\mu\text{g/ml}$ of CATH-1 showed significant increase of score of the necrosis area (3.00 ± 0.00) as compared to tumor control group ($P < 0.0001$) and showed a significant decrease in the score of giant cells (0.40 ± 0.24) and mitotic figures (0.60 ± 0.24) as compared to carcinoma control group ($P \leq 0.0001$).

Immunohistochemical investigation

The detection of Antiapoptotic Bcl2 expression in tumor sections in untreated Ehrlich solid tumor control and Ehrlich cotreated with different doses of CATH-1 (10, 20 & 40 $\mu\text{g/ml}$) are revealed in (Figure 13). The tumor section in the Ehrlich solid tumor untreated control group exhibited a strong positive reaction for Bcl2 expression. In contrast, mild to moderate positive reactions for Bcl2 expression were detected in Ehrlich cotreated with 10 & 20 $\mu\text{g/ml}$ of CATH-1. Interestingly, in cotreated group with 40 $\mu\text{g/ml}$ of CATH-1 showed few positive cytoplasmic brownish reactions for Bcl2 between extensive negative cells. The immune-staining analysis was achieved and summarised in (figure 14), there was a significant increase of the antiapoptotic BCL-2 in solid tumors grown in mice in control group (65.00 ± 1.58) as compared to group treated with 10 $\mu\text{g/ml}$ of CATH (45.40 ± 1.12 , $P < 0.0001$), group with 20 $\mu\text{g/ml}$ of CATH (32.60 ± 1.12 , $P < 0.0001$), and group treated with 40 $\mu\text{g/ml}$ of CATH (11.40 ± 0.87 , $P < 0.0001$). Interestingly, high dose of cathelicidin 20 & 40 $\mu\text{g/ml}$ revealed significant decrease of the BCL2 immuno-staining area percentage % for solid tumors as compared to control group ($P < 0.0001$, $P < 0.0001$, respectively).

Discussion

Various issues are prevalent with respect to cancer management. The first issue is the side-effects of the treatment methods used for cancer management. Cancer cells are subjected to cytotoxicity through several treatment strategies like radiation therapy, chemotherapy, or chemo radiotherapy (combination of the radiation and chemotherapy) (1,37-38). Besides these, cancer therapy may involve using RTK or kinase inhibitors or other specific inhibitors (available as small organic molecules or monoclonal antibodies) (39-41). Despite the effective treatment of various cancer types through these interventions, the non-specific mechanisms of these interventions result in side effects and delayed neurotoxicity.

Resistance is the next issue faced during cancer therapy. Various factors may be attributed to resistance development. The main problem with traditional anti-cancer agents is that these are mainly focused on inhibiting cancer cell growth with no focus on tumor penetration. Consequently, the growth-arrested hypoxic cells inside tumors show a decline in their sensitivity (42). Hence, innovative and more effective interventions are needed to be developed for cancer treatment. Various new cancer treatment targets have been recognized in numerous studies; these include mitochondria (43), anti-angiogenesis (44) and hybrid tubulin-targeting compounds (45). It is also possible to take cancer treatment to new heights by determining peptides that have anti-cancer properties and developing such peptides (46-48).

This study served as the pioneer in studying the anti-cancer role of chicken cathelicidin peptides against different cancer cell lines *in vitro* and *in vivo*. The mechanism of action of the interaction of cationic peptides with breast cancer cell line MCF-7 was also investigated. The current study considered 3 different concentrations of peptides at 3 different time intervals to gain an insight into the anticancer impact of these peptides on breast and colon cancer cell lines MCF-7 and HCT116, respectively. It was found during the study that exposure of cells to higher concentration of these peptides for 24 h led to about 95% inhibition of cell growth. At another time interval of 48h of treatment, the treated cells showed lesser inhibition of cell growth; however, despite this reduction, there was adequate level of killing efficacy when the cells were treated with high concentrations of cathelicidin-1 and cathelicidin -3 peptides. These outcomes correspond to the outcomes of other studies that suggest that cytotoxic character of LL-37 and its fragments is evident in different types of cancer cell (1,49-50).

It is important to consider that when the cells were subjected to incubation with chicken cathelicidin-1 for 72h, the breast and colon cancer cell lines did not show any growth. Similarly, cells subjected to high concentration of cathelicidin-2 and -3 peptides also showed negligible growth of 7% and 16% accordingly. As per the outcomes, chicken cathelicidin-1 peptides outperformed both the other peptides in all 3 concentrations and all 3 time intervals in terms of its anticancer activity when treating both breast and colon cancer cell lines. The outcomes of our study showed correspondence with other works that indicate the contribution of human cathelicidin antimicrobial peptide LL-37 during carcinogenesis. Anticancer properties of the Cathelicidin LL-37 gene in humans and its fragments and counterparts are evident in various cancer cell lines (1).

Various research works revealed therapeutic significance of a combination of human cathelicidin and oligo of Cytosine-phosphate-guanosine in terms of their antitumor properties during in-vivo experiments (50–100 µg/mL) (51). The combination was also discovered by Chuang et al. to stimulate activation and division of NK (natural killer) cells; this causes more DNA translocation and consequently greater potential anticancer efficacy besides promoting the production of interferon-α in plasmacytoid dendritic cells. Hence, human cathelicidin plays a crucial role in the therapy of ovarian cancer by acting as a target as well as a candidate (1). According to Lee et al. (52), both bacteria and tumor cell cultures depicted higher cytotoxicity of the chicken Nk-lysin peptides and their derivatives following the incubation.

The role of high Tumor Necrosis Factor TNF expression in modulation of immune system was highlighted by Parvy et al. (2). He discovered that when some of the tumor cells are treated, the phosphatidylserine (PS) develops sensitivity to the effects of tracheal and fat tissues secretions namely the *Drosophila diptericin* defensin; this exposure is mediated by TNF. *Drosophila diptericin* defensin stimulates cell death as well as regression of tumor by binding tumor cells in PS-enriched regions. Moreover, in vitro examinations showed stimulation of death of prostate and renal cancer cells with the expression of human beta-defensin-1 (53-54). It was also found in other studies that the patients with prostate, lung or colorectal cancers showed reduced survival rate when there was prevalent deletion of human defensin gene (55). Another study pertaining to this subject of beetle defensin revealed the anticancer effect of a combination of this gene with synthetic peptides on certain cancers (56). Peng et

al.(17-18) concluded that chicken cathelicidin-2 and -B1 have both antibacterial and immunomodulatory roles when they incubated with chicken macrophages cell line infected by pathogenic *E. coli* through neutralisation of certain bacterium and decreases inflammation response.

Our study revealed that chicken cathelicidin peptides can indirectly lead to death of cancer cells. The mechanism of action involves the action of these peptides on breast cancer cells to down regulate certain genes essential for G1/S phase transient and S/G2 phase in these cells which consequently causes “prometaphase arrest”; thus, inhibiting the cell growth and cell division and ultimately leading to death of the breast cancer cell. Similar results were found for 2-Methoxyestradiol that inhibited cancer cell growth by stimulating G2/M cell cycle arrest in MCF-7 cell line as well as MDA-MB-435s cell lines, consequently enhancing apoptotic pathways due to its possible interaction with cytoskeleton protein fibers particularly with microtubules (57-63).

Apoptotic cell death may be caused because of binding of microtubule inhibitors with tubulins or microtubules followed with suppression of microtubule dynamics and activities. This stimulates G2/M cell cycle arrest and ultimately causes apoptotic cell death (64-69). A study conducted by Wêsierska-G et al. (70) also depicted the same mode of action; he discovered that the G/M cell cycle arrest caused by roscovitine (CDK inhibitor) inhibited the cell division in MCF-7 cells while S/G phase transient was prevented by olomoucine (another CDK inhibitor).

This current study took 3 different chicken cathelicidin peptides with the intention to evaluate their efficacy in the treatment of breast cancer cells by stimulation of pro-apoptotic pathway and augmentation of the anti-proliferative activity. The study showed two- and three-times higher expression of the caspase-3, and -7 genes respectively in MCF-7 cells treated with chicken peptides (especially cathelicidin-2 and -3) relative to untreated cells.

The caspase-3 and -7 are executioner proteases. These findings corresponded to the findings of various other research works that suggested the stimulating effect of 20-40 μ M of human cathelicidins on caspase proteases pathways and elicited autophagy and apoptosis in case of colon cancer as indicated by (1,71-72). The testing of most of the human breast tumors revealed deficiency of caspase-3 gene expression. Even the apparently normal breast parenchyma of patients with breast cancer showed loss of expression of caspase-3 gene. Caspase-3 belongs to cysteine protease family which is recognized for its contribution in apoptosis execution. Moreover, it was discovered that chemotherapeutic agents, irradiation, and cytokines as well as other apoptotic stimuli can activate caspase-3 (73).

On the other hand, cell death was inhibited because of selective inhibition of caspase-3 gene (74-75). Hence, it may be concluded that breast cancer cells are likely to become resistant to the interventions like radiation therapy and chemotherapy due to lower expression of caspase-3 gene. This explains the insensitivity of the MCF-7 cells with low expression of caspase-3 gene to cisplatin, doxorubicin, and etoposide; this low expression may be attributed to functional deletion mutation in the caspase-3 gene. But the sensitivity to the mentioned medications and apoptotic stimuli can be restored by reconstituting the MCF-7 cells with caspase-3 gene (Figure 6) (76).

On the contrary, morphological apoptosis was seen in MCF-7 cells as they respond to various agents even in the absence of caspase-3 expression (77). This indicates that these agents have the property to stimulate caspase-independent cell death (as through AIF; 78) or caspase-dependent apoptosis through alternative downstream caspases, including caspase-6 and -7 (79).

The outcomes of our study indicated that before the treatment of MCF7 cells with peptides, the membranes of these cells were not disrupted; however, when the breast cancer cells were subjected to chicken peptide of 50 µg/ml concentration, the cell membranes were found to be disrupted which is evident from comparatively higher stain permeability than the untreated cells and extreme alteration and depolarization in cell morphology of the treated cells. Moreover, the shape of the mitochondria in treated cells is also elongated due to the effect of peptides on mitochondrial membrane. The nucleus of the treated cells undergoes fragmentation and is also smaller in size than in untreated cells.

The classical way of cancer's treatment from chemo-radio-therapies to surgical interference have significantly evolved which brings a great step to reduce the morbidity and less mortality caused by cancers. But it does not prevent inconvenience or undesirable effects. Nonetheless, new therapies have rapidly appeared such as immunotherapy which is classified as crucial alternative in various cases (80). Further, this therapy could impressively adapt with new faced challenges via enhancing new antitumor molecules which reinforcing the body's immune functions. A large spectrum of immunotherapies against cancer proved them efficient in several patients via exploring checkpoint blocker, monoclonal antibodies, vaccines, and immune cells-based therapies (81).

Recently, new studies are focused on finding out a novel and more accurate approaches to further develop the therapeutic strategies for cancers. Between the Recent used tools, the Antimicrobial peptides (AMPs) which partially belong to innate immune system in several species. Despite the fact that AMPs already characterized and evaluated as potential anti-infectious element for numerous infectious disorders, its remarkably noted that they mightily defined as an anti-cancer peptide (ACPs) directly in treatments or combined with further drugs as strategy to beat cancers (82-83).

Cathelicidin peptides possessed a large spectrum of functionality, including a direct antibiotic effects verses fungus, bacteria, parasites virus and microorganisms. Thus, they can deeply modulate the immune or inflammatory reaction. Further, they demonstrated a chemotactic function in neutrophils, monocytes, and T lymphatic cells by stimulating the damaged vascular or in re-epithelization of injured skin. And further, they are implicated in beating cancer as antitumor factors (84) and they possess the ability to interact with different effector of immune cells. These cells include monocytes, macrophages, dendritic cells, lymphocytes, epithelial cells, and neutrophils. Many of these functions are still not fully defined, but a range of different receptors are involved. These receptors include FPR2 (also known as FPRL-1), CXCR2, and P2X7R (Barlow et al. 2014). The helical cathelicidin peptides have received much attention as a potential therapeutic agent. Herein, the objective was to investigate the role of chicken cathelicidin in Ehrlich ascites cell (EAC) suppression as a tumor model after subcutaneous implantation

in mice. In addition, through this research work we would like to evaluate the therapeutic effect of chicken cathelicidin on the innate and adaptive antitumor immunity.

The INF- γ and TNF α population were measured in the serum of all study animal using flow cytometric technique. Notwithstanding, Interferon- γ (INF- γ) is ranged as pleiotropic cytokine which is principally expressed via cytotoxic T lymphatic cells and natural killer (NK) cells. It could activate several pathways to inhibit the tumor growth (85). Tumor necrosis factor alpha (TNF α) which has a role for variety of signaling events inside the cells and has demonstrated that during acute inflammation, macrophage and monocytes produce the inflammatory cytokines. TNF α is important for infection resistance and cancer and it exerts many of its action effects by binding to TNF receptor (TNFR) and this mechanism will cause necrosis or apoptosis (86).

Our data showed that chicken cathelicidin (CATH-1) enhance release of TNF α and INF- γ in treated mice groups and this finding in line with previous studies demonstrated that coadministration of CPG oligodeoxynucleotides with cathelicidin peptides result in enhanced activation of NK cells against ovarian cancer (87-88). This observation could be attributed to the effect of cathelicidin in stimulating of the innate immune system cells to release INF- γ and TNF α which eradicate cancer cells.

We assessed the effect of cathelicidin in the expression of granzymes as determined by RT-qPCR. Chicken cathelicidin-1 increased expression of granzyme K involved the induction of apoptosis. This partly explained that cathelicidin induce cytotoxic T cells and natural killer (NK) cells to release the pore-forming perforin together with a variety of granule-associated proteases including granzymes that mediate apoptosis via a cleavage of caspase-3 which result in DNA fragmentation (89) and this finding in line with previous study that demonstrated that cathelicidin induced leakage of granzymes from cytolytic granules in CD57BL/6 lymphocytes of mice treatment with LL-37(90). In parallel, the tumor size in untreated EAC-bearing group was significantly large and elevated. However, the volume of this tumor reduced in the treated EAC-bearing groups with chicken cathelicidin. Our findings are compatible with previous studies which showed that the intravenous administration of cathelicidin considerably mitigated the colonic tumor size in azoxymethane-and dextran sulfate-treated mice (91). Additionally, animals received high dose of cathelicidin-1 (40 μ g/ml) displayed an apical survival rate compared to untreated carcinoma control and animals which received low dose of cathelicidin (10 and 20 μ g/ml).

These results were supported by the results of histopathological examination of cancer tissues. Tumor of groups treated with chicken cathelicidin displayed high area of necrosis. Contrary, cancer section of untreated EAC-bearing mice showed high- grade malignant growth with minimal necrotic areas, and widely features of cancer cell proliferation. Moreover, the histological examination, immunohistochemical staining with Bcl2 marker revealed that the tumor section in Ehrlich solid tumor in untreated control exhibited a strong positive reaction for Bcl2 expression. In contrast, mild to moderate positive reactions for Bcl2 expression were detected in mice treated with 10 & 20 μ g/ml of cathelicidin. Interestingly, few positive reactions for Bcl2 between extensive negative cells were observed in mice taken 40 μ g/ml of cathelicidin. Similar findings were observed in a study showed apoptogenic effect of cathelicidin in colon

cancer cells (HCT116) and Jurkat human T leukemia cells via upregulation of Bax and Bak and downregulation of Bcl2 which confirmed by TUNEL assay and Annexin V/PI staining (50,92).

In the current study, solid tumors grown in mice treated with high dose of the Cathelicidin (40 µg/ml) exhibited significant increase of the necrosis area percentage and significant decrease of the BCL-2 immunostaining with significant decrease of the total histological scoring of the tumor mass. These results support the idea that the high dose of the chicken cathelicidin-1 has antitumor effect against solid tumors in mice. Our results provided further evidence of the potential role of cathelicidin as anti-cancer therapy and clearly increased apoptosis levels in EAC-bearing mice bearing. Our findings in line with previous studies that demonstrate that LL-37 can apply the anti-cancer effects and mediate apoptosis in several types of cancer including gastric cancer , hematologic malignancy, and colon cancer (93,94).

The different exploited experiments in this study demonstrated that variations in the cytotoxic nature of LL-37 may potentially related to peptide-mediated augmentation of innate immunity. These in vivo results showed that chicken cathelicidin exerted anti-proliferative and anti-cancer cytotoxicity verses cancer cells. The anti-tumor cytotoxicity of chicken cathelicidin was correlated with an upgraded survival of EAC-bearing mice, reducing the tumor size and induce apoptosis of cancer cells. Those finding are in consonance with these reached via numerous anticipants' research which showed that cathelicidin play the role of inhibitor against cancer cell proliferation. According to our observation, we can state that chicken cathelicidin induced a potential cytotoxic anti-tumor, and anti-proliferative effects verses cancer cells in EAC-bearing mice via enhancing antitumor immunity.

Materials And Methods

The experiments were carried out in multiples of three and were repeated at three different times. Negative controls in all experiments for determining the impact of chicken cathelidins peptides were cancer cells lines cultivated in their absence. The study protocol was approved by the Ethics Committee of King Abdulaziz University (Reference No 325-19). This study was conducted based on the health guidelines for completing animal experiments. Ether was used for euthanasia and anesthesia.

Peptides

The synthesis of mature peptides of chicken cathelidins peptides were carried out by (Peptide 2.0, USA) <http://www.peptide2.com>. HPLC was used to purify the three peptides up to 95%, and it was shown in mass spectrometry analysis, sequences of amino acid of these mature peptides are as shown in Table 1.

Invitro experiments

Cell culture and treatment

The breast (MCF-7) and colon cancer (HCT116) cells cell line were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained at 37°C in a 1:1 mixture of Dulbecco's modified Eagle's medium DMEM high glucose (4.5 g/L) supplemented with 10% FBS in Corning®T75 flasks

containing 1.2 g/l sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA), 2.5 mM L-glutamine (Invitrogen Life Technologies), 15 mM HEPES, and 0.5 mM sodium pyruvate supplemented with 400 ng/ml hydrocortisone (Sigma-Aldrich) and 10% fetal bovine serum (PAA Laboratories, Pasching, Austria) in a humidity incubator with 5% CO₂ and 95% air. The culture medium was refreshed every 2–3 days for a period of 5–7 days to allow for recovery from cryopreservation and for confluency to be reached. For cell subculturing, the cells were digested with 0.25% trypsin and 0.03% EDTA solution (Invitrogen Life Technologies). Upon reaching 90% confluency, cell numbers were counted. The density of cells was 5×10^3 cells/cm². After that, the MCF-7 cells were seeded into three 96 well plates and treated with three chicken cathelicidins peptides for three period of incubations (24h-48h-72h) (95).

Cell viability assay

To assess the altered cell viability, Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) assay was performed. Briefly, the cells were seeded in 96-well plates at 5×10^3 cells/well containing 180 µl of medium and cultured for up to 72 h. At the end of each experiment, 5 µl of CCK-8 solution (5 mg/ml) was added into each well, and the cells were incubated for 2 h at 37°C. The optical density value was measured by using BioTek microplate reader (Dynatech Laboratories Inc., Chantilly, VA, USA) at 450 nm. The median inhibition concentration (IC₅₀) values were calculated using the probity model, and the inhibition rate of cell proliferation was calculated as: inhibition rate (%) = $1 - A_{450}(\text{test})/A_{450}(\text{control}) \times 100\%$. Data were calculated from three independent experiments, each performed in triplicate (95).

RNA Isolation

Total RNA was isolated from MCF-7 cells after chicken cathelicidins treatment using e EZ RNA Clean Up Plus DNase Kit (EZ BioResearch, St Louis, MO, USA)) following the manufacturer's instructions. RT-PCR was performed by using 1 µg of total RNA samples in the Access RT-PCR System (Bioneer Corporation Co., Ltd, South Korea) under the following conditions: first-strand DNA was synthesized with 12 cycles at 37°C for 10 sec, 48°C for 4 min and 60°C for 30 sec and finally, heat inactivation at 95°C for 5 mins then denatured at 94°C for 5 min for the first cycle but for 30 sec for the additional 35 cycles; annealing according to Table. 2 for 30 sec and extension at 72°C for 2 min; and a final extension at 72°C for 10 min(95). The PCR products were then subjected to electrophoresis in a 1.2% agarose gel and stained with ethidium bromide (26).

For RT-qPCR, cDNA was synthesized by using 0.5 µg of total RNA with a SuperScriptIII Cells Direct cDNA Synthesis kit (Bioneer, Inc, Daejeon, Republic of Korea). The levels of cyclin D1, cyclin A1, caspase-3 and 7 mRNA were amplified in triplicate using the SYBR-Green Real-time PCR master mix (Biotool LLC, Houston, TX, USA) on a LightCycler®480 Real-Time PCR system (Roche, Basel, Switzerland). The level of glyceraldehyde-3-phosphate dehydrogenase GAPDH mRNA was used as an internal control in all the experiments. The primer sequences are listed in [Table I](#). The qPCR program was set to an initial denaturation at 94°C for 2 min; then 40 cycles of denaturation at 94°C for 10 sec, annealing at 60°C for

15 sec, and extension at 72°C for 30 sec; and a final extension at 72°C for 5 min. The relative levels of gene expression were quantified by using the comparative CT method of $^{-\Delta\Delta C_t}$ (96).

Cancer cell Membrane Damage with Fluorescent Microscopy

MCF-7 cells changes after treatment of chicken cathelicidins peptides was determined by incubating 5×10^3 cells/well. Fluorescent microscopy (Nikon, Carl Zeiss, Oberkochen, Germany) was used to observe the membranes and cell contents changes merely after stained with the DAPI and PI for 1 hour, after which 10 μ l of stained solution was added to the glass slide in dark area. This was then covered with over slip to observe the damaged Mcf7 cells membranes after synthetic Nk-lysin peptides were used to treat them.

***In vivo* experiments**

The Ehrlich tumor cell inoculation

The Ehrlich ascites cells (EAC) was supplied by Faculty of Pharmacy of male in King Abdulaziz University, Jeddah. The transplantation of EAC cells in the mice was implemented based on the technique adopted by (97). Ascitic fluid from EAC-bearing stock mice containing 8-10 days of ascitic tumor was obtained from the EAC cells. In order to cause carcinoma in mice for the experimental study, 9.9 ml of saline (dilution 1:50) was applied to 0.1 ml of EAC aliquot aspirated from stock mice and 0.1 ml of this diluted EAC (1×10^6 cells) was subcutaneously injected into the back of 28 mice for the experimental study.

***In vivo* treatment**

A total number of 35 male BALB/c mice were randomized divided into five groups ($n=7$ /each) as follows: the first group as negative control, the second group was the positive control with mice bearing carcinoma and finally three groups with mice bearing carcinoma and treated with three different dose of chicken cathelicidin-1 of 10,20 and 40 μ g/ml. All Administration was given after two days of tumor implantation and for three times a week. The administration injected was intravenously (IV) and locally (at tumor site) simultaneously and the doses determined according to the mice body weight.

Tumor Volume Measurement

Tumor volume was measured by caliper digital after six days of tumor induction in mice groups and every two days. The volume of tumor was calculated using the following formula : $V_T = L \times W^2 \times 0.52$; where W is tumor width and L is tumor length. In addition, the tumor size of five mice in each carcinoma groups were measured at second and third week via ultrasounds device (Vevo 2100 Imaging System, Canada) with the following equation:

$V_T = W \times L \times H \times 0.523$; where W is tumor width, L is tumor length, and H is tumor Height.

Measurement of INF- γ and TNF α by flow cytometry

Blood samples from each mouse were collected from the eyes by Sino-orbital puncture of mice using micro-capillary tubes. Blood samples were stored in clean and dry test tubes that contained ethylene diamine tetra acetic acid (EDTA). Red blood lysed with 1 ml of 1x BD FACS™ lysing solution (BD Bioscience, USA) for 10 minutes at room temperature and centrifuge at 500 x g for 5 minutes, discard the supernatant and re-suspend the pellet in 3 ml of PBS. We performed cell count and viability analysis with hemocytometer. Cells were transferred to FACS tubes (BD Bioscience, USA) and performed surface staining with 5 µl of mouse mAbs that are specific for mice epitopes: PerCP anti- CD3 (BD Bioscience, USA) and FITC anti-CD56 (Abcam, UK) for 30 minutes in the dark at room temperature, following fixed the cells with 100 µl of fixation buffer and permeabilized with 100 µl of Intracellular staining perm buffer. We performed intracellular staining with 5 µl of APC anti-TNFα (BD Bioscience, USA) and PE anti- INF-g (BD Bioscience, USA) for 30 minutes in the dark at room temperature. Flow cytometry was executed on an LSR III (BD Biosciences, USA) using Diva[®] software.

Animal Survival

Mice were euthanized after three weeks of treatment. The animals were monitored daily upon 21th day of tumor induction for signs of mortality. At the end of the experiment, the number of animals living in each group was determined and the survival percentage in each group was compared.

RNA extraction, cDNA synthesis and qPCR Analysis

Total cellular RNA was isolated from blood using GENEJET RNA purification kit (Invitrogen, USA) according to manufacturer's instructions(26). Total RNA concentration and purity were evaluated by measuring absorbance at 260 and 280 nm respectively, in a DeNovix DS-11 ä microvolume spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA). First strand supplementary DNA (cDNA) was synthesized from 1 µg of total RNA sample using the first strand cDNA synthesis kit (Promega, USA) transcription system according to the manufacturer's conventions. Each reverse transcriptase (RT) reaction was incubated in thermal cycler (Applied Biosystem, USA) for 5 mins at 25 °C, 60 mins at 42 °C, 15 mins at 70 °C, and finally was kept on hold at 4 °C. The resulted cDNA samples were then stored at - 20 °C. Real-time polymerase chain reaction (PCR) amplification and analysis were performed in an optical 48-well plates in Real-Time PCR Detection System (Applied Biosystem, USA) using SYBR[®] Green Master Mix (Qiagen, Germany). The intensification protocol comprised of 40 cycles (denaturation at 95 °C for 15s, annealing at 60 °C for 30s, and extension at 72 °C for 30s). The primers used for amplification of mouse granzyme B, mouse granzyme K and mouse Glyceraldehyde 3-phosphate dehydrogenase (GADPH) as housekeeping gene to normalize the expression data were depicted in Table 2 (98). Results were calculated by the difference between Ct value of gene of interest and Ct value of housekeeping gene. While $\Delta\Delta Ct$ was obtained by the difference between Ct value of treated and control groups. Finally, $2^{-\Delta\Delta Ct}$ was determined to represent the fold expression difference of gene of interest, between treated and control groups. $2^{-\Delta\Delta Ct} > 1$ indicates that gene expression in treated groups is higher than that in control groups(96).

Histological analysis

Mice were anaesthetized by ether and scarified at the end of the experiment on day 21. Cancer tissues were extracted and fixed in 10 % formalin and sliced into 5 µm thick sections. Then washed, dehydrated, cleared, and embedded in paraffin wax. Deparaffinized sections through incubated slides at 65 °C for 30 minutes and immersed slides in xylene, two changes of 10 minutes each. Then rehydrated through immersed slides in two changes of 100 % ethanol, 5 minutes each. Immersed slides in 95 % ethanol for two minutes, 70 % ethanol for two minutes, 50 % ethanol for two minutes. Rinsed slides in PBS for five minutes. Stained with 200 µl hematoxylin solution and incubated at room temperature for 5 minutes. Washed in distilled water and rinsed in PBS for 5 minutes. Stained with 400 µl of eosin solution for 30 seconds and washed with distilled water. Rinsed slides in PBS for 5 minutes. Dehydrated through 95 % of ethanol, two changes of 5 minutes each and cleared in two change of xylene, 5 minutes each. Mount and prepared the slides for light microscopic examination.

Immunohistochemistry analysis

The paraffin blocks of cancer tissues sliced into 5 µm thick sections and placed on positive charged glass slides, then incubated at 56 °C overnight, de-paraffinized in xylene, and placed for 10 minutes in room temperature. The excess liquid was drained, and the slides were then transferred to different concentrations of ethanol (100 %, 90 % then 70 %). Slides were immersed in distilled water for one minute, followed by PBS for 5 minutes. Slides were immersed in 3 % hydrogen peroxide for 5 minutes, followed by washing in PBS for 5 minutes. Ultra V block was applied and incubated for 5 minutes at room temperature to block nonspecific background staining. Slides were washed in PBS for 5 minutes. The anti-Bcl2 was applied to cover the tissue sections and the slides were kept in 37 °C for overnight. Slides were then immersed in PBS for 10-15 minutes. The slides were completely covered with biotinylated goat anti-polyvalent for 10 minutes at room temperature, immersed in PBS for 5 minutes and then completely covered with streptavidin peroxidase for 10 minutes, at room temperature. Slides were then immersed in PBS for 5 minutes, the chromogen was prepared by adding 1-2 drops (40 µl) DAB plus chromogen to 1 ml of DAB plus substrate, mixed by swirling. Tissue were completely covered with the chromogen for 10 minutes, at room temperature, rinsed with distilled water and the excess fluid was blotted around the tissues with filter paper. Tissue sections were counterstained with Mayer's hematoxylin, ready to use, for 5 minutes, followed by rinsing of slides with tap water, for 10 minutes. Dehydration of sections in ascending concentrations of ethyl alcohol (70 %, 90 %, and 100 %) for 10 minutes each was done. The slides were immersed in xylene, mounted, and covered with cover slips.

Statistical analysis

All data are expressed as the mean \pm standard deviation. Data analyses were performed using GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA). Significance was determined using Student's t-test for two groups and one-way ANOVA for multiple comparisons. The Kaplan-Meier function was calculated for survival and a log-rank test was used to assess the differences of mice survival. $P \leq 0.05$ was considered to indicate statistically significant differences.

Conclusion

Our study concluded that cancer cell lines growth was significantly inhibited (around 85-95%) when treated with high concentration of chicken cathelicidins (40 µg/ml) at 72 h of treatment which proves to be a strong anti-cancer agent. It was found that the untreated MCF-7 cell show expression of both the cyclin A1 and cyclin D while cells treated with chicken cathelicidins peptides do not depict clear expression of these genes. The cells treated in this way may sometimes even depict cell cycle division arrest in the cell cycle phases of G1/S and G2/S (prometaphase arrest) consequently inhibiting the growth of breast cancer cell line and ultimately causing death of cancer cell. Our finding shows that cathelicidin-1 induced a detectable level of caspase-3 gene expression which was 3 times greater than the expression of this gene in untreated cells whereas, treatment with cathelicidin-2 and -3 induced two times greater gene expression. The treatment with cathelicidin-2 and -3 resulted in two-fold caspase-7 gene expression than the expression of this gene in untreated MCF-7 cells. However, treatment of cancer cells with cathelicidin-1 did not show any change in caspase-7 gene expression. Our data showed that chicken (CATH-1) enhance releasing of TNF α , INF- γ and upregulation of granzyme K in treated mice groups, in parallel, the tumor size and volume was reduced in the treated EAC-bearing groups after cathelicidin administration compared to untreated EAC-bearing group. Additionally, animals received high dose of cathelicidin-1 (40 µg/ml) displayed an apical survival rate compared to untreated carcinoma control and animals which received low dose of cathelicidin (10 and 20 µg/ml). Tumor of mice groups treated with chicken cathelicidin displayed high area of necrosis compared to untreated EAC-bearing mice. Based on histological analysis and immunohistochemical staining revealed that the tumor section in Ehrlich solid tumor exhibited a strong Bcl2 expression in untreated control compared to mice treated with 10 & 20 µg/ml of cathelicidin. Interestingly, low expression of Bcl2 were observed in mice taken 40 µg/ml of CATH-1. The outcomes of our study indicated that administration of chicken cathelicidin peptides reduced the tumor growth and induce necrosis of cancer cells *in vivo* via releasing TNF α and granules enzymes which mediate apoptosis pathways.

Declarations

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Tables

Table.1. The amino acids sequence of the three chicken cathelicidins peptides

Peptide name	Amino Acid Sequence	M.W
Cathelicidin-1	RVKRVWPLVIRTVIAGYNLYRAIKKK	3399.92
Cathelicidin-2	RFGRFLRKIRRFKPKVTITIQGSARF	3419.16
Cathelicidin-3	RVKRFWPLVPVAINTVAAGINLYKAIRRK	3568.07

Table. 2. The primers list for detection and characterization of cell cycle division genes and caspases executioner genes

Gene	Forward Primer (5' to 3')	Reverse primer (5' to 3')	Annealing temperature (°C)
Cyclin A-1	ACC CCA AGA GTG GAG TTG TG	GGA AGG CAT TTT CTG ATC CA	53
Cyclin D-1	GTG CTG CGA AGT GGA AAC C	ATC CAG GTG GCG ACG ATC T	57
Caspase-3	CAAAC TTTTTCAGAGGGGATCG	GCATACTGT TTTTCAGCATGGCAC	55
Caspase-7	TGAGCCACGGAGAAGAGAAT	TTTGCTTACTCCACGGTTCC	53
GAPDH	GTA TTG GGC GCC TG G TCACC	CG C TCC TGG AAG AT G GTG AT G G	60
mouse granzyme B	TCGACCCTACATGGCCTTAC	GCTGGGTCTTCTCCTGTTCT	60
mouse granzyme K	TGAGCCCATGAAGCAGACAT	TGGCATTTGGTCCCATCTCT	60
Mouse GAPDH	TGTTTGTGATGGGTGTGAACC	CATGAGCCCTTCCACAATGC	60

Figures

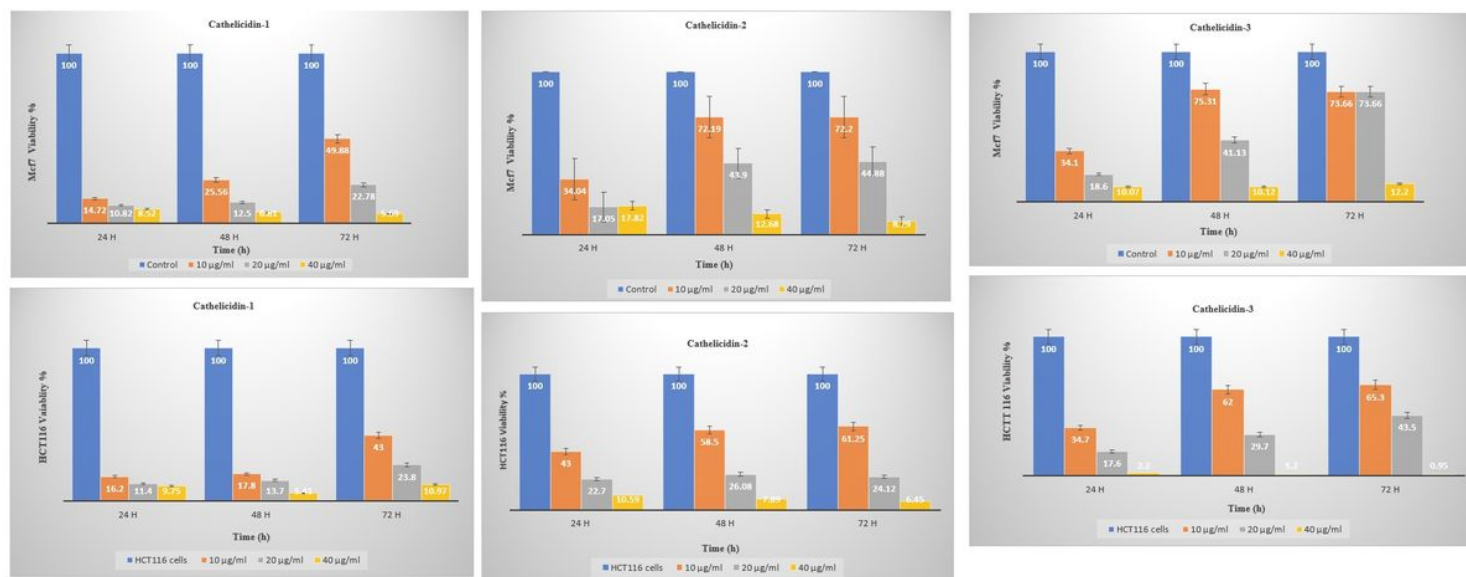


Figure 1

Anticancer activity of chicken cathelicidin peptides against breast cancer cells MCF-7 , colon cancer cell line (HCT116) and untreated cell (Control). Data presented as means (\pm SD) of three independent repeats in triplicate.

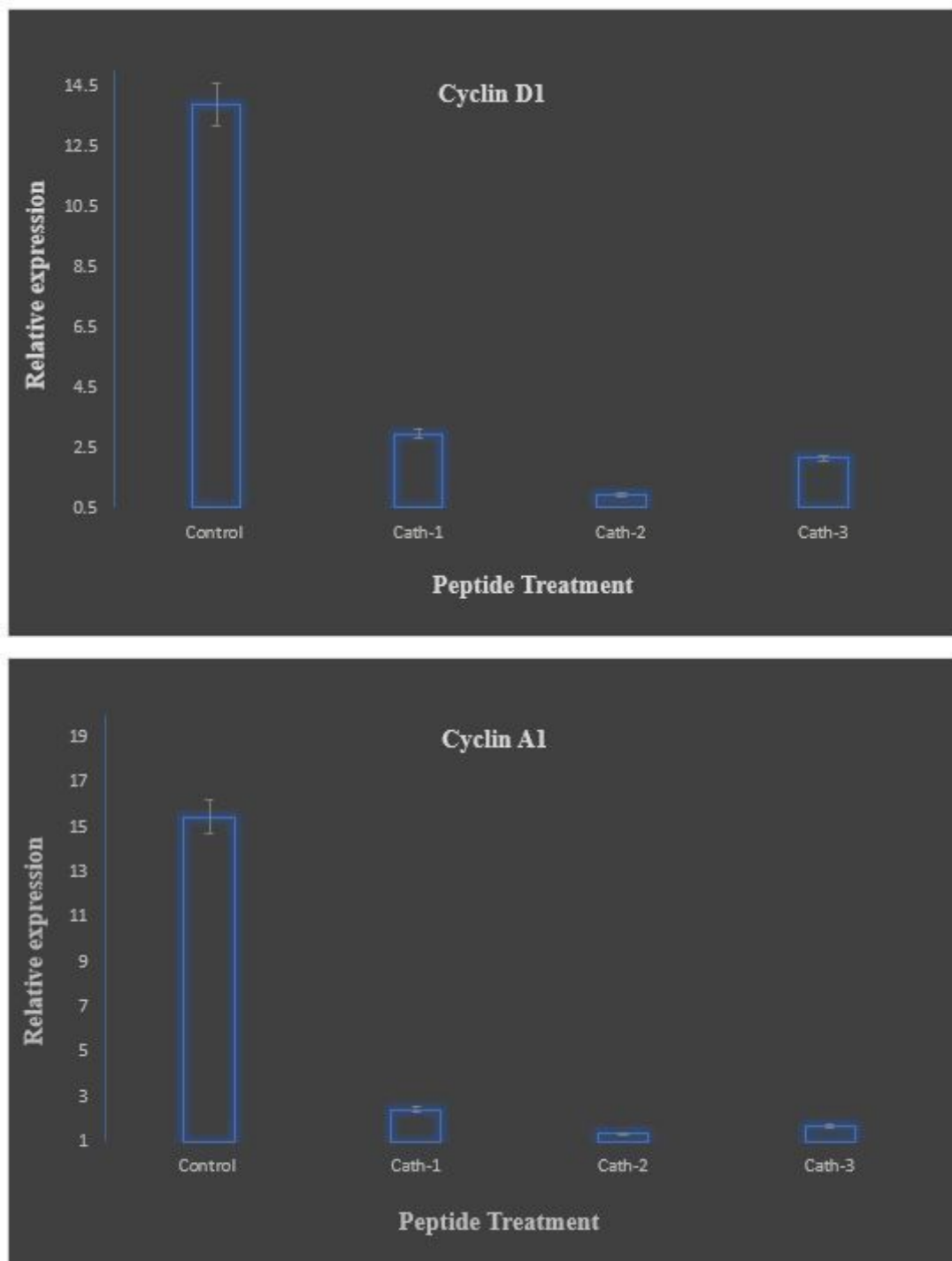


Figure 2

The expression patterns of Cyclin D1 and Cyclin A1 genes after challenge with chicken cathelicidin peptides and untreated cell(Control). The expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase as the reference gene. All assays were performed in 3 independent experiments, and each point is the mean \pm SD. The baseline is 1 or -1, since the range is defined to be between +X and +1 for the up-regulated genes, and between -1 and -X for the down-regulated genes.

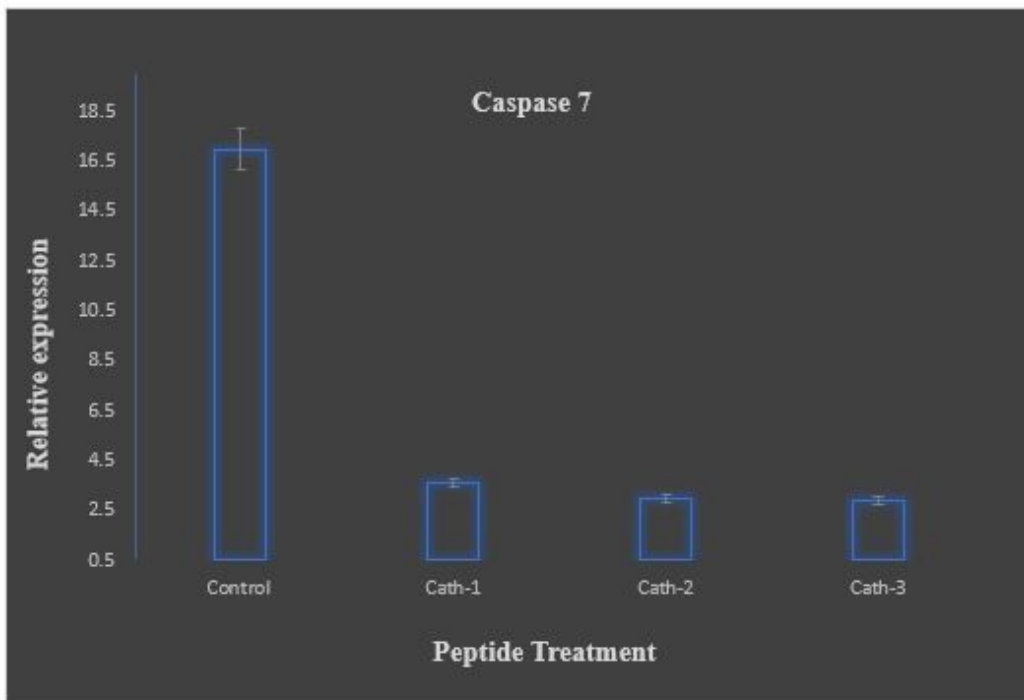
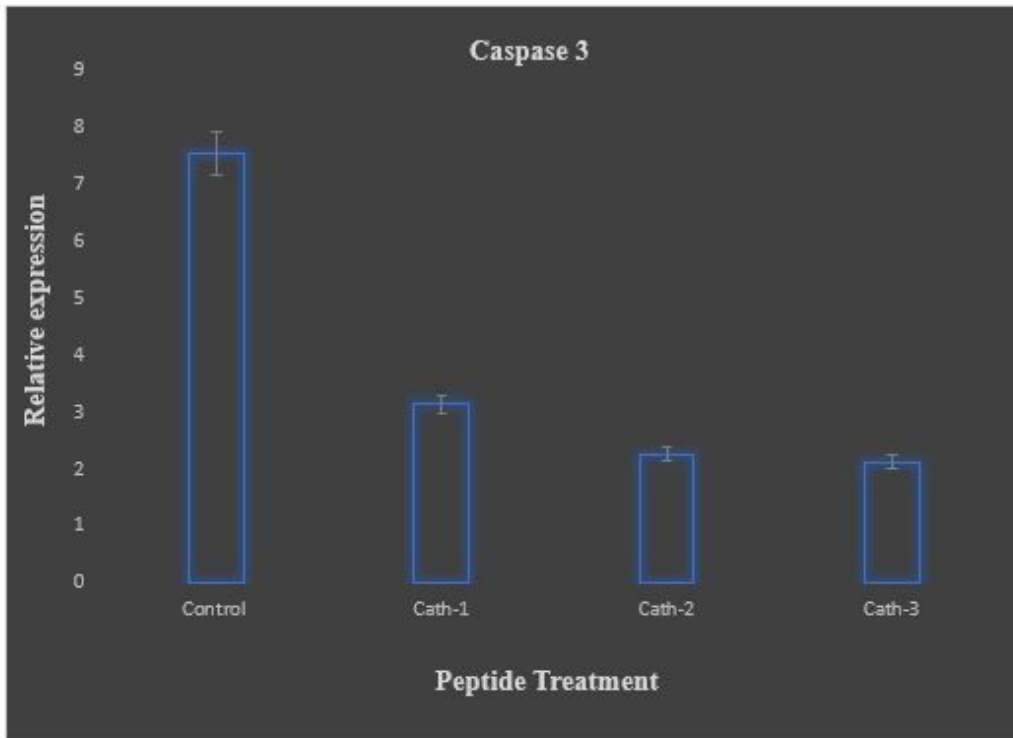
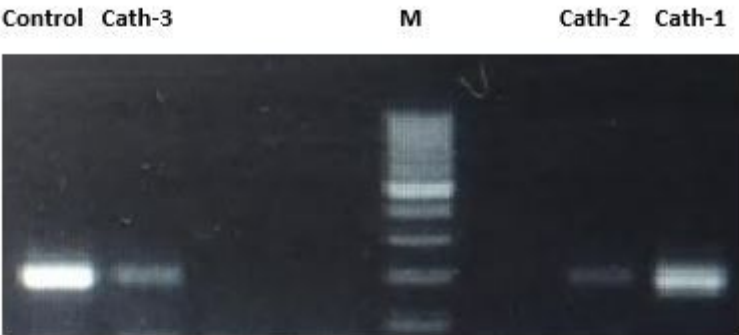


Figure 3

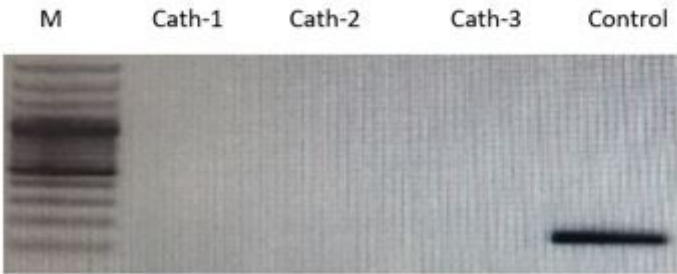
. The expression patterns of casepase-3 and -7 genes and Cyclin A1 genes in MCF-7 after challenge with chicken cathelicidin peptides and untreated cell(Control). The expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase as the reference gene. All assays were performed in 3 independent experiments, and each point is the mean \pm SD. The baseline is 1 or -1, since the range is

defined to be between +X and +1 for the up-regulated genes, and between -1 and -X for the down-regulated genes.

GAPDH



Cyclin D1



Cyclin A1



Caspase 3

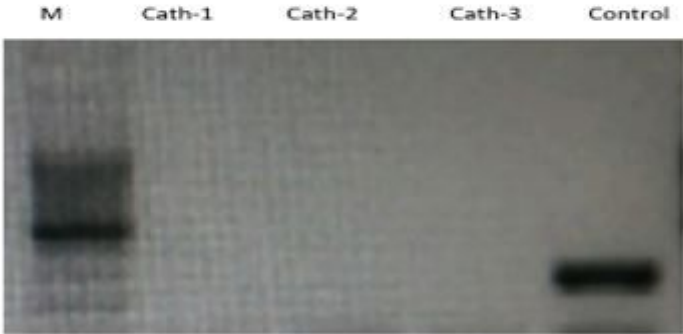
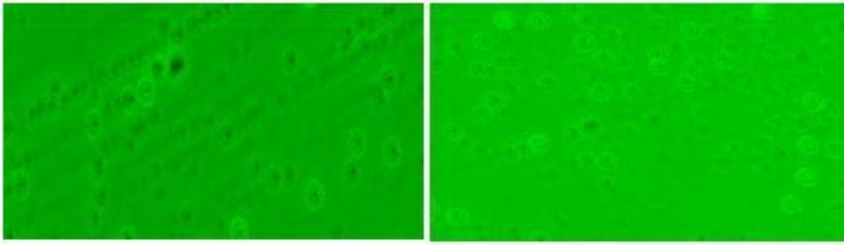


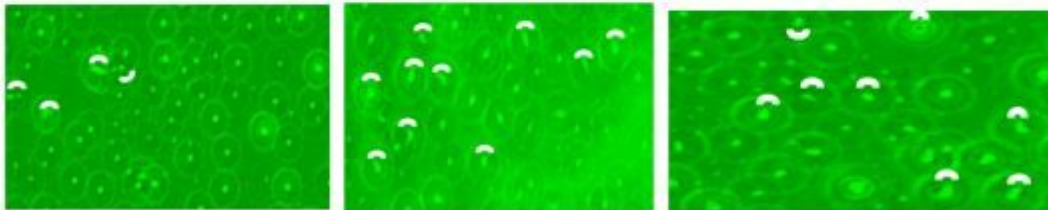
Figure 4

The gel electrophoretic images of amplified genes of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene , Cyclin D1, Cyclin A1 and casdpase-3 after challenge with chicken cathelicidin peptides and untreated cell(Control).

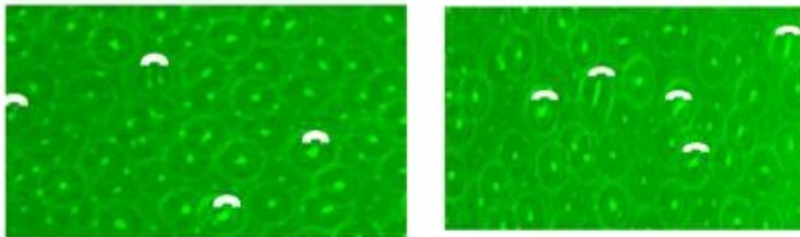
Untreated MCF-7 cells



Treated MCF-7 cells with Cath-1



Treated MCF-7 cells with Cath-2



Treated MCF-7 cells with Cath-3

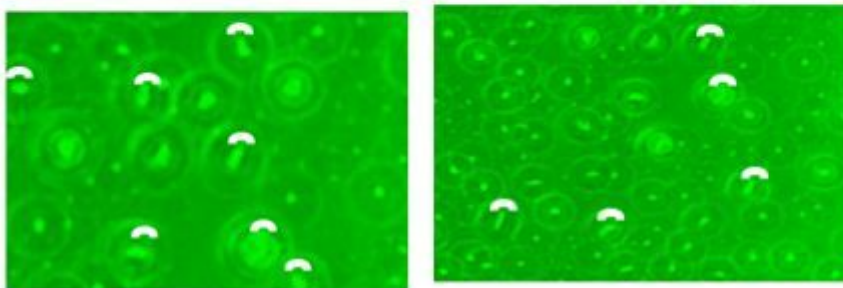
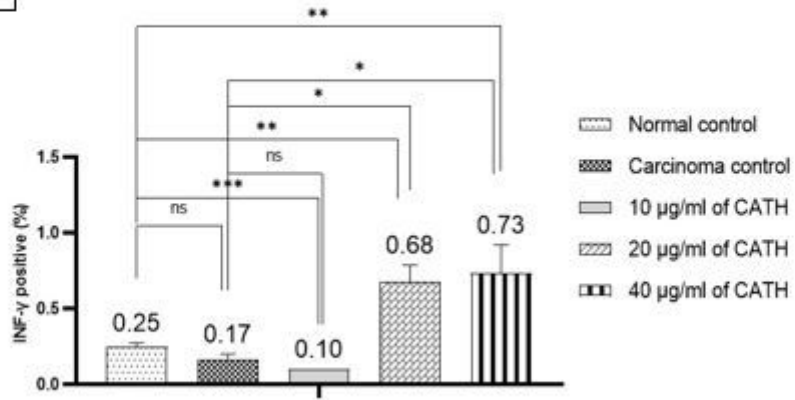
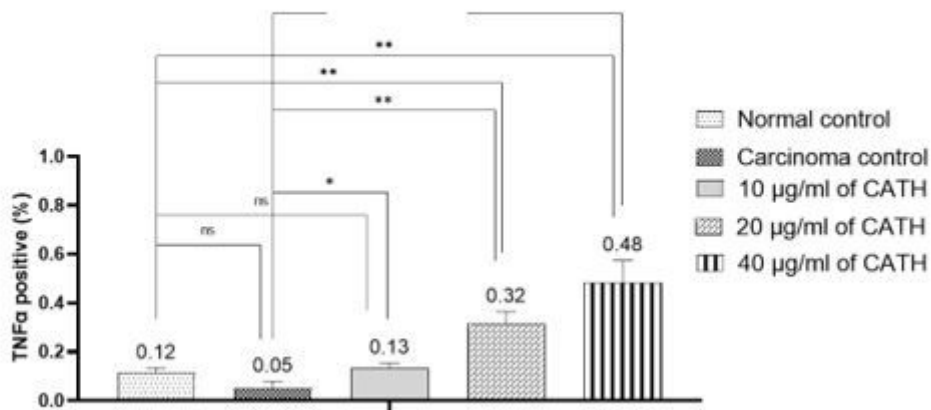


Figure 5

The cell membrane damage, prometaphase phase arrest, apoptotic cell death of breast cancer cells MCF-7 after treatment with chicken cathelicidin peptides and untreated cell (Control).

A**B****Figure 6**

Flow cytometric analysis of INF-γ and TNFα population percent of mice groups.). Values are given as mean ± SD; n = 7 per group. ns: non-significant, *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001 compared to control groups).

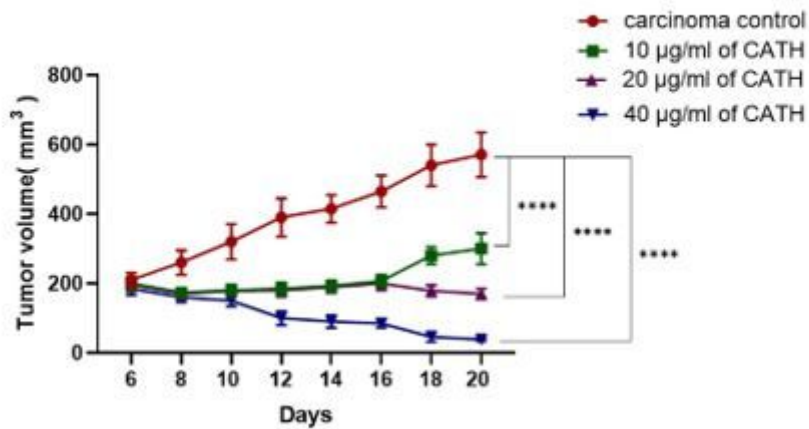


Figure 7

Tumor volume of EAC-bearing mice groups. Values are given as mean \pm SD; n = 7 per group. (****p \leq 0.0001 compared to untreated carcinoma control groups).

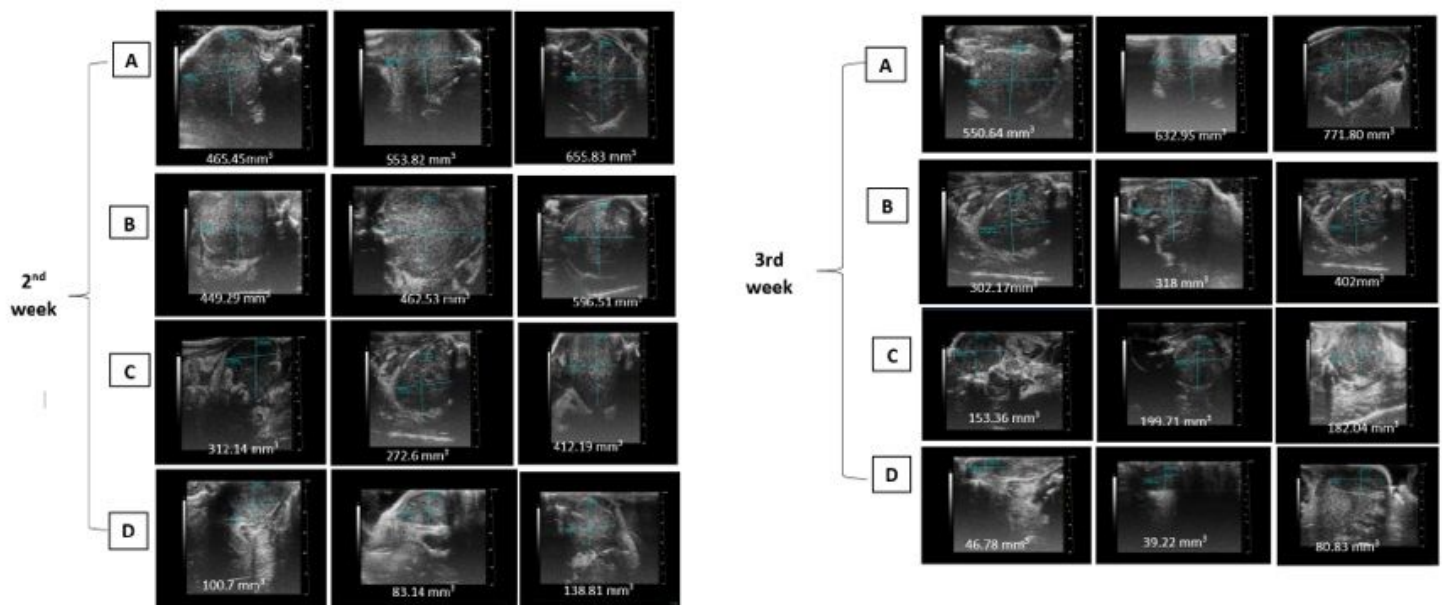


Figure 8

ultrasonography of tumor size in tumor mice groups at second and third week. A: untreated carcinoma mice, B: treated mice with 10 µg/ml of CATH-1, C: treated mice with 20 µg/ml of CATH-1, D: treated mice with 40 µg/ml of CATH-1.

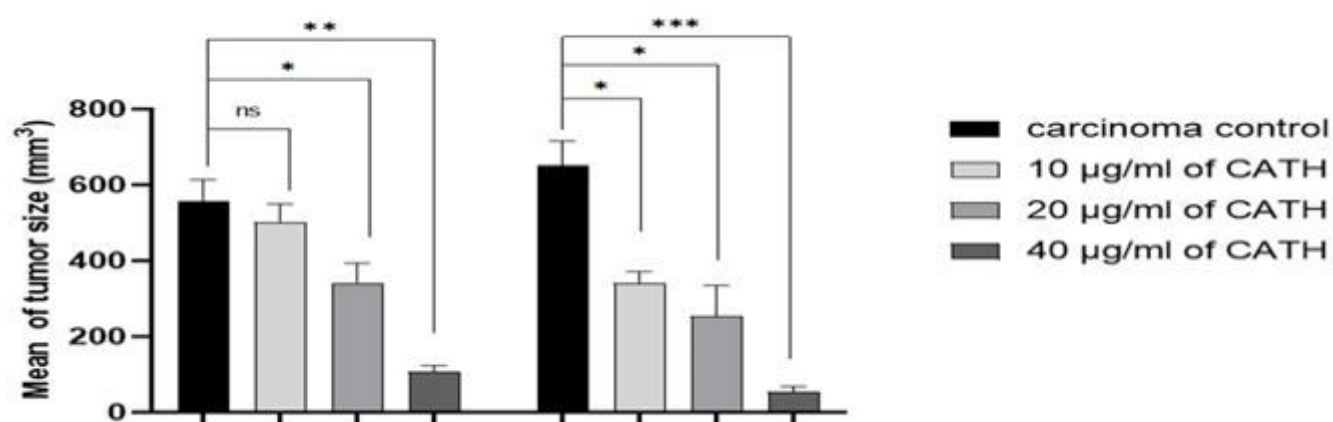


Figure 9

Mean of tumor size of tumor mice groups measured by ultrasound imaging. Values are given as mean \pm SD; n=3 per group. (ns = non-significant, *p \leq 0.05, **p \leq 0.01, ***p \leq 0.001).

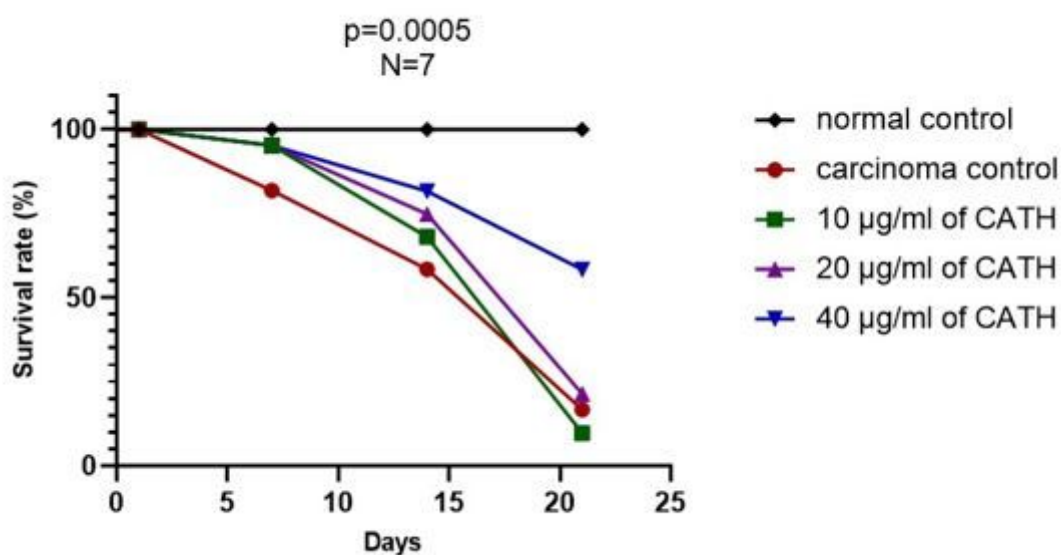
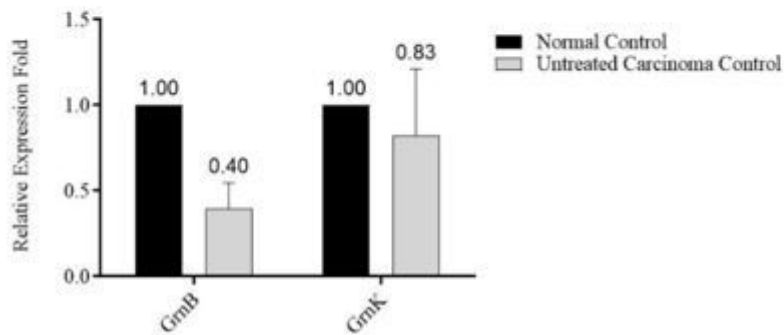


Figure 10

Survival rate of mice groups. Kaplan-Meier survival curves were used to estimate survival rate in five groups(n=7). Survival rate differences were analyzed by the log-rank test.

A



B

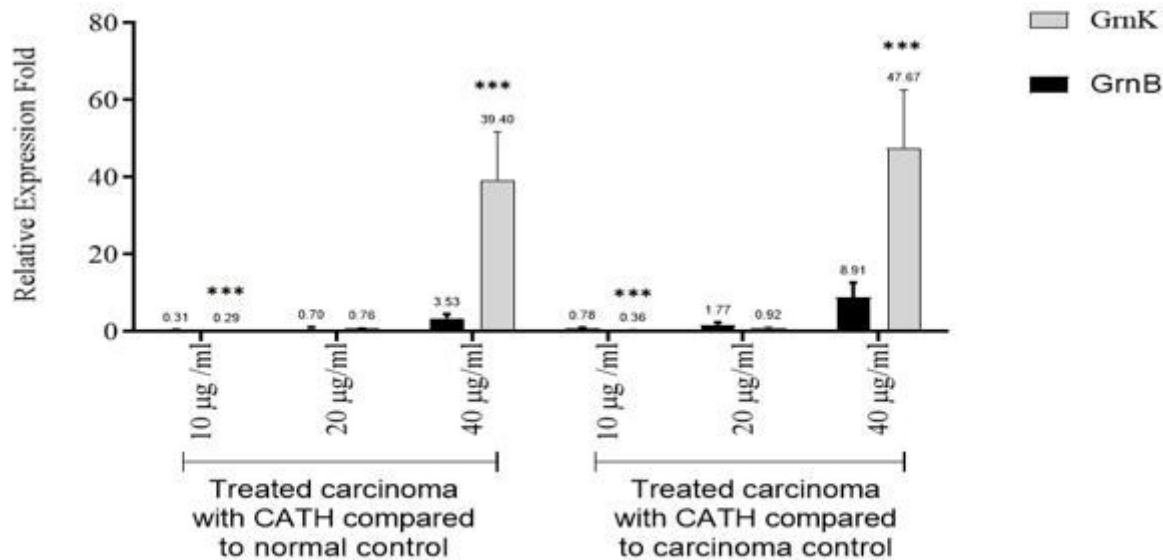


Figure 11

Expression level of granzyme B (GrnB) and granzyme K(GrnK) after chicken cathelicidin administration. Data was plotted using the $2^{-\Delta\Delta Ct}$ method (expression normalized to the house keeping gene GADPH). Fold expression and significance was calculated relative to control groups. Data presented as mean \pm SD; n=7 in each group. (***) $p \leq 0.001$ compared to control groups).

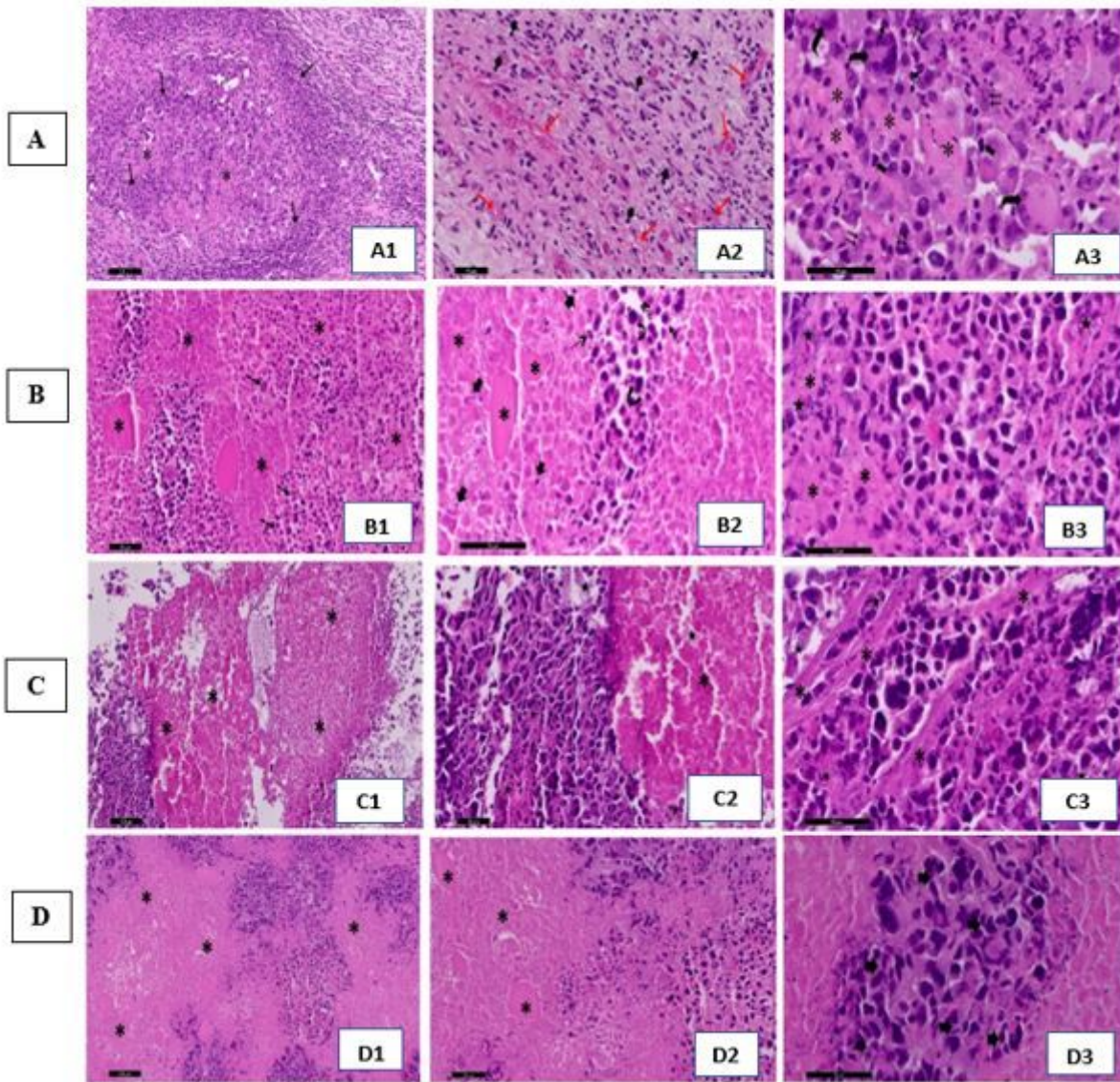


Figure 12

A: Photomicrographs represent subcutaneous Ehrlich solid tumor from untreated tumor mice stained with hematoxylin & eosin showing (A1) infiltration of subcutaneous tissue with tumor cells (arrow). Sheets of small, higher chromatophilic tumor cells of variable shape representing cell proliferation surrounding minimal microscopic necrotic areas(*) (H&E X 10), (A2) numerous newly formed blood capillaries (neovascularization) (red arrow) are seen in the surrounding tissue with leukocyte infiltration (arrowheads) (H&E X 20), (A3) apoptosis (bifid arrows), necrosis (*), giant multinucleated cells (curved arrow), mitosis (dot arrow), and binucleated cells (↑↑) (H&E X 40). B: Photomicrographs represent subcutaneous Ehrlich solid tumor in treated mice with 10 µg/ml of CATH showing (B1) cells with mitotic figures (dot arrow), and features of necrosis (*) (H&E X 20) , (B2) cells with mitotic figures (dot arrow), giant multinucleated cells (curved arrow), features of necrotic areas (*), and inflammatory cells infiltrations (arrowhead) (H&E X 40) ,(B3) features of necrosis (*) and chromatin dust are seen (star) (H&E X 40). (C): Photomicrographs represent subcutaneous Ehrlich solid tumor in treated mice with 20 µg/ml of CATH showing (C1) wide zones of necrosis (*) (H & E x 10), (C2) frequent necrosis and (*) and

wide chromatin dust (star) (H&E X 20), (C3) frequent necrosis (*) and chromatin dust (star) (H&E X 40). (D) Photomicrographs represent subcutaneous Ehrlich solid tumor in treated mice with 40 µg/ml of CATH showing (D1) good response in the form of wide zones of necrosis (*) (H&E X 10), (D2) marked wide necrosis (*) (H&E X 20), (D3) most of cells chromatin dust and fragmented nuclei (arrow)(H&E X 40).

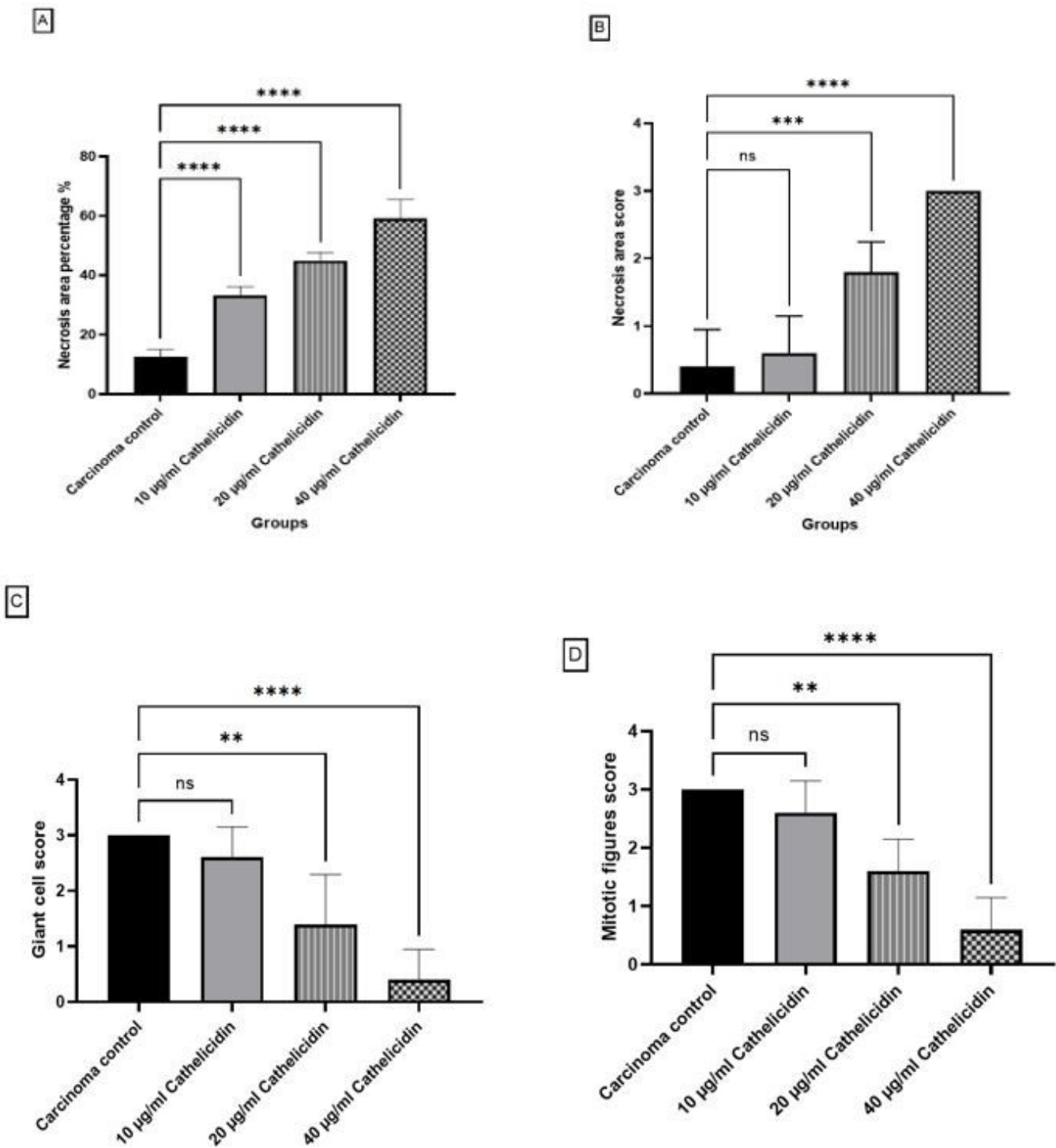


Figure 13

Column chart demonstrating the mean (A): Necrosis area percentage %, (B): Necrosis area score, (C): Giant cell score, (D): Mitotic Figure score. Data were expressed as mean \pm standard error (S.E.), N= number of the mice in each group, One-way ANOVA followed by Bonferroni's post-hoc analysis comparison tests. Non-significant (ns), ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

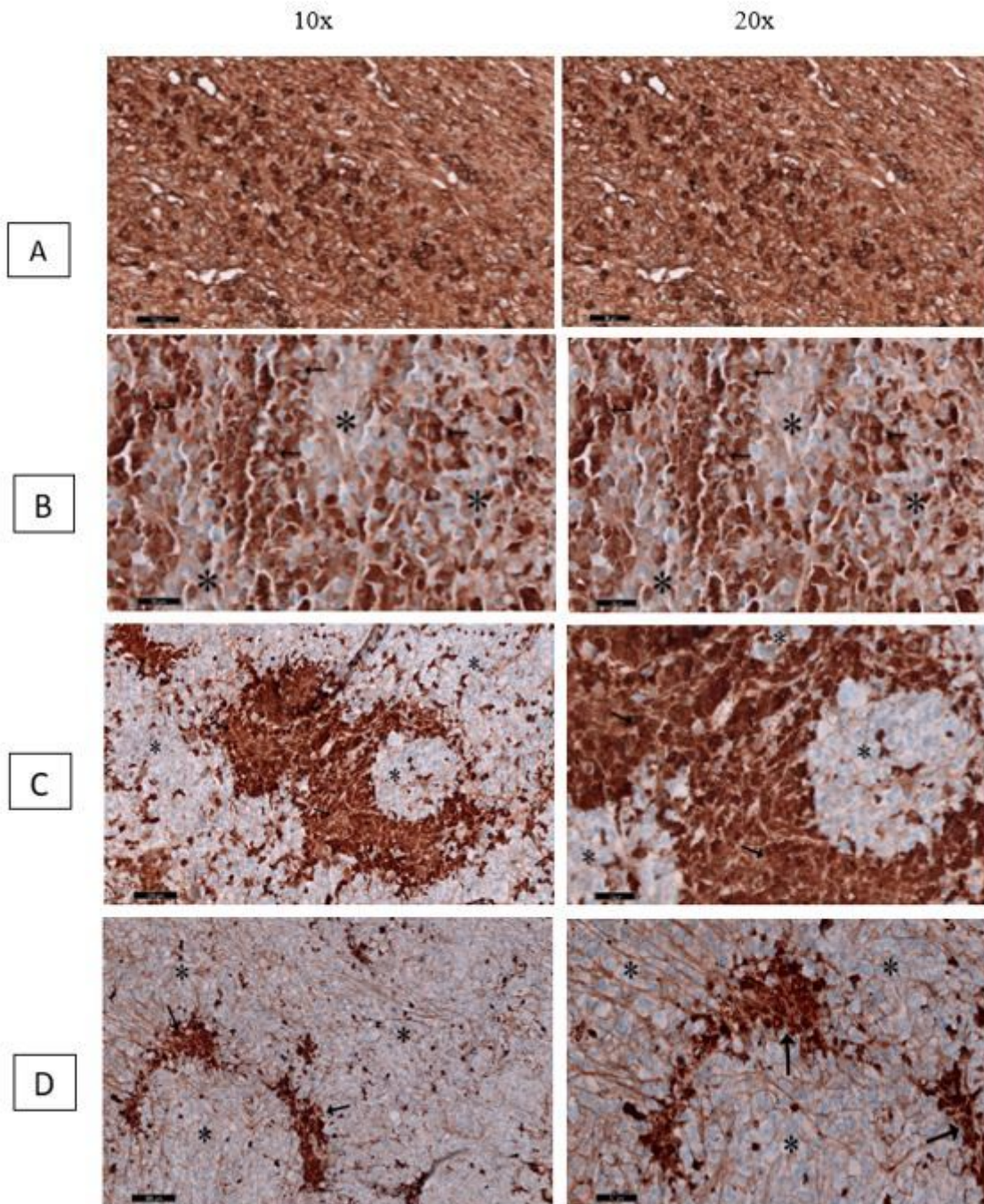


Figure 14

Photomicrographs of tumor sections EAC-bearing mice groups stained with antiapoptotic Bcl2 marker. (A) tumor section in Ehrlich solid tumor in untreated tumor control revealed strong positive cytoplasmic brownish reactions for Bcl2 (Immunohistochemistry Bcl2 X 10, 20). (B) tumor section in treated Ehrlich solid tumor with 10 µg/ml of CATH revealed moderate positive cytoplasmic brownish reactions (↑) for

Bcl2 in between marked negative cells (*) (Immunohistochemistry Bcl2 X 10,20). (C) tumor section in treated Ehrlich solid tumor with 20 µg/ml of CATH reveals moderate positive cytoplasmic brownish reactions (↑) for Bcl2 in between marked negative cells (*) (Immunohistochemistry Bcl2 X 10, 20). (D) tumor section in treated Ehrlich solid tumor with 40 µg/ml of CATH shows few positive cytoplasmic brownish reactions (↑) for Bcl2 in between extensive negative cells (*) (Immunohistochemistry Bcl2 X10,20).

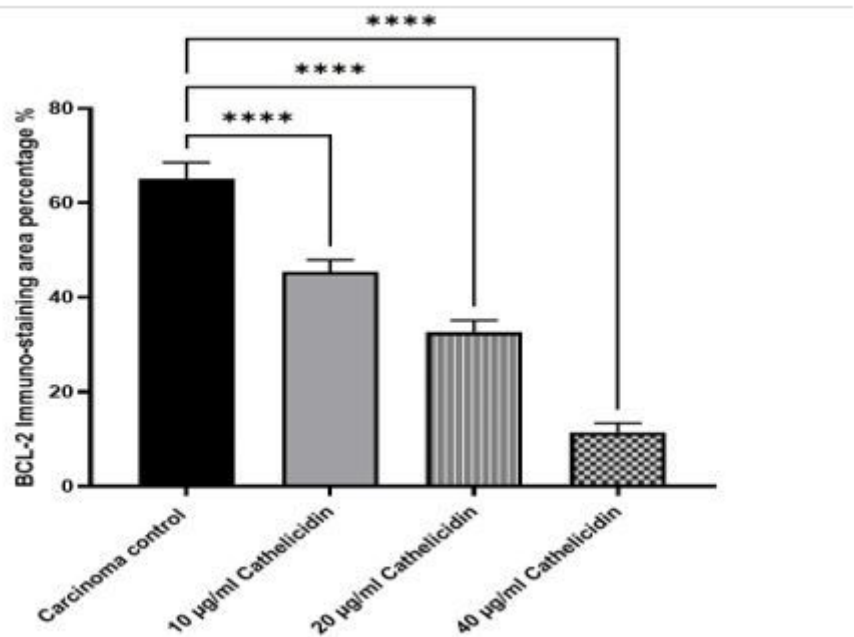


Figure 15

Column chart demonstrating the mean area percentage of BCL-2 immunohistochemical staining for solid tumors grown in mice groups. Data were expressed as mean± standard error (S.E.), N= number of the mice in each group, One-way ANOVA followed by Bonferroni's post-hoc analysis comparison tests. **** P < 0.0001.