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CYTOTOXIC EFFECT OF HEPCIDIN (TH1-5) ON HUMAN BREAST CANCER CELL LINE (MCF7)

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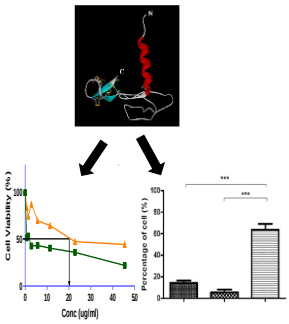
Graphical abstract



Abstract

Hepcidin (TH1-5) is a cysteine-rich antimicrobial peptide originally isolated from the freshwater fish *Oreochromis mossambicus*. A synthesized form of the peptide has been reported to exhibit cytotoxic activity against few human cancer cell lines. This study investigated the potential cytotoxicity of the peptide against human breast cancer cell line and normal mouse embryonic fibroblast cell line (NIH/3T3) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Morphological changes by acridine orange and propidium iodide (AO/PI) double staining were also studied to determine apoptotic evidences. Hepcidin (TH1-5) showed cytotoxic activity against MCF7 with an IC_{50} of 20 µg/mL but no significant effect against NIH/3T3. This outcome indicates hepcidin (TH1-5) to be a promising cytotoxic peptide that warrants further studies as a potential anticancer agent for breast cancer therapy.

Keywords: Hepcidin (TH1-5), antimicrobial peptide, cytotoxicity, MCF7

Abstrak

Hepcidin (TH1-5) yang asalnya diasingkan daripada ikan air tawar *Oreochromis mossambicus*. merupakan peptida antimikrob yang kaya dengan protein sistina. Peptida sintetik dilaporkan mempunyai aktiviti sitotoksik terhadap beberapa sel barah manusia. Kajian ini bertujuan menyelidiki potensi sitotoksik peptida berkenaan terhadap jujukan sel barah payu dara manusia dan sel normal embrio fibrolas tikus (NIH/3T3) dengan menggunakan kaedah cerakin 3- (4,5-dimetiltiazol-2-il)-2,5-difenilltetrazolium bromida (MTT). Perubahan morfologi turut dikaji menggunakan kaedah dwipewarnaan akridin jingga dan propidium iodida (AO/PI) bagi menentukan bukti apoptotik. Hepcidin (TH1-5) menunjukkan aktiviti sitotoksik ke atas MCF7 dengan IC_{50} 20 µg/mL, tetapi ianya tidakmemberi kesan yang ketara terhadap NIH/3T3. Hasil kajian menunjukkan hepcidin (TH1-5) berpotensi sebagai peptida sitoktoksik dan kajian seterusnya sebagai ejen antibarah berpotensi untuk terapi barah payudara harus dijalankan.

Kata kunci: Hepcidin (TH1-5), peptide antimikrob, sitotoksik, MCF7

1.0 INTRODUCTION

Antimicrobial peptides (AMPs) are cationic amphipathic molecules with short amino acid residues isolated from various organisms and constitute the innate immune system of the host [1]. In addition to the endogenous broad spectrum antimicrobial function, AMPs have been reported to possess several therapeutic properties including anticancer/antitumour, mitogenic, immunomodulatory, wound healing and as drug delivery vectors [2-3]. They are also used as food preservatives [4]. AMPs represent impending drug candidates largely due to their broad spectrum activities, low toxicity profile and decreased development of resistance by target cells [5]. Hepcidin was originally identified as a 25-amino acid peptide which is expressed mostly in the liver and responsible for iron homeostasis in humans and mice [6]. Closely related hepcidin genes (HAMP) and peptides also have been identified in a number of mammals, amphibians, and fish species [7-8]. Over 40% of known natural AMPs including hepcidin are cysteine-rich amphipathic peptides that contain multiple disulfide bonds, form stable β-sheets and kill cellular pathogens by enhancing membrane permeability [9-10]. Three different isoforms of hepcidin namely TH1-5, TH2-2 and TH2-3 of 88, 86 and 91 amino acids respectively, have been isolated from the tilapia fish *Oreochromis mossambicus* and they share amino acid similarity with the human hepcidin [11]. TH1-5 and TH2-3 have been reported for their antimicrobial and cytotoxic activities [11]. Synthetic forms of the hepcidin TH1-5 have shown cytotoxic effects in few studies. Chang *et al*. [12] reported that the peptide inhibited the proliferation of human cervical (HeLa), hepatocellular (HepG2) and fibrosarcoma (HT1080) cell lines. It also reduced colony formation in a soft agar assay. TH2-3 also showed potent antitumour activity against HT1080 [13]. It has been forecasted that cancer-related deaths, including breast cancer, will reach an estimated 11.5 million by the year 2030 [14]. Mortality has been linked to uncontrolled metastasis and the challenge of resistance faced by chemotherapy [15]. Many of these lapses and inadequacies of chemotherapy have prompted the unending investigation for newer agents with better therapeutic outcome. It is noteworthy to mention that, to the best of our knowledge, no study has reported cytotoxicity of hepcidin (TH1-5) towards human breast cancer cells (MCF7). In view of this, this study attempts to determine the potential cytotoxic properties and to investigate the mode of cell death of hepcidin (TH1-5) against MCF7.

2.0 EXPERIMENTAL

2.1 Hepcidin (TH1-5)

The synthetic peptide with the sequence *GIKCRFCCGCCTPGICGVCCRF* was purchased from Mimotopes The Peptide Company (Victoria, Australia). It was reconstituted in phosphate-buffered saline (PBS, pH 7.4) prior to use.

2.2 Cell Cultures

Human breast cancer cell line (MCF7) and normal mouse embryonic fibroblast cell line (NIH/3T3) were originally purchased from American Type Culture Collection (ATCC) and deposited at Animal Tissue Culture Laboratory Cell bank, Universiti Sultan Zainal Abidin (UniSZA), Malaysia. Both cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% foetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin. Optimum growth conditions of 37°C, 5% CO2 95% air in a humidified incubator was maintained for both cell lines.

2.3 Cell Viability Assay

MCF7 cells were seeded into 96-well plates at a density of 1x105 per well in 100 µL medium and incubated for 24 h in order to allow for cells adhere to the bottom of the wells. The cells were treated with hepcidin (TH1-5) in various concentrations by two-fold serial dilution and incubated for 72 h. Commercial anticancer drug; methotrexate (Sigma, USA) was used as the positive control while peptide-free cells was taken for the negative control. Freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye (5 mg/mL in PBS, 20 µL) (Nacalai tesque, Japan) was added to all the wells in the dark and the culture was further incubated for 4 h. After removal of the supernatant, 100 µL dimethyl sulfoxide (DMSO) was added to all wells to solubilize the formazan crystals formed and plate was incubated for 15 min. Absorbance (A) was measured at 570 nm using microplate reader (Tecan Infinite M200, Switzerland) and experiment was conducted in triplicates. Cytotoxicity was expressed as IC50, which is the concentration of sample that caused 50% inhibition of cell growth using the formula:

$$\% \text{ Cell viability} = \frac{\text{A sample (mean)}}{\text{A control (mean)}} \times 100\%$$

2.4 Mode of Cell Death

In order to investigate the mode of cell death following cell treatment with hepcidin (TH1-5), the MCF7 cells were subjected to acridine orange/propidium iodide (AO/PI) double staining according to the method by Tajudin *et al.*[16]. Cellswere seeded into 6-well plate (SPL Life Sciences,

Korea) at a density of 1x105 cells per well. Twenty four hours later, cells were treated with IC50 doses of hepcidin (TH1-5) and methotrexate for 24, 48 and 72 h. Untreated cells served as negative control. Cells were harvested by trypsinization, washed twice with PBS and centrifuged at 300 xg for 10 min. Cell pellet was allowed to dry before resuspended with acridine orange and propidium iodide mixture in the dark for 5 min. A volume of 10 µL of stained cell mixture was pipetted onto a microscopic slide and covered with coverslip. Stained cells were observed and analyzed under a fluorescence microscope (Nikon Eclipse, Japan). The viable, apoptotic and necrotic cells were counted in a population of 200 cells.

2.5 Statistical Analysis

GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA, USA) was used to analyze data from both experiments. Non-normal data from cell viability were analyzed using Mann-Whitney test while mode of cell death data were compared among all the groups in one-way analysis of variance (ANOVA). Statistical significance was observed at $p< 0.05$.

3.0 RESULTS AND DISCUSSION

Breast cancer still remain a major global health concern in spite of the recent advances in the diagnosis and treatment of the disease [17]. It is the most common type of cancer and cause of cancer-related morbidity among women worldwide [18]. Hepcidin (TH1-5) was screened for cytotoxicity properties against human breast adenocarcinoma cells and normal mouse embryonic fibroblast cell line was used as control to examine whether or not the peptide exerts significant cytotoxic effect on healthy non-cancerous cells with results shown in Tables 1 and 2. The IC50 which refers to the dose or concentration of a substance which caused 50% cell growth inhibition is extrapolated from the cell viability plot (cell viability versus concentration).

The MTT assay utilize colorimetric reaction based on the reduction of the tetrazolium salt from yellow to purple formazan crystal by dehydrogenase enzymes present only in the mitochondria of viable cells [19]. Result of the MTT assay showed that there was a dose-dependent cytotoxic effect in MCF7 cells treated with hepcidin (TH1-5) after 72 h incubation.

Table 1 Cytotoxic effect of hepcidin (TH1-5) on MCF7 and NIH/3T3 cell lines

% Cells viability	Concentration of hepcidin (TH1-5) (µg/mL)							
	0.00	0.710	1.420	2.841	5.681	11.36	22.73	45.45
MCF7	100.0 ± 0.03	84.1 ± 0.080	74.8 ± 0.080	87.5 ± 0.280	70.0 ± 0.13	64.8 ± 0.050	47.6 ± 0.080	44.6 ± 0.060
NIH/3T3	100.0 ± 0.03	82.2 ± 0.080	78.4 ± 0.150	64.8 ± 0.160	60.4 ± 0.120	60.3 ± 0.100	59.5 ± 0.130	45.5 ± 0.090

Values are expressed as mean ± SD for three replicate experiments

Table 2 Cytotoxic effect of methotrexate on MCF7 and NIH/3T3 cell lines

% Cells viability	Concentration of methotrexate(µg/mL)							
	0.00	0.710	1.420	2.841	5.681	11.36	22.73	45.45
MCF7	100.0 ± 0.11	51.6±0.020	53.6 ± 0.000	42.8 ± 0.010	43.6 ± 0.000	40.5 ± 0.020	36.0 ± 0.02	22.1 ± 0.000
NIH/3T3	100.0 ± 0.03	60.6 ±0.050	60.2 ± 0.020	57.9 ± 0.050	57.5 ± 0.030	57.5 ± 0.030	45.3 ± 0.020	43.4 ± 0.060

Values are expressed as mean ± SD for three replicate experiments

This is indicated by a decrease in percentage of cell viability as hepcidin (TH-5) dose increases. At zero concentration of the peptide there was 100% viable cells; and the cell viability decline up to the maximum concentration of the peptide used with just 44.6%viability count for MCF7 cells. At concentration of 20 µg/mL extrapolated from the graph (Figure 1), 50% of the cells were only viable and this represent the half maximal inhibitory concentration (IC50). The IC50 value of hepcidin (TH1-5) suggests the peptide to be a potential anticancer agent because its IC50 value is within the range of ≤ 30 µg/mL which is the cut-off value for potentially cytotoxic bioactive compounds as reported by the American National Cancer Institute [20] and there was statistical significant difference in the cell viabilities between hepcidin (TH1-5) and methotrexate-treated MCF7 cells. Cytotoxicity of hepcidin (TH1-5) was also evaluated on NIH/3T3 cell line. A similar dose-dependent toxicity was observed from the obtained values (Table 2). However, the degree of cytotoxicity was lower as compared to the effect on MCF7 judging by the high value of the IC50 (38 µg/mL) (Figure 2), which is nearly twice higher than that of hepcidin (TH1-5) on MCF7.

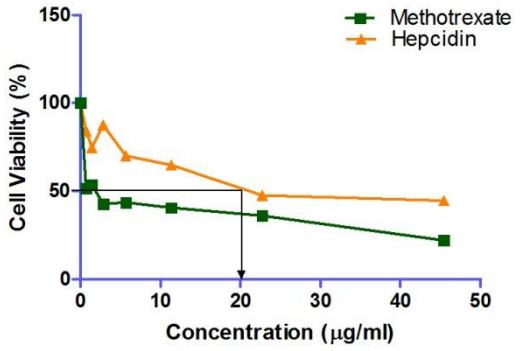


Figure 1 Cytotoxic effect of hepcidin (TH1-5) on CF-7 after 72 h of incubation with peptide. Each point represents the mean of three replicate data. Graph was plotted using Graphpad Prism v5.0

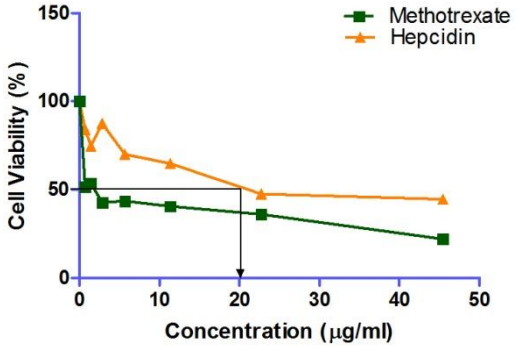


Figure 2 Cytotoxic effect of hepcidin (TH1-5) on NIH/3T3 after 72 h of incubation with the peptide. Each point represents the mean of three replicate data. Graph was plotted using Graphpad Prism v5.0

In the investigation of the mode of cell death induced by hepcidin (TH1-5) on MCF7, the result of cells subjected to AO/PI double staining after treatment at various time intervals is shown in Figure 3. Viable cells show a round shape and green nuclei with their DNA intact. The cells that undergo early apoptosis appeared with a bright green nuclei and display the features of apoptosis such as fragmented DNA, membrane blebbing and chromatin condensation. For late apoptosis cells, an orange colouration will be seen while necrotic cells are characterized by cell burst or rupture and red colour stained nuclei.

Exposure of cells to cytotoxic compounds yield variety of cell fates including necrosis, differentiation or apoptosis [21]. Hepcidin (TH1-5) induced apoptosis in MCF7 cells, and this could be seen through AO/PI double staining which could discriminate viable cells, apoptotic cells and necrotic cells. The intact plasma membrane of viable cells allowed the penetration of acridine orange but impermeable to propidium iodide owing to selective permeability phenomenon. The nucleus of viable cells stained greenish orange whereas in the case of necrosis the cell appear red due to loss of plasma membrane permeability and thus allowing propidium iodide to penetrate the cells [18]. Apoptotic cells appear green and smaller in size with features of membrane blebbing and chromatin condensation in their nuclei [22]. Approximately 200 cells were randomly counted and categorized into either viable, apoptotic (early and late) or necrotic cells. Untreated cells serve as the negative control whereas methotrexate (positive control) and the peptide hepcidin (TH1-5) were used at their respective IC50 doses. At 24, 48 and 72 h, the percentage of early apoptotic cells resulting from hepcidin (TH1-5) treatment were 65.67%, 63.67% and 61.33% respectively; while late apoptosis account for 9%, 16% and 18% respectively within the same time interval. When compared to necrotic cell count, the record shows a minimal range of values 0.33%, 3.67% and 7.67% necrotic cells at 24, 48 and 72 h respectively. This shows that majority of hepcidin (TH1-5) treated cells undergo early apoptosis with low tendency towards necrosis with increased treatment time. Result for methotrexate-treated cells shows early apoptotic cells in proportion of 6.33%, 5.67% and 8.67% at 24, 48 and 72 h respectively. Late apoptosis recorded a count 76%, 22% and 10% within the same time interval (Figure 4A-4C; EA). Figure 2 Cytotoxic effect of hepcidin (TH1-5) on NIH/3T3 after 72 h of incubation with the peptide. Each point represents the mean of three replicate data. Graph was plotted using Graphpad Prism v5.0 In the investigation of the mode of cell death induced by hepcidin (TH1-5) on MCF7, the result of cells subjected to AO/PI double staining after treatment at various time intervals is shown in Figure 3. Viable cells show a round shape and green nuclei with their DNA intact. The cells that undergo early apoptosis appeared with a bright green nuclei and display the features of apoptosis such as fragmented DNA, membrane blebbing and chromatin condensation. For late apoptosis cells, an orange colouration will be seen while necrotic cells are characterized by cell burst or rupture and red colour stained nuclei.

However, no necrotic cells were observed at 24 h of methotrexate treatment but prolonged incubation showed high values at 62% and 77% for 48 and 72 h respectively (Figure 4B-4C; NC). Necrotic cells count for methotrexate was higher than hepcidin TH1-5, which implies that it had induced necrotic events much more than apoptotic events. Hepcidin (TH1-5) as shown from the value induce apoptotic events with a combined value as high as 65-79% at the time interval. Hepcidin (TH1-5) appears to be a good cytotoxic agent since it induces cell death predominantly in the apoptotic mode. This feature is very desirable in the search for alternative anticancer agents and cell burst on the other hand usually affect the normal cells posing a very big challenge to chemotheraphy application.

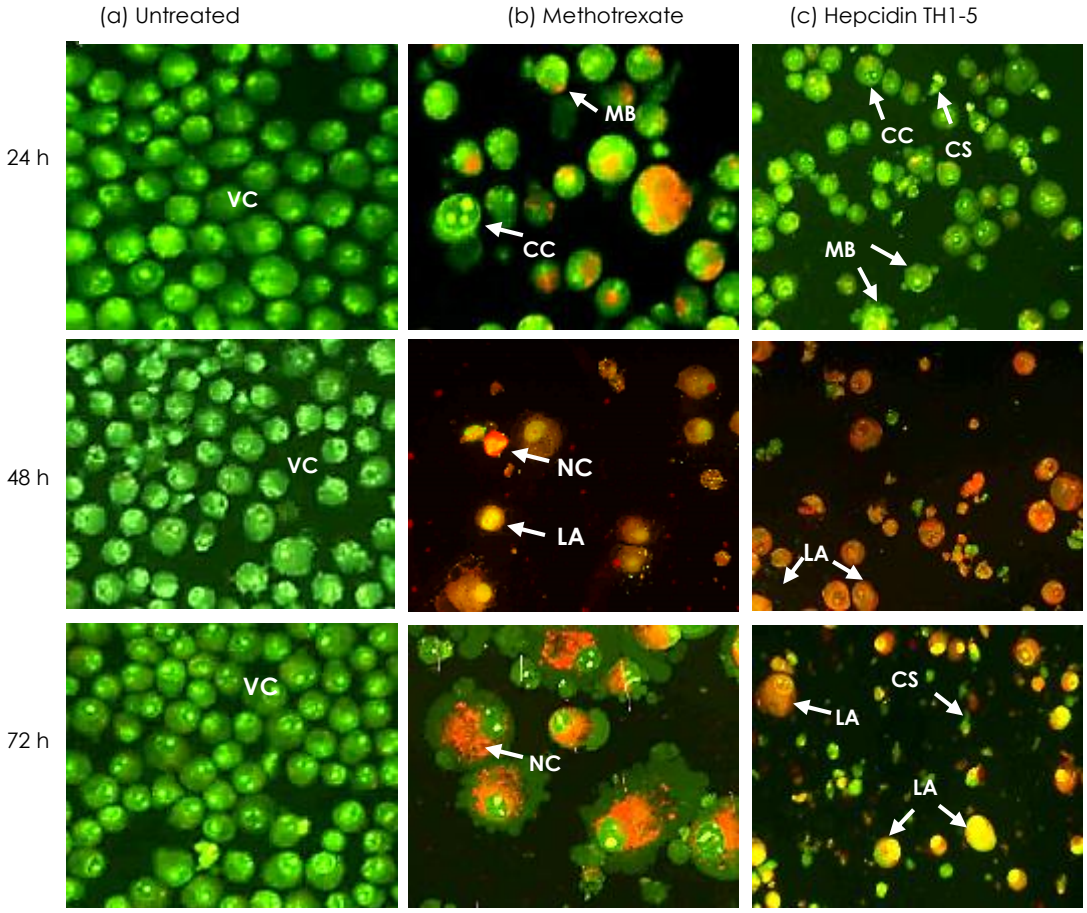
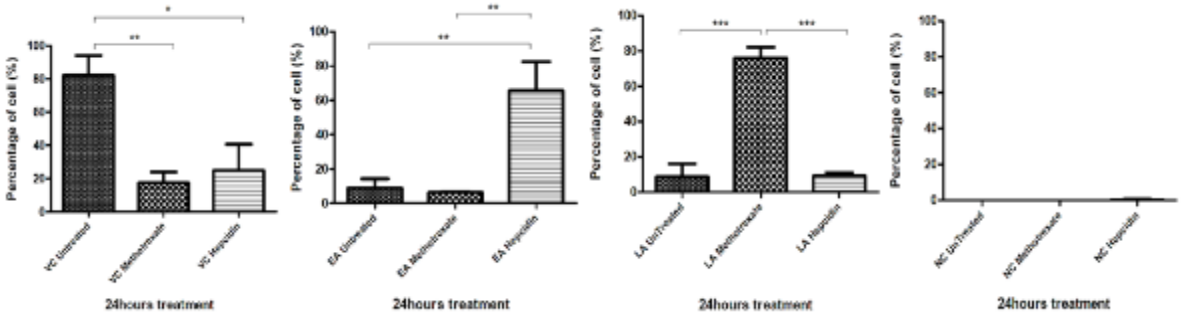


Figure 3 Morphological assessment of human breast cancer cell line MCF7 with untreated cells, methotrexate (1.8 µg/ml) and hepcidin (TH1-5) (20 µg/mL) after 24, 48 and 72 hours of treatment. Image was viewed under fluorescence microscope at 200x maginification. Abbreviation such as VC: viable cell, MB: membrane blebbing, CC: chromatin condensation, CS: cellular shrinkage, LA: late apoptosis and NC: necrosis were annotated.

(A)



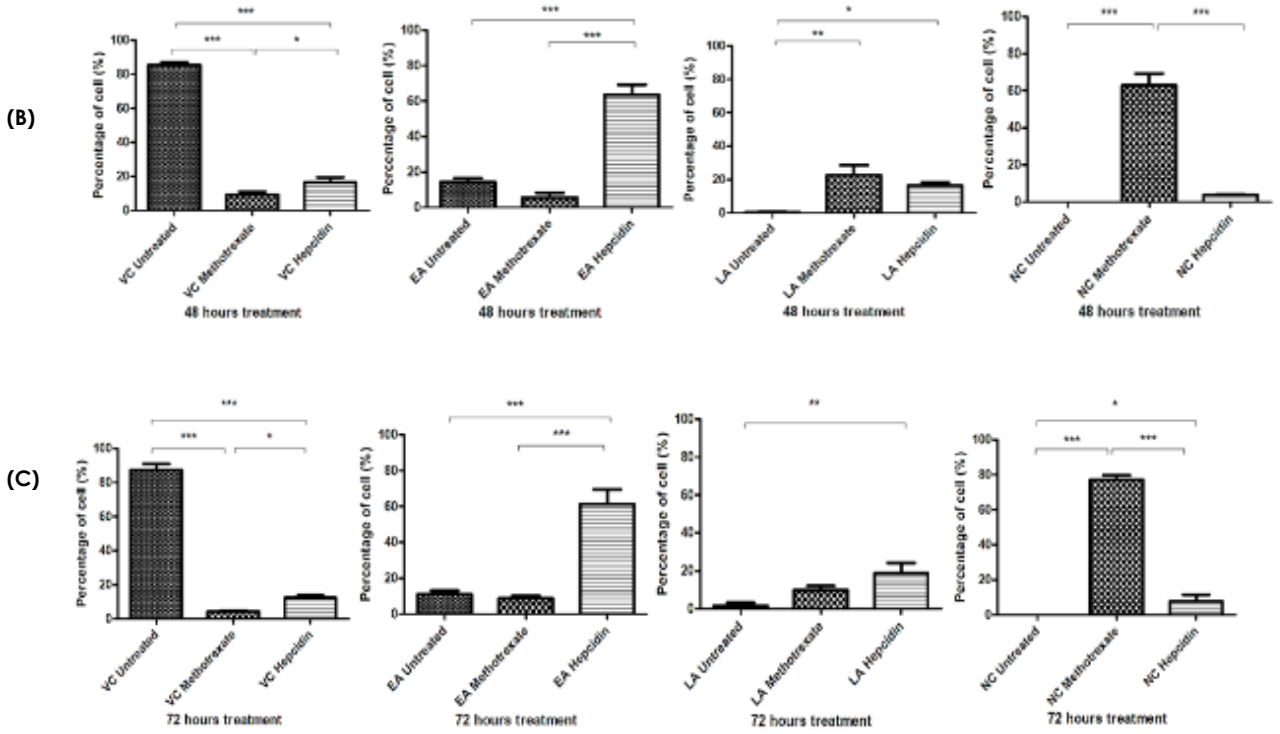


Figure 4 Mode of cell death following treatment of MCF7 cells with hepcidin (TH1-5) and methotrexate (positive control) after (A) 24 h, (B) 48 h and (C) 72 h treatments using their IC_{50} values respectively. Data represent as mean ± S.D from three replicates *p< 0.05, **p Abbreviation for VC: viable cell, EA: early apoptosis, LA: late apoptosis, NC: necrosis was annotated. Graphs were plotted using Graphpad Prism version 5.0

4.0 CONCLUSION

In conclusion, synthetic form of hepcidin (TH1-5)displayed cytotoxicity and induced apoptosis against human breast cancer cells (MCF7). This preliminary outcome suggests that hepcidin (TH1-5) possess the therapeutic potential for the treatment of breast cancer. However, further study is required to buttress these findings and also to unravel the mechanism and pathway behind the apoptosis induction.

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