# The Effect of Therapeutic Klotho Protein on Cell Viability in HT-29 Cell Line

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**Research Article**

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# Abstract

Several studies have shown that klotho protein plays an important role in tumorigenesis concerning cell viability, proliferation, senescence, survival, autophagy, and resistance to antitumor therapies. From the literature, there is no study has been analyzed that the klotho protein induces cytotoxic effects on human colorectal adenocarcinoma or healthy colon cells. We aimed to investigate the anticancer effects of klotho protein against the human colon adenocarcinoma cells and the selectivity using healthy colon cells by adding exogenously klotho protein to the medium of HT-29 cancer and the CCD 841 CoN healthy cell lines and analyzing its effect on cell viability and apoptosis process. The half-maximal inhibitory concentration (IC50) of the human klotho protein was analyzed by WST-8 assay. Annexin V-PI flow cytometric analysis and AO-PI fluorescent staining techniques were used to test the effect of klotho protein on cancer and healthy colon cells for measuring the percentage of cells undergo apoptotic. Our results have firstly demonstrated that klotho had a cytotoxic effect against colorectal adenocarcinoma cells in a dose-dependent manner. Annexin V-PI flow cytometric and AO-PI fluorescent analyses found that klotho induced quantitative and morphological changes that indicate the apoptotic induction in the human colorectal adenocarcinoma. This study results proved for the first time that klotho may be an effective potential therapeutic agent that may be used in adjuvant therapy in human colorectal adenocarcinoma because it does not affect selectively healthy colon cells and but leading cancer cells to apoptosis.


# Introduction

The klotho protein has come to the fore as a potential agent for cancer therapy [1, 2] many studies demonstrated that klotho protein contributes to the induction of apoptotic cell death [3]. The klotho protein was first identified as an aging-related gene by Kuro-o et al in an experiment with mice. This gene was named the klotho, inspired by klotho, one of the three goddesses of destiny in Greek mythology, who spun the thread of life. Mutations in this gene could cause soft tissue calcification, which is associated with early signs of aging, including atherosclerosis, skin atrophy, gonadal dysplasia, infertility, hypoglycemia, severe hyperphosphatemia, osteoporosis, emphysema, and a shorter lifespan [4, 5]. Studies have reported that the defects in klotho gene expression reduces insulin production and increase insulin sensitivity [6]. Many physiological functions of klotho protein, such as participation in intracellular signaling mechanisms, including p53/p21, cAMP, protein kinase C, and Wnt signaling have been determined [7–9]. Klotho protein plays a role as a cofactor in the fibroblast growth factor 23 (FGF23) signaling mechanism. It stimulates FGF23 signaling to maintain mineral and vitamin D metabolism. Moreover, increasing phosphorus intake with diet increases klotho gene expression [10]. Klotho is highly expressed in the proximal tubular epithelial cells of the kidney [11]. In human's serum, the klotho protein level decreases after the age of 40, this decrease in klotho levels contributes to various damages such as cancer, and kidney diseases [12].

Some recent studies have revealed that dysregulation in the klotho protein does not only contribute to the aging process but also the tumor formation mechanisms. Therefore, the klotho protein is recommended

as a potential agent to develop therapeutic interventions in cancer [1, 2]. It has been determined that the expression of the klotho gene is decreased in cancer cells [3]. Also, in tumor tissues, the klotho gene expression is lower than in non-tumor regions, and loss of klotho gene expression causes malignant formation in some cancers, including colon [1].

Accordingly, this study aims to determine the apoptotic dose of klotho protein in human colorectal adenocarcinoma cell lines (HT-29) and the healthy colon cell lines (CCD 841 CoN), to evaluate the effects of klotho protein as a potential antiproliferative and an apoptotic inducer factor on HT-29 cell lines.

## Materials And Methods

# Cell culture and morphological analysis

HT-29 (ATCC® HTB-38) and CCD 841 CoN (ATCC® CRL-1790 ™) cells were commercially available from the American Type Culture Collection (ATCC, Washington DC, USA). After thawing the vial both cell lines were cultured in Dulbecco's Modified Eagle's Medium Dulbecco's Modified Eagle's Medium (DMEM) (Biochrom, Holliston, MA, USA) containing heat-inactivated 10% fetal bovine serum (FBS; Invitrogen/GIBCO, Grand Island, NY, USA) and %0.2 primosin (Invivogen, USA) and %1 (ml/ml) Glutamax (Sigma-Aldrich, St Louis, MO, USA) in 75 $cm^2$ flasks. They were plated at the density of HT-29 cells ($7.5x10^5$ cells/flask) and CCD 841 CoN cells ($5x10^5$ cells/flask) and incubated at $37^0$C in a humidified atmosphere containing 5% $CO_2$ and 5% $O_2$. The adherent cells grown to 70% confluency were defined as passage zero (P0) cells. Complete medium was changed every other 3 days during a 6–7 day passage period. For each passage, the cells were plated similarly and grown to a confluency of 70%.

# WST-8 viability assay

The human recombinant α-klotho (rα-klotho) protein was supplied from Sigma (Sigma-Aldrich, Darmstadt, Germany, Catalog no: SRP3102-20UG). The effect of klotho protein on cell viability and proliferation of HT-29 and CCD 841 CoN cells, was tested by 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST)-8 (AAT Bioquest, California, USA) [13].

The assay was performed as following: $7x10^3$ cells per well of HT-29, and $5x10^3$ cells per well of CCD 841 CoN, in 100µl medium were seeded into 96-well culture plates. Klotho protein was treated in a dose range [14–17] ranging from 0.04µg/ml to 0.31 µg/ml (0.04µg/ml, 0.08µg/ml, 0.16µg/ml, and 0.31µg/ml).

After 24 and 48 hours, 10µl/well of WST-8 solution was added, subsequently, cells were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$ for 3 days. Then the absorbance values were measured at 460 nm in a microplate reader with a monochromator system (BIOTEK ELx808IU, Vermont, USA). Next, the viability of the klotho-treated cells was calculated, assuming the viability of untreated cells to be 100%. The following formula was used to calculate the percent viability:

[(OD klotho treated cells-OD blank)/(OD untreated cells-OD blank)]x100

The OD refers to the optical density. The IC50 dose was determined and accordingly the next analyzes were performed.

# Annexin V-PI analysis with flow cytometry

Annexin V/PI staining (cell meter™ live cell caspase 3/7 and phosphatidylserine detection kit, AAT Bioquest, California, ABD) was performed to detect apoptosis in CCD 841 CoN, and HT-29 cells. Cells were treated with the human klotho protein, and then after 48 hours, cells were suspended by treatment with trypsin-EDTA, then it was washed twice with DMEM containing 10% FBS to remove trypsin-EDTA. The cell pellet was formed by centrifugation at 300xg for 5 minutes. 1:100 dilution of Annexin V-iFluor 488 ™, and 1:500 dilution of propidium iodide (PI) were added. Incubation was performed at 5% CO2, 95% relative humidity, 37°C and for 1 hour. Then the cells were centrifuged at 300xg for 5 minutes. And washed with 1mL of washing solution and centrifuged at 300xg for 5 minutes. 500µl washing solution was added onto the pellet and Accuri™ C6 (BD Biosciences) was read in a flow cytometry device. Analyzes were performed on the BD Accuri ™ C6 program (BD Biosciences).

# Acridine orange-propidium iodide (AO-PI) fluorescent labeling assay

HT-29 ($7x10^3$ cells/well) and CCD 841 CoN ($5x10^3$ cells/well) each cell line was seeded separately in 100µl medium into 96-well culture plates. The IC50 of klotho was the considered concentration, which is 0,31µg/ml for 24 hours and 48 hours, two repeats were performed for each day. After that, the medium containing klotho was gently aspirated. Cells were subsequently detached and centrifuged at 180xg for 5 min., and the pellet was stained with AO (100 µg/ml) and PI (100 µg/ml) mixture at a dilution of (1:1, v/v). The morphology of HT-29 cells was observed immediately at 20x magnification under a fluorescent microscope within five minutes. Micrographs were taken from 10 randomly selected areas for morphometric analysis. The cells in these 10 photographs were counted and the average was taken for quantitative analysis of cell viability and the cells which undergoe apoptosis.

# Statistical Analysis

Three independent experiments were conducted at different times for all experiments. Results are expressed as mean ± SE. SPSS 20.0 was used for statistical analysis. Data were analyzed using one-way ANOVA-Tukey's post-hoc and paired *t* test. Differences between experimental and control groups were considered statistically significant when $p < 0.05$.

# Results

### *Morphologic Analysis*

During the culture period, the CCD 841 CoN and HT-29 cells at early and late passages were examined daily with phase-contrast microscopy, and photographs were obtained (**Figure 1**). In microscopic examinations, CCD 841 CoN cells attached to culture flasks as clusters, and most cells displayed a

fibroblast-like, pindle-shaped morphology during the days of incubation. These cells began to proliferate in approximately 4–5 days and gradually grew as in diffuse monolayer morphology **(Figure 1A and B)**. HT-29 cells attached to culture flasks sparsely, and most cells displayed an epithelial-like, polygonal-shaped morphology during the days of incubation. These cells began to proliferate in approximately 6–7 days and gradually grew as non-homogenized and monolayer islets to form wide and big colonies **(Figure 1C and D)**. Throughout 15 passages, no changes in their morphology, growth patterns, or immunophenotype were observed **(Figure 1A–D)**.

## The Detection of Cell Viability and Apoptosis

### *WST-8 assay*

As a result, the half maximal inhibitory concentration (IC50) of the human klotho protein was 0.31 µg/mL for both 24 and 48 hours (***$p$<0.001 in 24 hours and 48 hours). The viability and proliferation of colon cancer cells (HT-29) are presented in table 2 and in Figure 3. But klotho had no effect on the viability and proliferation of healthy colon cells (CCD841 CoN cells) at all treated doses, the results presented in table 1 and in Figure 2.

### *Annexin V-PI analysis with flow cytometry*

According to our Annexin V-PI flow cytometric results, for the healthy colon cells (CCD 841 CoN), in the control group (cells were treated with no rα-klotho): The cell viability was 97.63%, early apoptosis 0.39%, late apoptosis 0.61% and necrosis 1.38%. for the cells treated with klotho for 48 hours: the cell viability was 93.47%, early apoptosis 0.79%, late apoptosis 2.36%, and necrosis 3.39%.

In colorectal adenocarcinoma cells (HT-29), in the control group (cells were treated with no rα-klotho): The cell viability was 89.57%, early apoptosis 1.11%, late apoptosis 3.64%, and necrosis 5.69%. for the cells treated with klotho for 48 hours: The cell viability was 83.32%, early apoptosis 2.53%, late apoptosis 7.69%, and necrosis 6.47%, all the above results are shown in Figure 4A. While the viability of HT-29 colorectal adenocarcinoma cells treated with klotho decreased compared to the control group, however, this decrease did not show a significant difference (**Figure 4B**). HT-29 colorectal adenocarcinoma cells treated with klotho showed a significant increase in apoptosis rate compared to the control group (*$p$<0.05), while the apoptosis rates of CCD 841 CoN healthy colon cells showed a moderate increase in comparison with the control group **(Figure 4C).**

### *Acridine orange-propidium iodide (AO-PI) fluorescent labelling assay*

Acridine orange-propidium iodide (AO-PI) fluorescent labelling assay were done for quantitative analysis of cell viability and the cells which undergoe apoptosis.

Each of HT-29 and CCD 841 CoN cells was labelled with acridine orange and propidium iodide (AO-PI) florescent dyes, and nuclear changes were observed (**Figure 6**). AO is an intercalating dye that can permeate both live and dead cells. AO stains all nucleated cells and generates a green fluorescence, while

PI can only enter cells with permeable membranes, generating red fluorescence. Bright green live cells with normal nuclei were seen in HT-29 cells without klotho (**Figure 6, D**). At the end of 48 hours, condensations of nuclear material and bright orange dead and living cells with different sizes of apoptotic nuclei were observed in both klotho treated HT-29 cells (**Figure 6, E and F)**.

# Discussion

In this study, we aimed to determine the apoptotic dose of exogenous klotho protein on the human colon adenocarcinoma cell line HT-29, to estimate the half maximal inhibitory concentration (IC50) values by cell viability assay, and to assess the morphological changes in the treated cells using microscopic methods. However, the potential role and molecular mechanisms for klotho protein in colorectal adenocarcinoma remain unclear.

Klotho protein is a humoral factor that regulates the activity of oxidative stress, growth factor, receptors, and ion channels [4, 7, 18]. Many studies have reported that klotho gene expression in tumor tissues was lower than non-tumor regions, and the loss of klotho gene expression caused malignant formation in some cancers including colon cancer [1, 11, 19]. This is the result of hypermethylation of the promoter region of the klotho gene [3]. It has been determined that mutations in the klotho gene play a role in the formation process of cervical [20], stomach [21], liver [22], breast [23], and colon [3] cancers. Increasing the expression of klotho, suppresses cell proliferation in breast, lung, and cervical cancer cell lines [8, 24].

While klotho protein function contributes to apoptotic cell death in normal cells, it acts as an inducer of apoptosis in cancer cells. The function of klotho protein in cancer is characterized by its effect on the proliferation of cancer cells along with multiple signaling pathways. The effect of klotho on tumor metastasis has not been extensively studied, but only a few studies have examined the relationship between klotho gene expression and tumor invasion and metastasis. For example, klotho protein has been found to reduce the invasion of melanoma cells [25]. The mutation or hypermethylation of the klotho in breast cancer suggests that this gene can be used as a marker in the early stage of cancer [2]. Studies have suggested that klotho is significantly reduced in the cell's cytoplasm of the colorectal cancer tissues compared to healthy colon tissues. Accordingly, klotho protein loss can be used as a biomarker in the early diagnosis of colorectal cancer [1, 3].

Klotho protein is partially or completely silenced in colorectal cancer tissue and colorectal cancer cell lines. In studies conducted with HT29, HCT116, DLD1, LS180, SW620, SW480, and Colo-320 colorectal cancer cells, in-vitro klotho treatment significantly slows down the proliferation of cancer cells and induces apoptosis [1, 3, 26]. In HepG2, SMMC-7721, LY1, and LY8 cancer cell lines, it has been determined that klotho reduces the viability and proliferation of cancer cells [14–17].

The present study shows that klotho protein has a dose-dependent cytotoxic effect during 24- and 48-hour incubation periods and the optimum apoptotic dose is 0.31 µg/ml. It was revealed that the changes in cell viability, apoptosis, and necrosis rates after the addition of klotho to HT-29 cells were more

effective after 24 hours than after 48 hours at the applied klotho dose. In AnnexinV-PI flow cytometric analysis, it was observed that klotho had no significant effect on cell viability, apoptosis, and necrosis in healthy colon cells. On the other hand, it has been determined that klotho protein treated to apoptosis-resistant HT-29 colorectal adenocarcinoma cells, significantly reduces cell viability and induced apoptosis and necrosis in cancer cells. These results support the notion that klotho is a potent inducer of apoptosis.

Acridine orange is an intercalating dye that can permeate both live and dead cells. AO stains all nucleated cells and generates a green fluorescence, while propidium iodide (PI) can only enter cells with permeable membranes, generating red fluorescence [27]. The results of AO/PI fluorescence in this study showed the presence of a concentration of nuclear material and apoptotic bodies of different sizes in HT-29 treated cells (Fig. 6). In addition, examples of bubbling and nuclear margination were found in the klotho treated cancerous cells. The noticeable apoptotic morphological changes, including chromatin condensation, further confirmed the ability of klotho protein to induce apoptosis in the HT-29 cell line.

Taken together with the above findings, this study confirms the cytotoxic activity of klotho protein, achieved by decreasing cell viability, and necrosis, and apoptotic cell death induction. In conclusion, klotho protein has a strong apoptotic effect in apoptosis-resistant colorectal adenocarcinoma cells and may be effective in overcoming the apoptosis resistance observed in colorectal adenocarcinoma treatment and presenting more efficient treatment options to experimental small animal's cancer model studies *in vivo.*

# Declarations

### *Author' note*

Preliminary findings of our manuscript were previously published as a summary in the abstract book of EUROASIA International Congress on Scientific Researches and Recent Trends-VIII held in the Philippines on August 2-4, 2021.

### *Author Contributions*

*Ayla Eker Sariboyaci is the main author in this study and contributed to the study conception and design. Material preparation, data collection and analysis were performed by Sibel Gunes and Merve Nur Soykan. The first draft of the manuscript was written by Onur Uysal. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.*

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***Ethics committee:*** This study is not approval required for the ethics committee.

***Declaration of interest:*** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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# Tables

**Table 1.** Different doses of klotho (0.04 µg/ml, 0.08 µg/ml, 0.16 µg/ml, and 0.31 µg/ml) application on CCD 841 CoN cells viability at the end of 24 and 48 hours after performed IC50 analysis. The experiments were repeated 3 times.

| Viability values of CCD 841 CoN Cells (%) (after IC50 analysis) | | | | |
|---|---|---|---|---|
| | 24 hours | | 48 hours | |
| Klotho (µg/ml) | mean±SD | *p* value | mean±SD | *p* value |
| 0,04 µg/ml | 95,3±1,8 | - | 89,7±1,9 | - |
| 0,08 µg/ml | 97,9±2,9 | - | 96,5±3,4 | - |
| 0,16 µg/ml | 97,0±2,4 | - | 105,2±3,7 | - |
| 0,31 µg/ml | 88,8±0,5 | - | 89,8±2,6 | - |

**Table 2.** Different doses of klotho (0.04µg/ml, 0.08µg/ml, 0.16µg/ml, and 0.31µg/ml) application on HT-29 cells viability at the end of 24 and 48 hours after performed IC50 analysis. The experiments were repeated 3 times.

| Viability values of HT-29 cells (%) (after IC50 analysis) | | | | |
|---|---|---|---|---|
| | 24 hours | | 48 hours | |
| Klotho (µg/ml) | mean±SD | *p* value | mean±SD | *p* value |
| 0,04 µg/ml | 81,7±1,6 | ***$p<0.001$ | 92,6±1,9 | - |
| 0,08 µg/ml | 84,2±1,6 | ***$p<0.001$ | 75,1±0,5 | ***$p<0.001$ |
| 0,16 µg/ml | 68,8±1,4 | ***$p<0.001$ | 83,8±2,2 | ***$p<0.001$ |
| 0,31 µg/ml | 60,1±0,9 | ***$p<0.001$ | 75,2±1,4 | ***$p<0.001$ |

# Figures

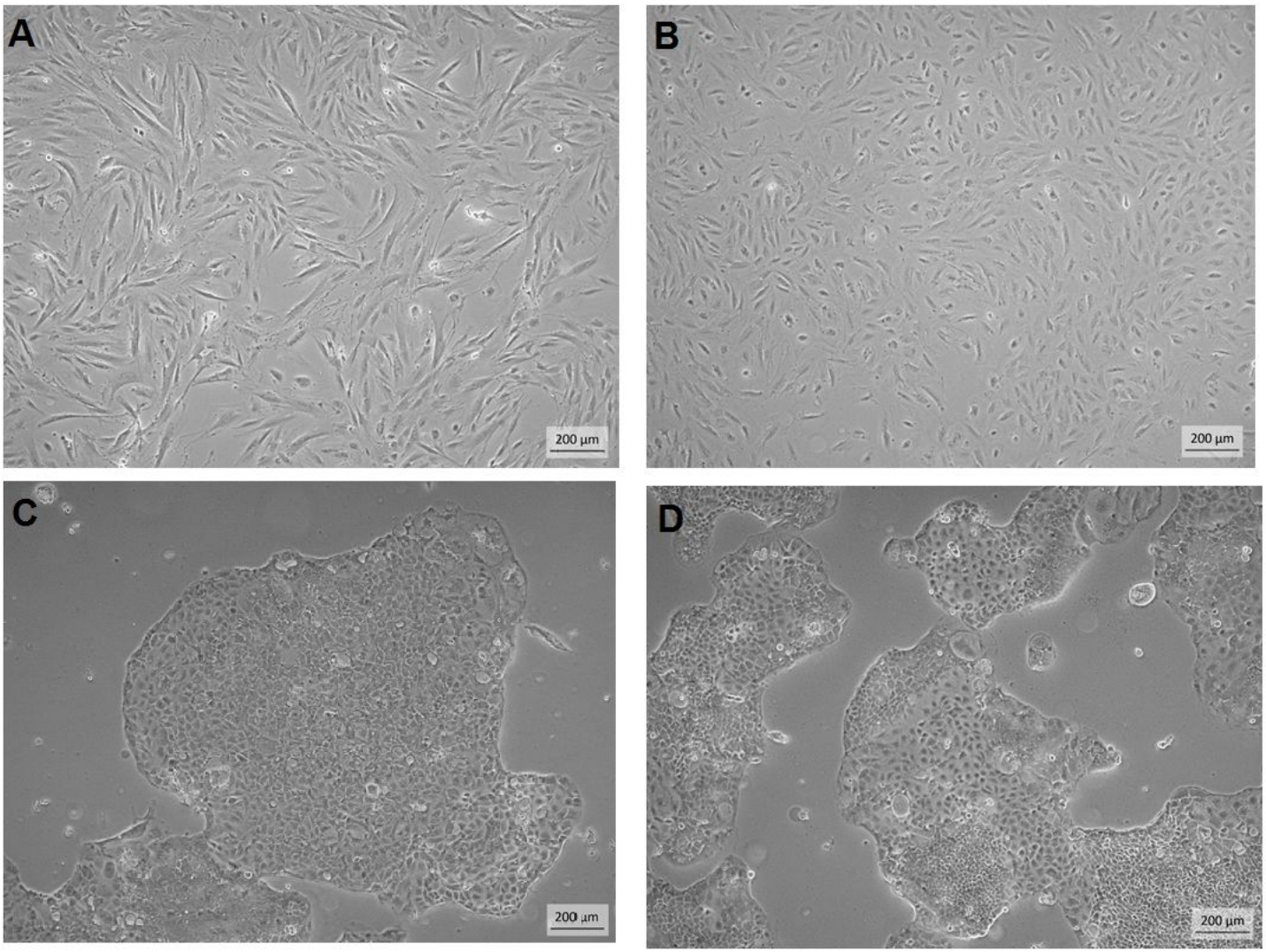
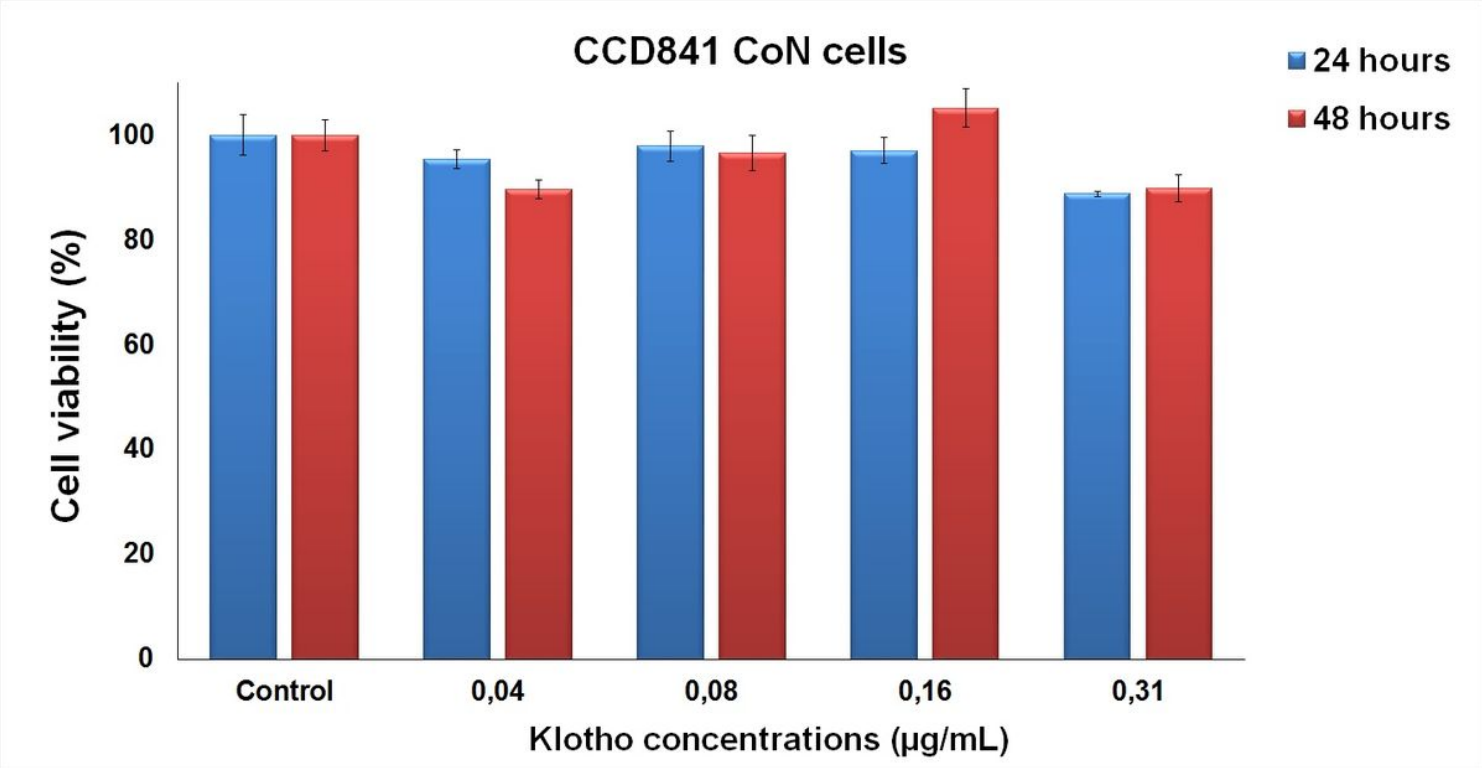



**Figure 1**

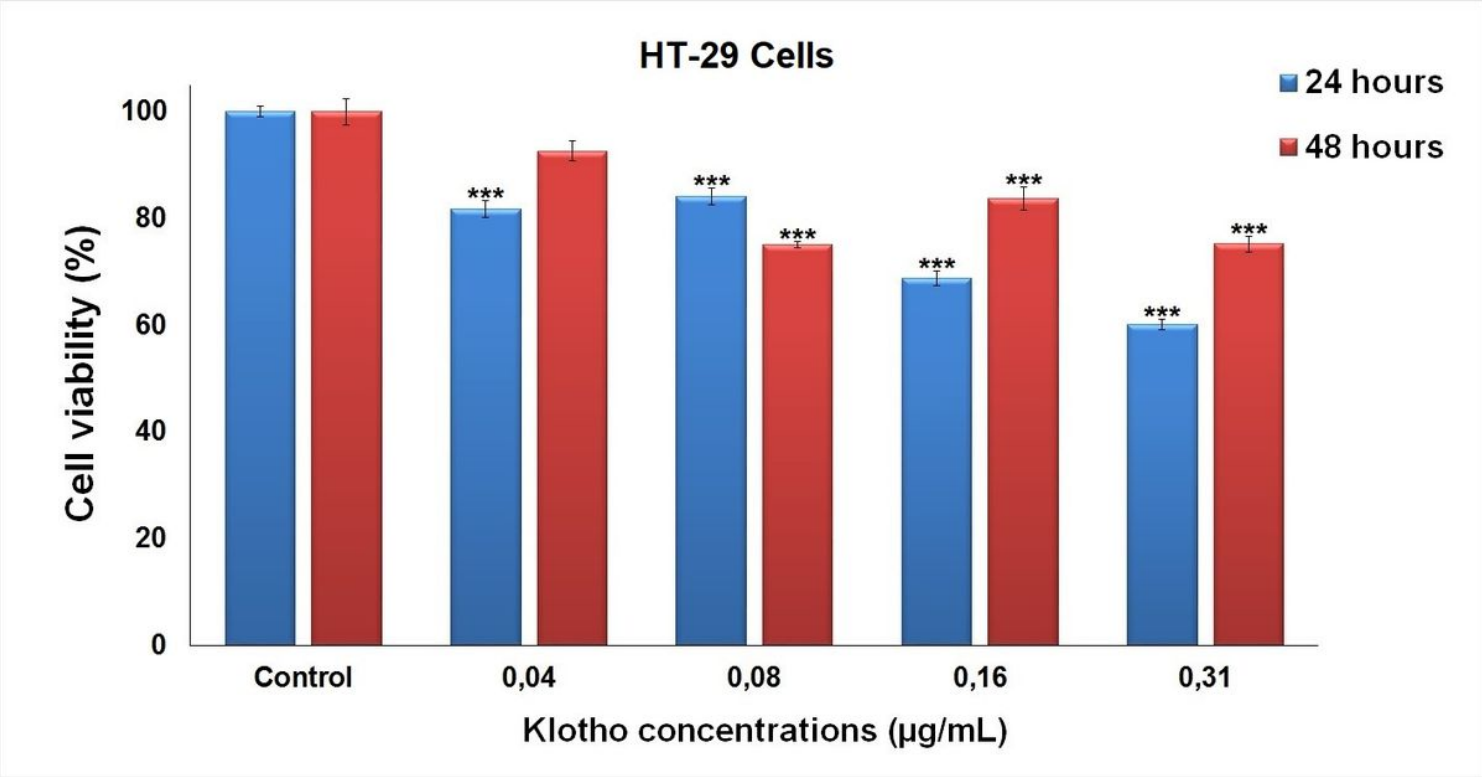
Morphological characteristics of CCD841 CoN and HT-29 cells at different passages in culture **(A-D)**. Phase-contrast microscopic views of HT-29 cells in early and late passages in culture **(A and B)**. **(A)** 3rd passage, 14th day. **(B)** 13th passage, 8th day. Phase-contrast microscopic views of CCD841 CoN cells at

early and late passages in culture **(C and D)**. **(C)** 4th passage, 5th day. **(D)** 12th passage, 13th day (scale bars: A-D 200µm).



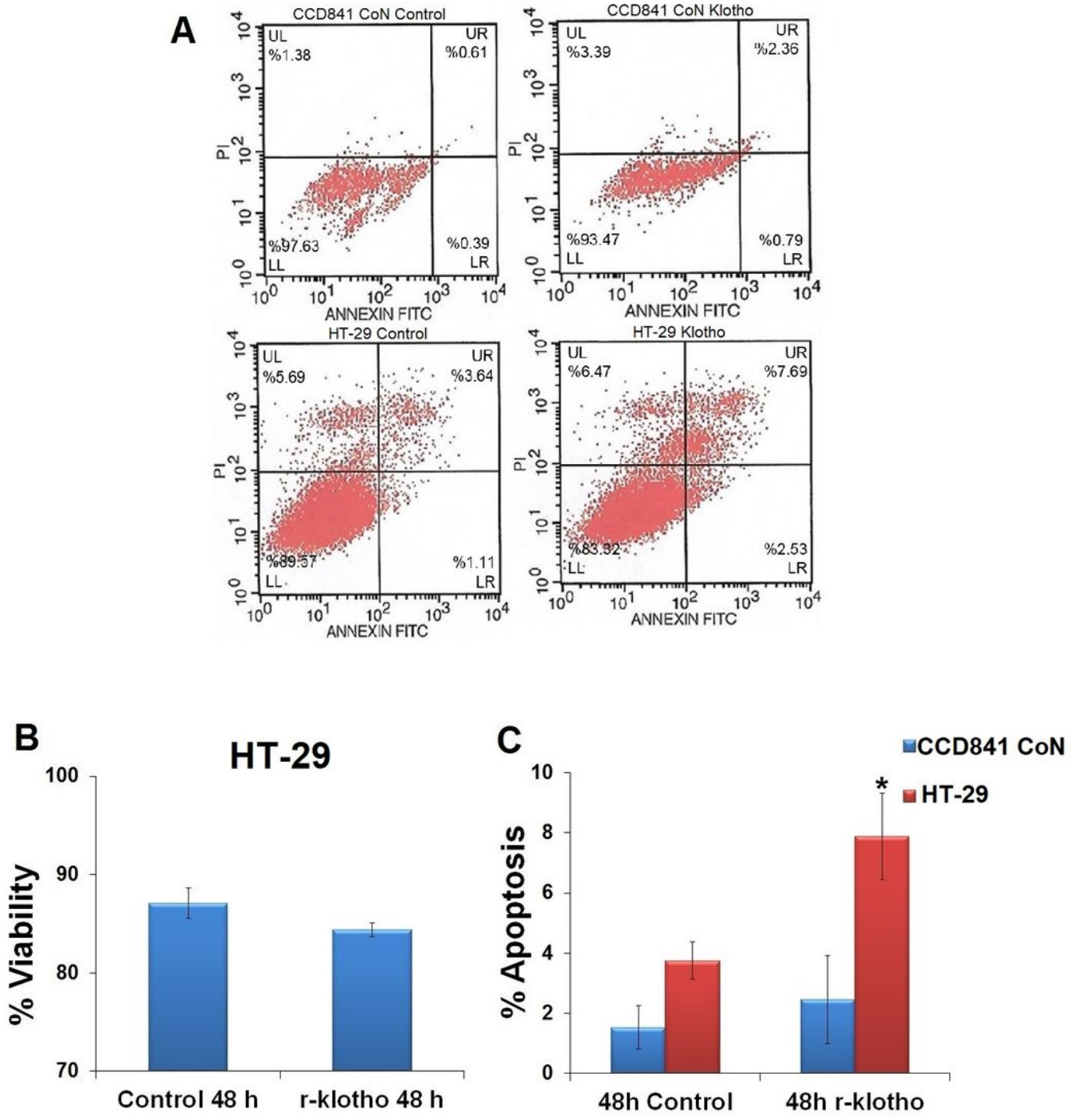

## Figure 2

Detection of the effect of the applied klotho (0.04 µg/ml, 0.08 µg/ml, 0.16 µg/ml and 0.31 µg/ml) to the CCD841 CoN cells on cell viability after 24 and 48 hours by WST-8 viability assay. Both in the first 24th and 48th hours it was determined that the cell viability did not changed significantly compared to control group (n= 3).




## Figure 3

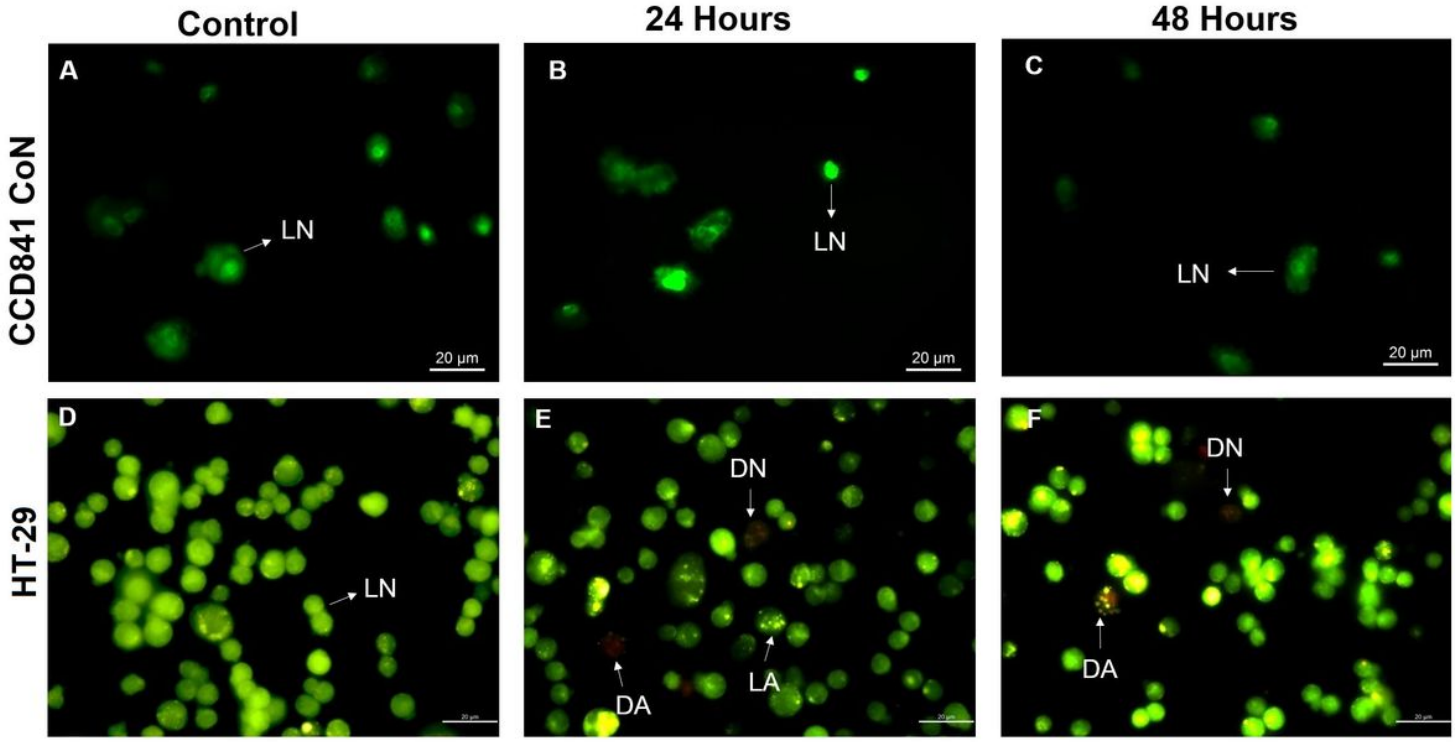
Determining the effect of the applied klotho (0.04 µg/ml, 0.08 µg/ml, 0.16 µg/ml, and 0.31 µg/ml) to HT-29 cells on cell viability after 24 and 48 hours by WST-8 viability assay. Both in the first 24th and 48th hours it was determined that cell viability decreased significantly compared to the control group (n= 3, *$p$<0.05, **$p$<0.01 ***$p$<0.001).




## Figure 4

Monitoring the effects of klotho on apoptosis of HT-29 cancer and CCD841 CoN healthy colon cells based on AnnexinV-PI. Annexin V-PI flow cytometry analysis of both type cells treated with the 0,31 µg/ml of klotho for 48 h. Control cells no treated with klotho. **(A)** Representative flow cytometry histograms showing the viable (LL), necrotic (UL), early apoptosis (LR), and late apoptosis (UR) rates of CCD841 CoN and HT-29 cells treated with or without 0.31 µg/ml klotho through 48 h. **(B)** According to Annexin V-PI

flow cytometric analysis, viability of HT-29 colorectal adenocarcinoma cells treated with klotho decreased compared to the control group, but this decrease did not show statistically significant. **(C)** HT-29 colorectal adenocarcinoma cells treated with klotho showed a significant increase in apoptosis rate compared to the control group. Data represent mean ± SE from three independent experiments (n= 3, **p*<0.05, ***p*<0.01 ****p*<0.001).




## Figure 5

Detection of apoptotic morphology in cells by acridine orange-propidium iodide (AO-PI) fluorescent staining at the end of 24 and 48 hours after 0.31µg/ml klotho treatment to CCD 841 CoN and HT-29 cells. CCD 841 CoN and HT-29 cells untreated with klotho control cells **(A and D)**, klotho treated for 48 hours **(B, C, E, and F)**. LN: Bright green living cells with normal nuclei. LA: Bright green living cells with apoptotic nucleus. DN: Bright orange dead cells with normal nucleus. DA: Bright orange dead cells with apoptotic nucleus (scale bars in A-F: 20 µm).