

## Gemini Curcumin suppresses gastric cancer AGS cells proliferation through modulation of lncRNA CCAT2 and c-Myc genes

**Short title:** Gemini Curcumin against AGS cancer cells.

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### Abstract

**Objectives:** Gemini surfactant nano curcumin (Gemini-Cur) is a novel formulation of curcumin with dramatic suppressive effects on cancer cells. Here, we investigate the anticancer effects of Gemini-Cur on AGS cancer cells through evaluation of the expression of lncRNA CCAT2 and its downstream c-Myc as known oncogenic modulators of tumorigenesis.

**Materials and Methods:** The AGS cells were treated with Gemini-Cur and pure curcumin in a time- and dose-dependent manner. The toxicity of Gemini-Cur was studied by using MTT and scratch tests. Furthermore, real-time PCR and western blotting techniques were employed to evaluate the expression of genes.

**Results:** Gemini-Cur significantly affected the viability of AGS cells in a dose- and time-dependent manner with IC<sub>50</sub> values of 59.32, 40.88, and 19.63  $\mu$ M during 24, 48, and 72 h, respectively. Our findings showed that Gemini-Cur effectively decreased the expression levels of lnc-CCAT2 and c-Myc genes. Western blotting analysis also confirmed the down-regulation of c-Myc in treated samples compared to controls.

**Conclusion:** Gemini-Cur attenuates the proliferation of AGS cells partly through modulation of the lncCCAT2-related pathway.

**Keywords:** AGS cells, Gastric cancer, Gemini Curcumin, Metastasis, Lnc-CCAT2 and c-Myc.



## 1. Introduction

The term cancer refers to a complex disease that has the main characteristic is unregulation of cell growth, aggression and spreading from the original place to the other organs of the body.<sup>[1]</sup> Gastric cancer is the fourth most common tumor malignancy worldwide and is the second cause of mortality.<sup>[2]</sup> However, the widespread of gastric cancer is higher in developing countries.<sup>[3]</sup> Colon cancer-associated transcript-2 (CCAT2) gene is a member of the long non-coding RNAs (lncRNAs), which is notably overexpressed in microsatellite-stable colorectal cancer and promote oncogenesis, metastasis, and chromosomal instability.<sup>[4, 5]</sup> In the area with high expression, the number of point mutations and centromeric displacements increases dramatically.<sup>[6]</sup> The expression of this gene enhances the number of fragile chromosomes in the body,<sup>[7]</sup> on the other hand, it causes fragile x and Huntington's diseases.<sup>[8]</sup> A comprehensive literature survey revealed that CCAT2 gene is a causative agent in infections related to fragile chromosomes.<sup>[4]</sup> The gene Accession number: (nr\_109834.1) has a total of one exon, which categorize in the lncRNAs classification.<sup>[9]</sup> Various studies also confirm that CCAT2 gene is up-regulated in a variety of cancers including ovarian, colon, gastric, liver and lung.<sup>[10, 11]</sup> Guo *et al.* realized that CCAT2 directly enhanced the c-Myc expression in glioma cells.<sup>[12]</sup> On the other hand, micro-RNA-33b prevents osteosarcoma cell invasion, proliferation and migration by targeting the c-Myc expression,<sup>[13]</sup> so that the understanding of the dynamic lncRNAs-MYC network is found to be more complicated.<sup>[14]</sup> On this occasion, Yan *et al.* concluded that CCAT2 overexpression significantly increases the LATS2 and c-Myc expression in osteosarcoma cells.<sup>[15]</sup> Therefore, finding the new bioactive resources or modify them to suppress the expression of lnc-CCAT2 and its downstream c-Myc genes are of particular interest for researchers as tumor suppressor candidates. Curcumin (Cur) is a diarylheptanoid natural product isolated from the rhizomes of *Curcuma longa* L. (family Leguminosae).<sup>[16]</sup> Extensive studies show that Cur can effectively modulate many cancer symptoms, including anti-invasive behavior, uncontrolled cell proliferation, cancer-associated inflammation, cell death, angiogenesis, and metastasis.<sup>[17]</sup> Particularly, Cur can inhibit epithelial-to-mesenchymal transition (EMT)/metastasis via various pathways and mechanism in human tumors.<sup>[18]</sup> However, its weak solubility in water, metabolism and rapid excretion from the body are the main obstacles that limit the use of Cur as an anti-cancer therapeutic compound.<sup>[19]</sup> Different methods have been developed to increase the effectiveness of Cur, one of which is the employment of Gemini Surfactant nanoparticles.<sup>[20]</sup> This special structure provides some advantages such as low critical micelle concentration (CMC), high solubility and low cost for these nanoparticles, which has been considered as drug carriers.<sup>[21]</sup> Therefore, Cur coating with Gemini surfactant nanoparticles (Gemini-Cur) may increase the cellular absorption of Cur and increase its anti-cancer effects. In this study, we aim to evaluate the anticancer properties of Gemini-Cur on gastric cancer AGS cells via the expression of lncRNA CCAT2 and its downstream c-Myc, a well-known oncogenic transcription factor linked to tumorigenesis in most types of cancers.

## 2. Materials and Methods

### 2.1. Chemicals and reagents

Curcumin and Gemini surfactant nanoparticles were a kind gift from Dr. Farhood Najati. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder and RPMI-1640 medium (R5886) were purchased from GIBCO Co. (USA). Dimethyl sulfoxide (DMSO, 99.9%) and Hoechst were purchased from Merck (Germany). Fetal bovine serum (FBS), phosphate-buffered saline (PBS 1X) and trypsin (0.25% EDTA solution) were obtained from Gibco (Taiwan).

### 2.2. Gemini Curcumin preparation

Gemini-Cur was prepared by single-step nano-precipitation method described in our previous works.<sup>[22]</sup> Briefly, 6 mg of curcumin and 100 mg of ethoxyl-poly urethane gemini surfactants (both of them as a gift by Dr. Farhood Najafi, Institute for Color, Science and Technology, Tehran, Iran) were dissolved in 3 mL of methanol. After evaporation of methanol in rotary evaporator at room temperature for at least 6 h, the Gemini-Cur were lyophilized and stored at 4°C until use. The characterization of Gemini-Cur was studied according to our recent works.<sup>[21, 23]</sup>

### 2.3. Cell Culture

The human gastric cancer cell line AGS was acquired from the National Cell Bank of Iran (Pasteur Institute of Tehran, Iran). These cells were cultured in the Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 10% fetal bovine serum (FBS) and 0.5% penicillin/streptomycin and kept in a humidified cell culture incubator containing 5% CO<sub>2</sub> at 37°C.

### 2.4. Cell Viability Assay

Cytotoxicity of free curcumin and Gemini-Cur on AGS cells was evaluated using MTT assay. In brief, 1×10<sup>4</sup> cells were seeded in 200 µL of media at a 96-well plate for 24 h. Having sufficient density, AGS cells were treated with various concentrations ranging from 0 to 100 µM of curcumin in free and nano forms in 5% FBS-RPMI media and incubated for 24, 48, and 72 h. At the end of incubation time, 20 µL of MTT solution (5 mg/mL) was added to each well and then incubated for 3 h. Finally, the MTT containing media was replaced with 100 µL DMSO and furthermore incubated for 30 min. The absorbance at 570 nm was read. The inhibitory effect on AGS cells was measured using the following formula:

$$I\% = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

Where  $A_{blank}$  is the absorbance of the control reaction and  $A_{sample}$  is the absorbance of the test compound. By means of the obtained inhibition percentages, cytotoxicity was expressed as IC<sub>50</sub> (the concentration causing 50% inhibition).<sup>[24]</sup>

### 2.5. Scratch Test

Cell migration ability was examined by using scratch assay. After scratching by yellow tip head in the middle of the monolayer, AGS cells were seeded in 6-well plates and then treated with 40.88 µM Gemini-Cur. The plates containing cells were photographed at the cleft site at zero times, immediately after scratching, and after 24, 32, and 48 h, using an Invert microscope under a 10X magnification lens.<sup>[25]</sup>

### 2.6. RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA from cultured cells was extracted using BRIZOL reagent (Fara gene, Iran) following the phenol guanidinium thiocyanate method and its manufacturer's protocol. The quality and quantity of RNAs were checked using agarose gel electrophoresis and Picodrop spectrophotometer (Termo Fischer, USA). DNase I treatment was employed to eliminate any DNA contamination and then, complementary DNA (cDNA) synthesis was accomplished by cDNA synthesis kit (Thermo Scientific, USA). The reaction was applied by SYBR Green master mix (AMPLICON, Iran) and appropriate primers. The primers were planned using Oligo7 software (Table 1). In this study, the total volume for real-time PCR reaction reached 10 µL consisted of 5 µL of SYBR Green, 1 µL of forward and reverse primers, 1 µL of cDNA template, and 3 µL of ddH<sub>2</sub>O. Step One TM Real-Time PCR System quantitative PCR was done to evaluate the expression of Lnc-CCAT2 and c-Myc genes. All tests were done at least twice with the accordance situation: 95°C for 10 min, 95°C for 15 s and 60°C for 30 s. The

melting curve was specified in 60°C to 95°C. Relative gene expression was computed as  $2^{-\text{ddcT}}$  [26]

### 2.7. Western blotting assay

Having lysed the treated AGS cells, the total protein from established cells was extracted and stored in the freezer -20°C. Based on the difference in light absorption at 630 wavelengths in the device, the protein concentration was determined by Bradford protein assay. Then, the prepared protein samples must be concentrated before pouring into the well and mixed with the sample buffer. The electrophoresis was performed on SDS plate gel. After western blotting or immunostaining, in which c-Myc proteins are detected by specific antibodies, the samples are electrically transferred from the gel to the surface of PVDF paper. The paper is mixed and diluted with the primary  $\beta$ -actin antibody (sc-47778, 1:300) and incubated. In the secondary antibody incubation phase, the paper is quenched with Anti Rabbit (1:1000) for all primary antibodies at room temperature. This diagnosis was used by the Advanced ECL Reagent Kit, which includes skim milk and reagents A and B used in this study.

### 2.8. Statistical Analysis

The difference between groups was analyzed by the student's t-test. Results are presented as mean value  $\pm$  standard deviation (SD). Statistical significance was considered as  $p < 0.05$ . For the cytotoxicity test, the inhibitory concentration (IC<sub>50</sub>) was calculated using program Curve Expert 1.3 (Cure Expert statistical software).

## 3. Results

### 3.1. Cell viability

The cytotoxicity of free curcumin and Gemini-Cur on AGS cells was analyzed by MTT assay (Fig.1). Gemini-Cur treatment for 24, 48, and 72 h demonstrated cytotoxicity on AGS cells in a time- and dose-dependent manner. However, we did not detect any significant toxicity of free curcumin in similar doses. The viability of AGS cells was minimized and achieved almost 80% after treating with 20  $\mu\text{M}$  ( $p = 0.0004$ ) of Gemini-Cur for 48 h. Interestingly, incubation of AGS cells with 20  $\mu\text{M}$  of Gemini-Cur for 72 h, meaningfully decreased the cell viability and reached around 50%. Hence, the IC<sub>50</sub> values were calculated to be 59.32, 40.88, and 19.63  $\mu\text{M}$  during 24, 48, and 72 h, respectively.

### 3.2. Cell scratch Test

The assessment of cell metastasis and the width of the scratch were measured under an Invert microscope under a 10X lens at 0, 24, 32, and 48 h. It showed that the scratches of the treated cells were wider than the control group at the same time (Fig. 2 A). This indicated that non-treated AGS cells invade scratched parts in a time-dependent manner. However, cells treated with 40.88  $\mu\text{M}$  showed fewer cells in scratched spaces ( $p < 0.0001$ ). Considering all factors, our data show that Gemini-Cur inhibits invasion of gastric cancer AGS cells (Fig. 2B).

### 3.3. Lnc-CCAT2 and c-Myc expression studies

Gemini-Cur affects the expression of lnc-CCAT2 and its sub-gene c-Myc in both gene and protein levels. Real-time PCR data showed that CCAT2 is significantly down-regulated in treated cells rather than c-Myc ( $p < 0.01$ , Fig. 3). Western blotting also confirmed that c-Myc expression is decreased in treated cells compared to control ( $p < 0.01$ , Fig. 4).

## 4. Discussion

Gastric cancer ranks the second leading cause of cancer-related death.<sup>[27, 28]</sup> CCAT2 expression is significantly elevated in gastric cancer tissues compared to the adjacent non

tumoral gastric specimens.<sup>[29]</sup> The level of CCAT2 expression is also positively correlated with the lymph node involvement and distance metastasis<sup>[30]</sup> and served as an independent predictive factor for shorter overall survival in gastric cancer patients.<sup>[31]</sup> These findings indicate that up-regulation of CCAT2 is correlated with gastric cancer development and metastasis, and might function as a potential prognostic biomarker for stratifying gastric cancer patients with different clinical outcomes.

It is worth mentioning that previous researches indicated that pure Cur shows a significant inhibitory effect on AGS cell lines in a dose- and time-dependent manner.<sup>[32, 33]</sup> Meanwhile, our results of MTT assay showed that Gemini-Cur reduces the viability of the same cell lines in a dose- and time-dependent manner. These outcomes are in accordance with Karimpour *et al.*<sup>[4]</sup> and Zibaei *et al.*<sup>[5]</sup> studies regarding the enhancement of toxicity of Cur in the form of Gemini-Cur nanoparticles' on breast and colon cancer cells.<sup>[21, 23]</sup>

Considering the capacity of CCAT2 in the migration and invasion of AGS cells, which is an imperative process for metastasis, the results showed that Gemini-Cur at a concentration of 40.88  $\mu\text{M}$  not only has no cytotoxic impact on normal cells but also ceases the migration of AGS cells. It effectively reduces the expression of CCAT2 gene and its downstream c-Myc as well (Fig. 2). Kim *et al.* examined the expression of CCAT2 gene in pancreatic cancer patients and healthy individuals in the American community by real-time PCR. The authors concluded that CCAT2 expression was increased when compared to control. Moreover, the CCAT2 gene plays an important role in pancreatic cancer.<sup>[34-36]</sup> Also, Wang *et al.* studies in hepatocellular carcinoma demonstrated the oncogenic role of this gene and concluded that it plays a major role in cell proliferation and cancer cell migration.<sup>[37, 38]</sup> All the findings of the present experiments were in agreement with the previous data.

In a study on the expression of CCAT2 gene in gastric cancer and its effect on invasion and metastasis, Wang *et al.* concluded that the CCAT2 expression increases in gastric cancer, which is directly related to invasion and metastasis.<sup>[39]</sup> Given that cell migration is an essential process for metastasis, our results manifest that the Gemini-Cur at concentration of 40.88  $\mu\text{M}$  significantly suppresses the metastasis. It is worth mentioning that the present findings confirm that naturally occurring metabolites such as curcumin, interrupt the metastasis by affecting multiple pathways.<sup>[40]</sup> Therefore, the plant preparations and natural drug combinations can act as an effective therapeutic approach for tumor suppressors without having toxic side effects on healthy and common tissues.<sup>[41-43]</sup> Of note, there have been no significant reaction of treated cells at 32 and 48 h, in other words, the drug will act only dose-dependently. Xin *et al.* examined the levels of CCAT2 gene expression by real-time PCR in normal and ovarian cancer tissue and cells. The authors concluded this gene was more expressed in cancer tissues than normal specimens, and the higher CCAT2 expression, the shorter the cells survived.<sup>[29]</sup> In the present study, the expression of CCAT2 gene in AGS gastric cancer cell line was evaluated and a significant correlation was observed with  $p < 0.001$ . These findings indicate not only an important role of CCAT2 gene in gastric cancer but a direct relationship between the CCAT2 gene expression and cell metastasis.

## 5. Study limitations

Future cancer researches especially pathway and whole genome studies are warranted to validate the findings of the present study by using clinically relevant animal models for the diverse therapeutic uses of Gemini-Cur. Besides, a comprehensive analysis of biocompatibility and toxicity of Gemini-Cur should be done using appropriate cell lines *in vitro* and *in vivo*.

## 6. Conclusion

In this study, gastric cancer cell line AGS was treated with a nano form of Cur (Gemini-Cur).

The results of our study were in line with the former studies performed on other cancer cell lines. To the best of our knowledge, the significant effect of Gemini-Cur in the modulation of CCAT2 gene expression and its underlying c-Myc was observed, which is also associated with preventing metastasis. Scratch test revealed a decreasing effect of metastasis in Gemini-Cur treated AGS cells indicating an anti-cancer potency of Gemini-Cur in appropriate concentration and time. This phenomenon can potentially reduce both cell proliferation and cell migration in AGS gastric cancer cell line. **Finally**, yet importantly, the further deep investigation of Gemini-Cur and its various applications are strongly recommended to strengthen the claims of being curcumin related nanoparticles as anti-cancer agents.

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### **Conflict of interest**

No potential competing interest was declared by the authors.

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**Figures and Legends:**

**Figure 1:** Cellular viability of AGS cells treated with free curcumin (A) and Gemini-Cur (B) in three-time intervals of 24, 48 and 72 h.

**Figure 2:** A) Evaluation of the effect of Gemini-Cur on the migration of treated and non-treated AGS cells using the scratch test. B) Percentage of migrated cells. Data analysis indicated a significant reduction in number of cells in scratched spaces in treated samples compared to control. \*\*\*\*:  $p < 0.0001$

**Figure 3:** Evaluation of the expression of CCAT2 and c-Myc in treated AGS cells compared to non-treated cells (control) in a time-dependent manner. \*\*:  $p < 0.001$

**Figure 4:** Western blotting data of c-Myc protein expression compared to non-treated cells (control). As proteins bands (A) and diagram (B) show, c-Myc is down-regulated in treated cells after 48 hours. \*\*:  $p < 0.001$

Figure 1

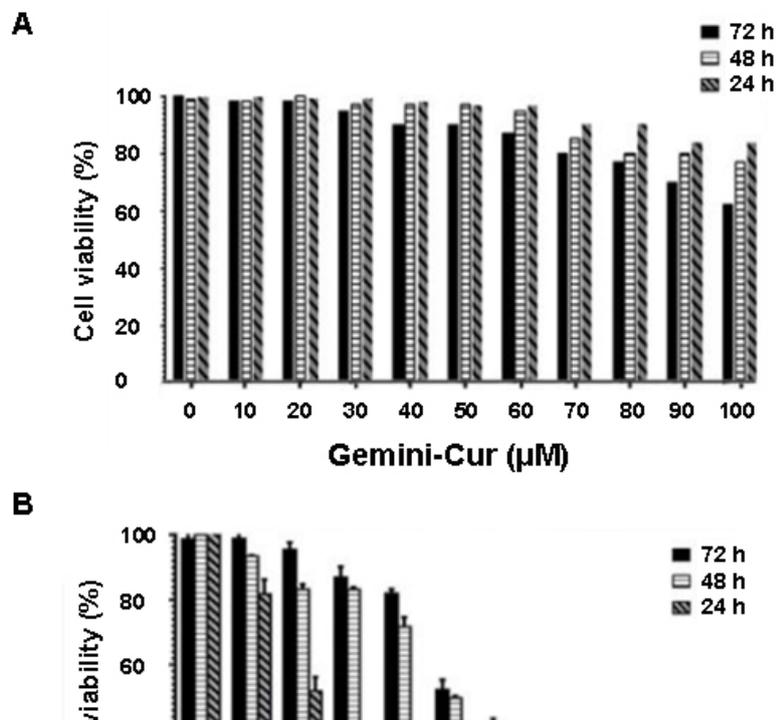


Figure 1

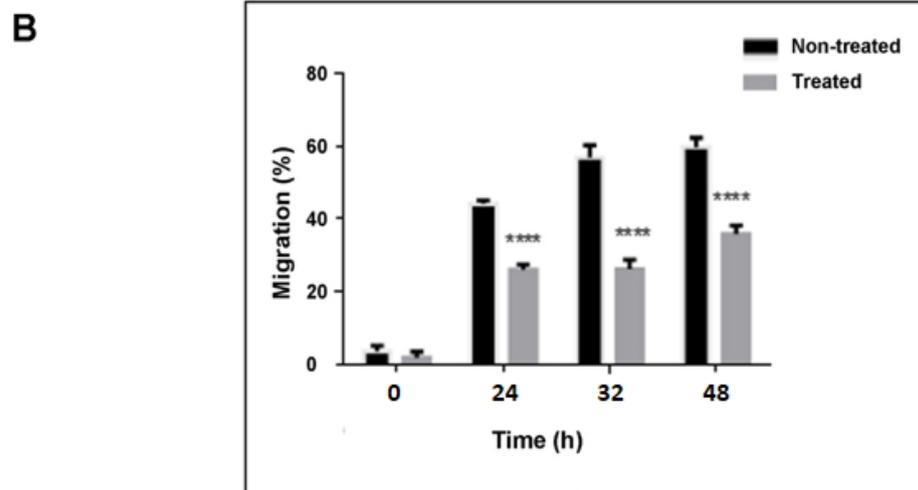
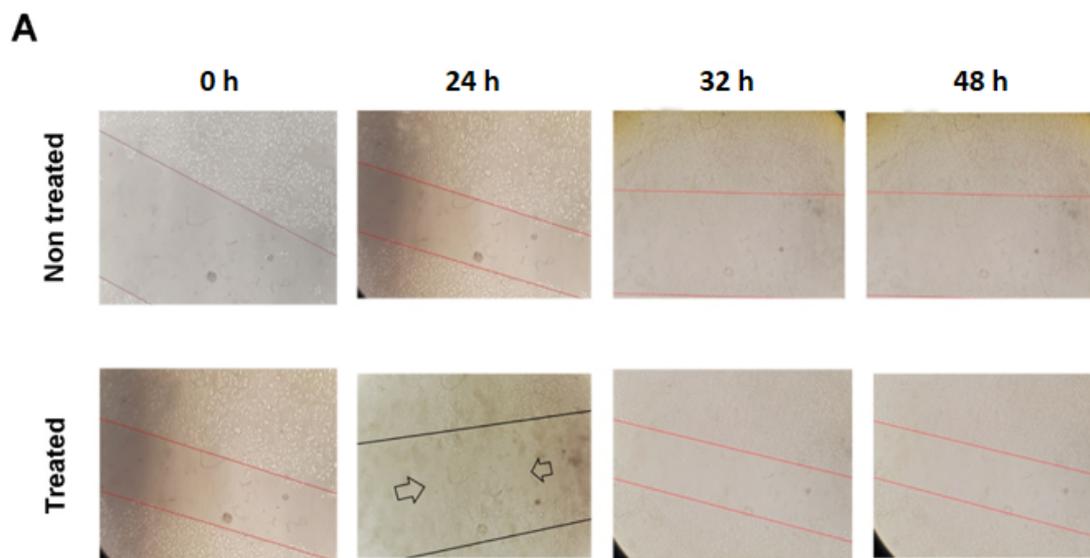
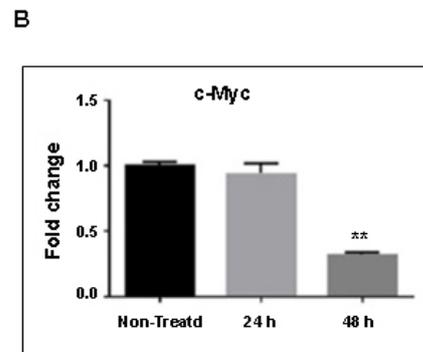
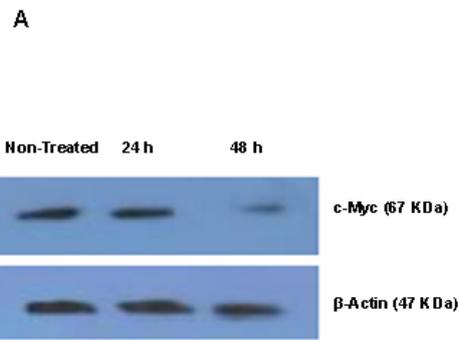
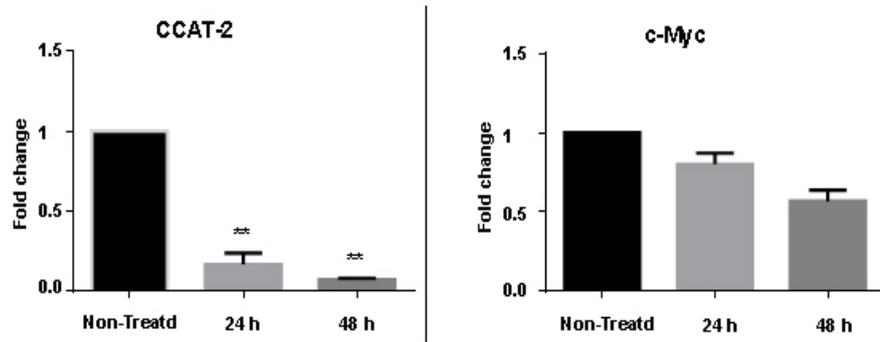


Figure 2

**Figur**



**Figure 4**

**Table 1:** Sequences of primers used in real-time PCR.

**Table 1**

<b>Genes</b>	<b>Sequences (5'→3')</b>	<b>T<sub>m</sub> (°C)</b>
CCAT2	F: 5'-CTACCAGCAGCACCATTTCAG-3'	59.2°C
	R: 5'-CACCAGATACACCCCAGAGAG-3'	
c-Myc	F: 5'-CTCGGTTTCTCTGCTCTCCTC-3'	59.8°C
	R: 5'-TTCCTCATCTTCTTGTTCTCC-3'	
β-actin	5'-AGAGCTACGAGCTGCCTGAC-3'	57°C
	5'-AGCACTGTGTTGGCGTACAG-3'	