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ORIGINAL ARTICLE

Gemini Curcumin Suppresses Gastric Cancer AGS Cell Proliferation Through Modulation of *lncRNA CCAT2* and *c-Myc* Genes

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ABSTRACT

Objectives: Gemini surfactant nanocurcumin (Gemini-Cur) is a novel formulation of Curcumin (Cur) with dramatic suppressive effects on cancer cells. Here, we investigated the cancer effects of Gemini-Cur in a human gastric adenocarcinoma cell-line (AGS) through the evaluation of the expression of long non-coding RNAs colon cancer-associated transcript-2 (*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 Cur in a time- and dose-dependent manner. The toxicity of Gemini-Cur was studied using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and scratch tests. Furthermore, real-time polymerase chain reaction 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 inhibitory concentration 50 values of 59.32, 40.88, and 19.63 μM 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.

Key words: AGS cells, gastric cancer, gemini curcumin, metastasis, *lnc-CCAT2* and *c-Myc*

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.[1] Gastric cancer is the fourth most common tumor malignancy worldwide and is the second cause of mortality.[2] However, the widespread of gastric cancer is higher in developing countries.[3] Colon cancer-associated transcript-2 (*CCAT2*) gene is a member of the long non-coding RNAs (*lncRNAs*), which are notably overexpressed in microsatellite-stable colorectal cancer and promote oncogenesis, metastasis, and chromosomal instability.[4,5] In the area with high expression, the number of point mutations and centromeric displacements increases dramatically.[6] The expression of this gene enhances the number of fragile chromosomes in the body,[7] on the other hand, it causes fragile X and Huntington's disease.[8] A comprehensive literature survey revealed that the *CCAT2* gene is a causative agent in infections related to fragile chromosomes.[4] The gene accession number (nr_109834.1) has a total of one exon, which is categorized in the *lncRNAs* classification.[9] Various studies also confirmed that the *CCAT2* gene is up-regulated in various cancers including ovarian, colon, gastric, liver and lung.[10,11] Guo et al.[12] realized that *CCAT2* directly enhanced the *c-Myc* expression in glioma cells. On the other hand, micro-RNA-33b prevents osteosarcoma cell invasion, proliferation and migration by targeting the *c-Myc* expression,[13] so that the

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understanding of the dynamic *lncRNAs-MYC* network is found to be more complicated.[14] On this occasion, Yan et al.[15] concluded that *CCAT2* overexpression significantly increased the LATS2 and *c-Myc* expression in osteosarcoma cells. Therefore, finding the new bioactive resources or modifying 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. (Zingiberaceae).[16] 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.[17] Particularly, Cur can inhibit epithelial-to-mesenchymal transition/metastasis *via* various pathways and mechanisms in human tumors.[18] 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.[19] Different methods have been developed to increase the effectiveness of Cur, one of which is the employment of gemini surfactant nanoparticles (Gemini-Cur).[20] This special structure provides the advantages such as low critical micelle concentration, high solubility, and low cost for these nanoparticles, which have been considered drug carriers.[21] Therefore, Cur coating with Gemini-Cur may increase the cellular absorption of Cur and increase its anti-cancer effects. In this study, we aimed 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.

MATERIALS AND METHODS

Chemicals and reagents

Gemini-Cur was 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).

Gemini curcumin preparation

Gemini-Cur was prepared by single-step nano-precipitation method described in our previous works.[22] Briefly, 6 mg Cur 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 methanol. After the evaporation of methanol in a rotary evaporator at room temperature for at least 6 h, Gemini-Cur was lyophilized and stored at 4°C until use. Characterization of Gemini-Cur was studied according to our recent works.[21,23]

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% FBS and 0.5% penicillin/streptomycin and kept in a humidified cell culture incubator containing 5% CO_2 at 37°C.

Cell viability assay

Cytotoxicity of free Cur and Gemini-Cur on AGS cells was evaluated using the MTT assay. In brief, 1×10^4 cells were seeded in 200 µL of media in a 96 well plate for 24 h. Having sufficient density, AGS cells were treated with various concentrations ranging from 0 to 100 µM of Cur in free and nanoforms in 5% FBS-RPMI medium 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, MTT containing media was replaced with 100 µL DMSO and furthermore incubated for 30 min. The absorbance was at 570 nm. 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 using the obtained inhibition percentages, cytotoxicity was expressed as inhibitory concentration 50 (IC_{50}) (the concentration causing 50% inhibition).[24]

Scratch test

The cell migration ability was examined using a scratch assay. After scratching by the 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.[25]

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 a Picodrop spectrophometer (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 with 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 the real-time PCR (RT-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_2O. Step one TM real-time PCR system quantitative PCR was performed 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 as 60°C to 95°C. Relative gene expression was computed as 2^{-ddcT}.[26]

Western blotting assay

Having lysed the treated AGS cells, total protein from established cells was extracted and stored in the freezer -20°C. Based on the difference in light absorption at 630 nm wavelength in the device, the protein concentration was determined by the Bradford protein assay. Then, prepared protein samples must be concentrated before pouring into the well and mixed with the sample buffer. The electrophoresis was performed on sodium dodecylsulphate plate gel. After Western blotting or immunostaining, in which *c-Myc* proteins were detected by specific antibodies, the samples were electrically transferred from the gel to the surface of the polyvinylidene fluoride paper. The paper was mixed and diluted with the primary *β*-actin antibody (sc-47778, 1:300) and incubated. In the secondary antibody incubation phase, the paper was 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.

Statistical analysis

The difference between groups was analyzed by the student's *t*-test. Results were presented as the mean value ± standard deviation. Statistical significance was considered $p<0.05$. For the cytotoxicity test, IC_{50} was calculated using the program Curve Expert 1.3 (Cure Expert statistical software).

RESULTS

Cell viability

Cytotoxicity of free Cur and Gemini-Cur on AGS cells was analyzed by MTT assay (Figure 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 Cur at similar doses. Viability of AGS cells was minimized and achieved almost 80% after treatment with 20 μM (p= 0.0004) of Gemini-Cur for 48 h. Interestingly, incubation of AGS cells with 20 μM of Gemini-Cur for 72 h, meaningfully decreased the cell viability and reached around 50%. Hence, IC_{50} values were calculated to be 59.32, 40.88, and 19.63 μM during 24, 48, and 72 h, respectively.

Table 1. Sequences of primers used in real-time polymerase chain reaction

Genes	Sequences (5'→3')	T_m (°C)
CCAT2	F: 5'-*CTACCAGCAGCACCATTTCAG*-3'	59.2°C
	R: 5'-*CACCAGATACACCCCAGAGAG*-3'	
c-Myc	F: 5'-*CTCGGTTTCTCTGCTCTCCTC*-3'	59.8°C
	R: 5'-*TTCCTCATCTTCTTGTTCCTCC*-3'	
β-actin	5'-*AGAGCTACGAGCTGCCTGAC*-3'	57°C
	5'-*AGCACTGTGTTGGCGTACAG*-3'	

Cell scratch test

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

Lnc-CCAT2 and c-Myc expression studies

Gemini-Cur affects the expression of *lnc-CCAT2* and its subgene *c-Myc* at both the gene and protein levels. RT-PCR data showed that *CCAT2* is significantly down-regulated in treated cells rather than *c-Myc* ($p<0.01$, Figure 3). Western blotting also confirmed that *c-Myc* expression is decreased in treated cells compared with controls ($p<0.01$, Figure 4).

DISCUSSION

Gastric cancer is the second leading cause of cancer-related death.[27,28] *CCAT2* expression is significantly elevated in gastric cancer tissues compared to with adjacent non-tumoral gastric specimens.[29] The level of *CCAT2* expression is also positively correlated with the lymph node involvement and distance metastasis[30] and serves as an independent predictive factor

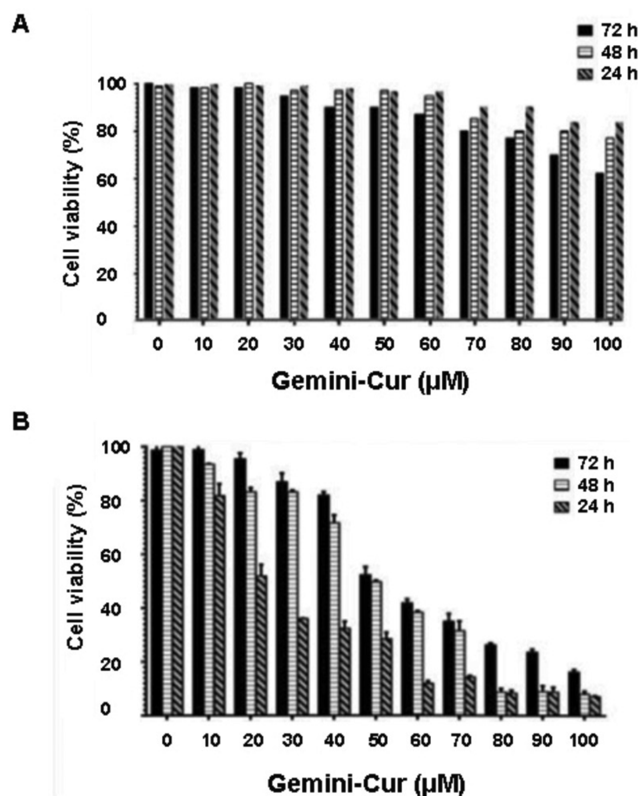

Figure 1. Cellular viability of AGS cells treated with free curcumin (A) and Gemini-Cur (B) at three-time intervals of 24, 48, and 72 h

Gemini-Cur: Gemini surfactant nanocurcumin

for shorter overall survival in gastric cancer patients.[31] 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 research indicated that pure Cur shows a significant inhibitory effect on AGS cell lines in a dose- and time-dependent manner.[32,33] Meanwhile, our results of MTT assay showed that Gemini-Cur reduces viability of the same cell lines in a dose- and time-dependent manner. These

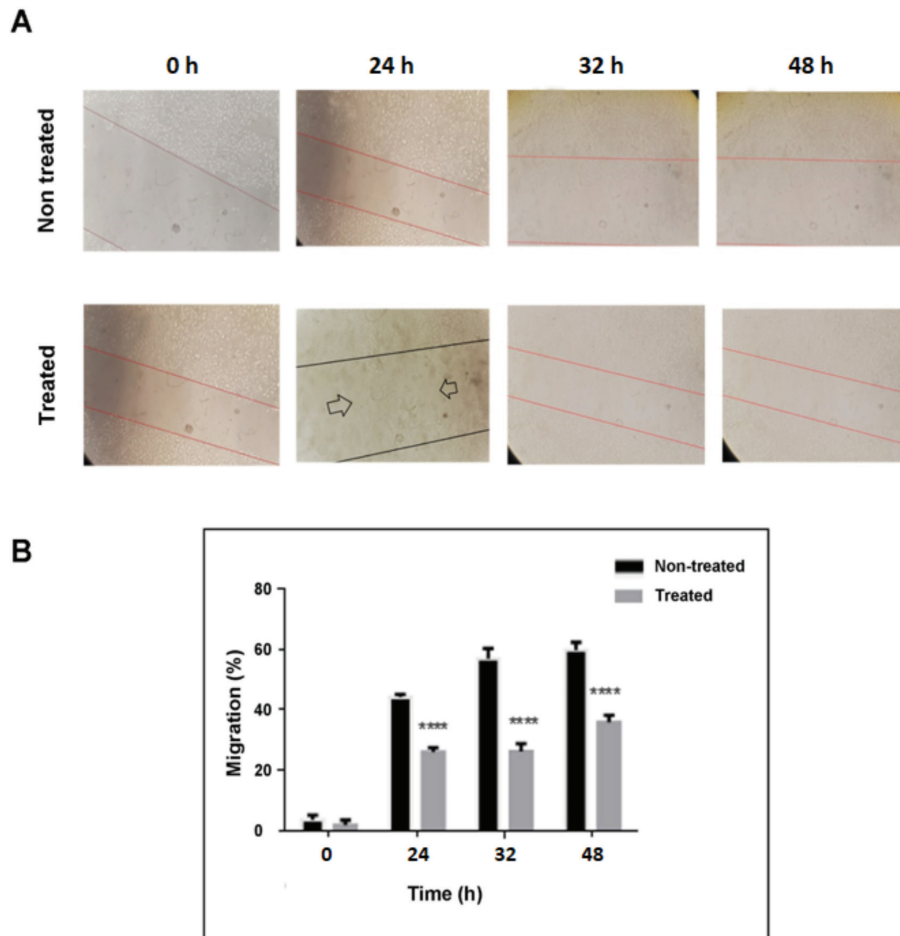

Figure 2. A) Evaluation of the effect of Gemini-Cur on the migration of treated and non-treated AGS cells using the scratch test. B) Percentage of migrated cells. Data analysis indicated a significant reduction in the number of cells in scratched spaces in treated samples compared to control

****: $p<0.0001$, Gemini-Cur: Gemini surfactant nanocurcumin

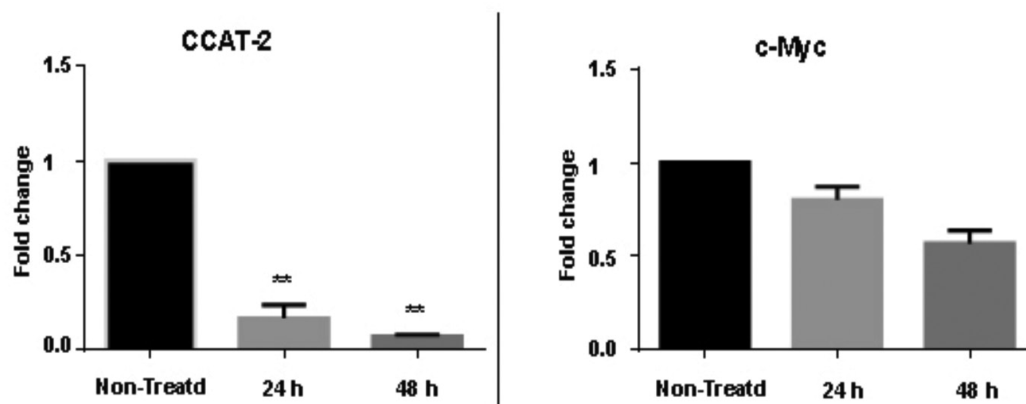

Figure 3. Evaluation of the expression of *CCAT2* and *c-Myc* in treated AGS cells compared with non-treated cells (control) in a time-dependent manner

**: $p<0.001$, *CCAT2*: Colon cancer-associated transcript-2

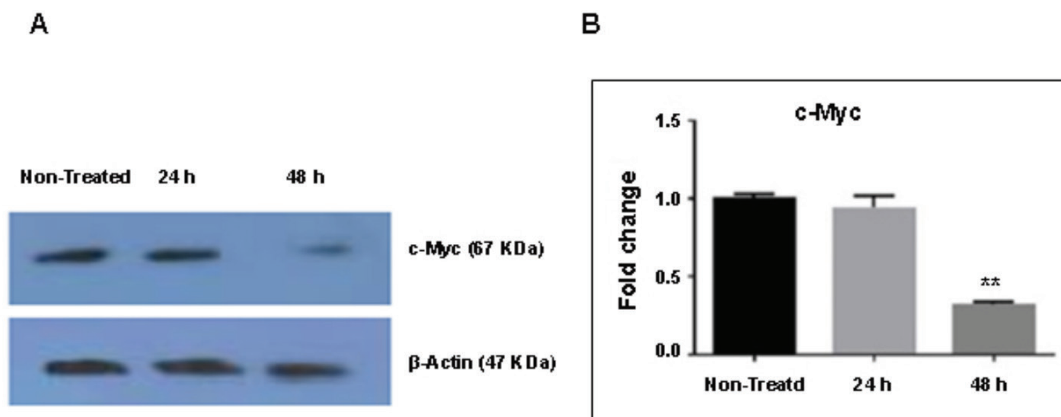

Figure 4. Western blotting data of *c-Myc* protein expression compared with non-treated cells (control). As protein bands (A) and diagram (B) show, *c-Myc* is down-regulated in treated cells after 48 hours
**: $p<0.001$

outcomes are in accordance with that of Karimpour et al.[23] and Zibaei et al.[21] studies regarding the enhancement of toxicity of Cur in the form of Gemini-Cur nanoparticles on breast and colon cancer cells.

Considering the capacity of *CCAT2* in the migration and invasion of AGS cells, which was an imperative process for metastasis, the results showed that Gemini-Cur at a concentration of 40.88 µM not only had no cytotoxic impact on normal cells but also ceased the migration of AGS cells. It effectively reduces the expression of *CCAT2* gene and its downstream *c-Myc* as well (Figure 2). Kim et al.[34] examined the expression of *CCAT2* gene in pancreatic cancer patients and healthy individuals in the American community by RT-PCR. The authors concluded that *CCAT2* expression was increased compared to control. Moreover, the *CCAT2* gene plays an important role in pancreatic cancer.[34-36] Also, Wang et al.[37,38] studies in hepatocellular carcinoma demonstrated the oncogenic role of this gene and concluded that it played a major role in cell proliferation and cancer cell migration. All the findings of this experiment 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.[39] concluded that *CCAT2* expression increased in gastric cancer, which was directly related to invasion and metastasis. Given that cell migration is an essential process for metastasis, our results manifest that the Gemini-Cur at a concentration of 40.88 µM significantly suppresses the metastasis. It is worth mentioning that the present findings confirm that naturally occurring metabolites such as Cur interrupt the metastasis by affecting multiple pathways.[40] Therefore, the plant preparation and natural drug combinations can act as an effective therapeutic approach for tumor suppressors without having toxic side effects on healthy and common tissues.[41-43] Of note, there has 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.[29] 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. In this study, the expression of *CCAT2* gene in the 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 the *CCAT2* gene in gastric cancer but a direct relationship between the *CCAT2* gene expression and cell metastasis.

Study limitations

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

CONCLUSION

In this study, gastric cancer cell line AGS was treated with a nanoform of Cur (Gemini-Cur). The results of our study were in line with the former studies conducted on other cancer cell lines. To the best of our knowledge, a significant effect of Gemini-Cur on the modulation of *CCAT2* gene expression and its underlying *c-Myc* was observed, which is also associated with preventing metastasis. Scratch tests 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 reduce both cell proliferation and cell migration in the 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 Cur-related nanoparticles as anti-cancer agents.

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Ethics

Ethics Committee Approval: The study was approved by the graduate studies committee at the University of Tabriz (3/11242/100).

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: N.J., E.B., S.H.G., Design: N.J., E.B., Data Collection or Processing: N.J., H.S., Analysis or Interpretation: N.J., B.M.H., E.B., Literature Search: N.J., H.S., B.M.H., Writing: N.J., S.H.G., E.B.

Conflict of Interest: No conflict of interest was declared by the authors.

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