Evaluation the effect of Iron oxide nanoparticles functionalized by glucose and conjugated with Coumarin (Fe3O4@Glu-coumarin NPs) on expression of CASP8, CASP9, p53, mTOR1, and MAPK1 genes in liver cancer cell line

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Abstract

Background: Due to the high morbidity and mortality of liver cancer, many researchers are seeking novel anticancer formulations. Complex iron oxide containing compounds could be used for site directed drug delivery using a magnetic force. Due to the anticancer property of coumarin, this study aimed to study the effect of iron oxide nanoparticles functionalized by glucose and conjugated with Coumarin ($\text{Fe}_3\text{O}_4@\text{Glu-coumarin NPs}$) on the expression of cell cycle and apoptosis regulating genes in a liver cancer cell line.

Methods: $\text{Fe}_3\text{O}_4@\text{Glu-coumarin NPs}$ were synthesized and their physicochemical properties were evaluated by FT-IR, EDS mapping, and electron microscope imaging. Anti-proliferative activity of the NPs on HepG2 cells was studied by MTT assay. The effect of $\text{Fe}_3\text{O}_4@\text{Glu-coumarin NPs}$ on the expression of $\text{CASP8}$, $\text{CASP9}$, $\text{p53}$, $\text{mTOR1}$, and $\text{MAPK1}$ genes was investigated by quantitative PCR.

Results: The synthesized NPs were spherical, with a moderate level of aggregation and in a size range of 26-46 nm. FT-IR and EDS mapping confirmed the proper synthesis and purity of the synthesized NPs. According to the MTT assay, $\text{Fe}_3\text{O}_4@\text{Glu-coumarin NPs}$ showed a considerable anti-proliferative effect on liver cancer cells, and the 50% inhibitory concentration was determined 120µg/mL. Quantitative PCR assay showed that the NPs significantly increased the expression of $\text{CASP8}$, $\text{p53}$, and $\text{MAPK1}$ genes by 1.94, 4.87, and 3.87 folds, respectively, while the $\text{mTOR1}$ gene was reduced by -6.33 folds. The expression of the $\text{CASP9}$ had an insignificant reduction by 0.06 folds.

Conclusions: Our results showed that $\text{Fe}_3\text{O}_4@\text{Glu-coumarin NPs}$ could induce cell apoptosis by interfering with the expression of the cell regulatory genes.

Introduction

Liver cancer is a very prevalent cancer that causes a large number of deaths, worldwide. As the 3rd leading cause of cancer associated deaths, treatment of the disease faces several difficulties which are mainly related to poor diagnosis and inefficient therapeutic options [1]. Similar to other cancers, treating drug resistant liver cancer is hardly achieved and toxic side effects of current anticancer drugs is the main obstacle in cancer chemotherapy. In this regard, a large number of researchers are seeking efficient novel anticancer formulations with improved biocompatibility.

The emergence of nanotechnology has attracted research interest in the design and characterization of nano-scale formulations for cancer diagnosis and treatment [2]. Extensive studies have been conducted on the anticancer features of metal oxide nanoparticles (NPs). Despite the considerable anticancer and anti-tumor properties of metal oxide NPs, they rarely reach clinical trials which is mainly due to their low biocompatibility and considerable toxic side effects [3]. However, owing to their satisfactory biocompatibility and stability, iron oxide NPs have received great attention in cancer medicine [4]. In addition, due to the magnetic property, iron oxide NPs could be employed to drive therapeutic
pharmaceuticals to the tumor site using an external magnetic force, which could avoid blind distribution of the drug in the body and as a result, decrease toxic side effects of cancer chemotherapy [5].

Coumarin (2H-1-benzopyran-2-one) and its derivatives have recently received considerable attention due to their pharmaceutical properties, including antioxidant, antimicrobial, and anti-inflammatory features [6]. In addition, a wide range of studies has reported the anticancer and apoptosis inducing potentials of natural and synthetic coumarins [6–8]. It was found that coumarin could reduce viability, arrest cell cycle, and induce apoptosis in several cancer cell lines [6–7]. However, the molecular basis of the anticancer feature of coumarin derivatives has been rarely studied. In this regard, the present study was performed to synthesize Iron oxide nanoparticles functionalized by glucose and conjugated with Coumarin (Fe₃O₄@Glu-coumarin NPs) and investigate the effect of the NPs on the expression of several regulatory genes, including CASP8, CASP9, p53, mTOR1, and MAPK1 in a liver cancer cell line.

**Materials And Methods**

**Synthesis of NPs**

In the first step, Fe₃O₄ NPs were synthesized as follows: 7.57 g of FeCl₃.6H₂O and 3.17 g of FeCl₂.4H₂O were added to 300 mL distilled water and heated 80°C. After 60 min, NH₃ solution was added and the mixture was additionally heated for 3 h to obtain Fe₃O₄ NPs. The synthesized NPs were harvested, washed with distilled water and ethanol, and finally, dried at 70°C for 6h [9].

Synthesized Fe₃O₄ NPs were functionalized by glucose as follows: 0.5 g of glucose and 1 g of Fe₃O₄ were suspended in 60 mL of distilled water and the suspension was subjected to sonication for 30 min. Next, the suspension was heated at 180°C for 3h to obtain Fe₃O₄@Glu NPs.

To synthesize Fe₃O₄@Glu-coumarin, 1 g of dried Fe₃O₄@Glu and 0.1 g of coumarin were suspended in distilled water in a total volume of 50 ml. The suspension was sonicated for 30 min and then, was shaken at room temperature for 24 h. Finally, Fe₃O₄@Glu-coumarin NPs were harvested by centrifugation, washed, and dried by lyophilization.

**Physicochemical properties of Fe₃O₄@Glu-coumarin NPs**

To evaluate the proper synthesis, the synthesized NPs and coumarin were subjected to an FT-IR analysis using a Perkin-Elmer FT-IR spectrophotometer. The analysis was performed in a wave range of 500–4000 cm⁻¹ and the resulting spectrogram was used to evaluate the functional groups of the particles. In addition, EDS mapping analysis was performed on Fe₃O₄@Glu-coumarin NPs to investigate the elemental composition and purity of the synthesized particles. Scanning and transmission electron microscopy (SEM and TEM) were also performed to study the morphological, size, and aggregation level of the synthesized Fe₃O₄@Glu-coumarin NPs.

**Cell culture and MTT assay**
The liver cancer cell line (HepG2) was purchased from the Pasteur institute of Iran. Cell culture was performed using Dulbecco’s modified Eagle medium (DMEM) medium supplemented with fetal bovine serum and penicillin-streptomycin. After preparation of a monolayer of HepG2 cells in a 25 cm² plate, the cells were seeded in 96-well plates to conduct MTT (2-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) assay. In brief, liver cancer cells were treated with a gradient concentration of Fe₃O₄@Glu-coumarin NPs (0-500 µg/mL) for 24 h at 37°C. Then, MTT solution (0.5 mg/mL) was added to the wells, incubated for 4h and then, the medium was aspirated from the wells. Next, 200 µL of DMSO was added to each well, incubated for an additional 30 min, and finally, the OD₅₉₀ of the wells was measured by a microplate reader (Bio-Rad, Hercules). The 50% inhibitory concentration (IC₅₀) of the NPs for HepG2 cells was calculated using the following formula [10–11].

\[
\text{Inhibition (%) } = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{Test}}}{\text{Abs}_{\text{control}}} \times 100
\]

**Quantitative PCR**

Quantitative PCR was used to study the effect of Fe₃O₄@Glu-coumarin NPs on the expression of the \( \text{CASP8, CASP9, p53, mTOR1, and MAPK1} \) genes. In brief, HepG2 cells were treated with the NPs (at IC₅₀ concentration) for 24 h, and then, the cells were harvested and subjected to RNA extraction using the TriZol™ (ThermoFisher) RNA extraction kit, according to the instruction.

Next, complementary DNA (cDNA) molecules were synthesized by Yekta Tajhiz (Iran) cDNA synthesis kit, according to the manufacturer’s protocol. The relative expression of the \( \text{CASP8, CASP9, p53, mTOR1, and MAPK1} \) genes in the control and NPs treated cells was investigated by qPCR using the gene specific primers that were presented in Table 1. The gene amplification was performed by 35 cycles of denaturation at 94°C for 30s, annealing at 57°C for 15s, and extension at 72°C for 20s. Reaction microtubes contained 12.5µL of SinaSYBR Blue HS-qPCR mix, 0.5 µL (300 nM) of forward and reverse primers, and 1 µL of cDNA template in a final volume of 25 µL. \( \text{GAPDH} \) gene was used as the internal control gene and the \( 2^{-\Delta\Delta t} \) method was used to calculate the relative expression of each gene [13].
Table 1
Sequence of the primers that were used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASP8-F</td>
<td>TTATTCAGGCTTGTCAGGGG</td>
<td>This study</td>
</tr>
<tr>
<td>CASP8-R</td>
<td>ATGTACCAGGTTCCCTCTGC</td>
<td></td>
</tr>
<tr>
<td>CASP9-F</td>
<td>ACATGCTGGCTTCGTTCCTG</td>
<td>This study</td>
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<td>CASP9-R</td>
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</tr>
<tr>
<td>p53-F</td>
<td>AGGTTGGCTCTGACTGTACC</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>mTOR-F</td>
<td>GGACCTCTGCTACACACCA</td>
<td>This study</td>
</tr>
<tr>
<td>mTOR-R</td>
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<td></td>
</tr>
<tr>
<td>MAPK1-F</td>
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<td>This study</td>
</tr>
<tr>
<td>MAPK1-R</td>
<td>CAGAATGCAGCCTACAGACC</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis

The assays were performed in three replicates. The SPSS software version 16.0 was used to study the significant difference between treatment and control groups using the *one-way ANOVA* analysis. A *p*-value of less than 0.05 was also considered statistically significant.

Results

Physicochemical properties

FT-IR spectrogram of coumarin and Fe₃O₄@Glu-coumarin NPs were presented in Fig. 1. According to the results, the FT-IR spectrogram of coumarin showed some peaks at 755, 827, and 886 cm⁻¹ that are related to the C-C, C-H, and = CH bonds, respectively. In addition, a sharp peak was observed at 1397 cm⁻¹ that seems to be related to the C-C bond of the benzene ring. Also, three peaks at 1608, 1723, and 3427 cm⁻¹ appeared that are associated with the C = C, C = O, and –OH bonds. Considering the FT-IR spectrogram of Fe₃O₄@Glu-coumarin, the peaks observed at 453 and 602 cm⁻¹ are associated with the Fe²⁺ and Fe³⁺ of the Fe-O bond, respectively, indicating the formation of Fe₃O₄ structure. Comparing the FT-IR spectra of coumarin and Fe₃O₄@Glu-coumarin, it could be concluded that Fe₃O₄@Glu-coumarin was synthesized correctly.

EDS mapping of Fe₃O₄@Glu-coumarin showed that the synthesized particles contained C, O, and Fe atoms and no elemental impurity was detected (Fig. 2). Electron microscopy imaging of the Fe₃O₄@Glu-
coumarin revealed that the particles were spherical and had a moderate level of aggregation. In addition, the particles were in a size range of 26–46 nm. Figure 3 displays the SEM and TEM images of Fe₃O₄@Glu-coumarin NPs.

**Inhibitory effect of Fe₃O₄@Glu-coumarin on liver cancer cells**

The inhibitory concentration of Fe₃O₄@Glu-coumarin on the liver cancer cell line was evaluated by MTT assay. The results indicated that the NPs were considerably toxic in concentrations greater than 31.25µg/mL. In a dose dependent trend, the relative viability of liver cancer cells was reduced by 22–82% at the concentrations of 31.25–500 µg/mL. The IC₅₀ of Fe₃O₄@Glu-coumarin for liver cancer cells was calculated 120µg/mL that indicated significant toxicity of the NPs (Fig. 4).

**Gene expression**

The relative expression of CASP9, CASP8, p53, mTOR, and MAPK1 genes in nanoparticle treated and control cells were studied by qPCR assay. Our results showed that treating liver cancer cells with Fe₃O₄@Glu-coumarin induced the expression of CASP8, p53, and MAPK1 genes, while attenuated the CASP9 and mTOR1 genes. The expression of CASP8 and p53 genes was increased by 1.94 and 4.87 folds, respectively. In addition, the expression of MAPK1 gene in treated cells increased to 3.87 folds. In contrast, the relative expression of the mTOR1 gene in the cells that were treated with Fe₃O₄@Glu-coumarin significantly reduced to -6.33 folds. Moreover, an insignificant decrease in the expression of the CASP9 gene in NPs treated cells was observed. Figure 5 presents the relative expression of the studied genes.

**Discussion**

Treating liver cancer, especially drug resistant cases, faces difficulties, that is mainly associated with the drug inefficiency and toxic side effects of the current pharmaceuticals. Many researchers are seeking for novel therapeutic formulations to combat cancer and nano-sized molecules have gained attention in this issue. Due to the biocompatibility and magnetic property, the use of iron oxide NPs in the formulation of novel anticancer agents has received attention [4]. Owing to the anticancer property of coumarin, this study was performed to study the anticancer mechanism of the Fe₃O₄@Glu-coumarin NPs in a liver cancer cell line through the evaluation of the expression of apoptosis regulating genes, CASP8, CASP9, p53, mTOR1, and MAPK1.

A significant anti-proliferative effect of the Fe₃O₄@Glu-coumarin on liver cancer cells was observed which could be mainly associated with the presence of coumarin molecules. The anticancer activity of coumarin on several cancer cell lines has been reported which is in agreement with our results [13–14]. It was reported that coumarin and its derivatives could block the cell cycle and induce cell apoptosis [6, 15].
In agreement with the literature, molecular analysis of the genes involved with the regulation of cell apoptosis showed that Fe$_3$O$_4$@Glu-coumarin NPs favor triggering cell apoptosis. We found that treating with the synthesized NPs considerably increased the expression of the CASP8, p53, and MAPK1 genes. Caspases are a group of cysteine proteases that are the central component of apoptotic responses [16]. The initiator caspases, including Caspase 2, Caspase 8, Caspase 9, and Caspase 10, are responsible for the activation effector caspases, such as Caspase 3, which results in the activation of apoptotic signaling pathways [16–17]. In humans, Caspase 9 and Caspase 8 are considered the main mediators of the intrinsic and extrinsic apoptotic pathways, respectively [16, 18–19]. In response to an intrinsic death stimulus, the release of mitochondrial membrane proteins, such as cytochrome c, results in the formation of a multicomponent apoptosome which activates caspase 9. In contrast, the extrinsic apoptosis response is mediated by the activation of caspase 8 upon the binding of an extracellular ligand to its membrane receptor [16]. In this work, the expression of the CASP8 gene was significantly induced in NPs treated cells, while a considerable change in the expression of the CASP9 gene was not observed. Therefore, it could be hypothesized that the activation of the extrinsic apoptotic response is the major mechanism of apoptosis induction and cell death by Fe$_3$O$_4$@Glu-coumarin NPs in liver cancer cells.

P53 is a well-known tumor suppressor in human cells that plays important roles in cell cycle regulation and induction of cell apoptosis [20]. This protein is a transcription factor that is able to bind specific sites on the DNA molecule and activate the genes responsible for cell apoptosis. It was found that upon the binding of the P53 protein to the regulatory regions of Bcl-2 family proteins, it could trigger the apoptotic responses through the increase of the pro- to antiapoptotic Bcl-2 proteins which favor the activation of apoptotic proteins, such as caspases [20]. It was found that modulation of the p53 gene could be an efficient approach in cancer chemotherapy and induction of p53 could exert anti-proliferative effects on cancer cells. In this study, Fe$_3$O$_4$@Glu-coumarin NPs significantly increased the expression of the p53 gene in liver cancer cells. Therefore, in agreement with other results, the synthesized NPs could trigger apoptotic signaling in cancer cells.

The mammalian target of rapamycin (mTOR) regulates cell metabolism, survival, growth, and proliferation. The mTOR1 is mainly involved with the regulation of cell growth and metabolism [21]. It was found that mTOR plays a critical role in tumor formation and development and therefore, many researchers are seeking mTOR inhibitors to be used for cancer treatments [21–22]. In this study, we observed that the expression of the mTOR1 was significantly decreased upon treating the cancer cells with Fe$_3$O$_4$@Glu-coumarin NPs. It could be concluded that the synthesized NPs could efficiently inhibit the activation of mTOR signaling pathways and inhibit the proliferation and development of cancer cells. In addition, the MAPK1 gene was significantly reduced in treated cells that suggest the induction of apoptosis pathways in NPs treated cells.

**Conclusion**
In this work, the effect of Fe$_3$O$_4$@Glu-coumarin NPs on the expression of some genes that are involved with the regulation of cell cycle and apoptosis, including CASP8, CASP9, p53, mTOR1, and MAPK1, in liver cancer cells was studied. We found that the synthesized NPs could induce apoptotic signaling pathways through the inhibition of the p53, mTOR1 and, CASP8 genes, and the upregulation of the MAPK1 gene. In conclusion, Fe$_3$O$_4$@Glu-coumarin NPs could be considered an apoptosis inducing agent to be used against liver cancer cells, after further characterizations.

**Declarations**

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**Data availability statement**

The datasets generated during the current study are available from the corresponding author on reasonable request.

**Ethics approval**

Not applicable.

**Consent to participate**

Not applicable.

**Author contributions**

Conceptualization: A. Salehzadeh and M. Zaefizadeh; Methodology: A. Salehzadeh and M. Zaefizadeh; Formal analysis and investigation: A. Salehzadeh and M. Zaefizadeh; Writing Original Draft Preparation: A. Salehzadeh and F. Shokrollahi; Editing: A. Salehzadeh and F. Kafilzadeh; Resources: F. Shokrollahi.; Supervision: A. Salehzadeh, M. Zaefizadeh and F. Kafilzadeh

**References**


**Figures**
Figure 1

FT-IR spectra of a) coumarin and b) Fe₃O₄@Glu-coumarin NPs
**Figure 2**

EDS mapping of Fe$_3$O$_4$@Glu-coumarin NPs

**Figure 3**

SEM (a) and TEM (b) images of Fe$_3$O$_4$@Glu-coumarin NPs
Figure 4

Anti-proliferative activity of different concentrations of Fe$_3$O$_4$@Glu-coumarin NPs on liver cancer cells
Figure 5

Relative expression of the a) *CASP9*, b) *CASP8*, c) *p53*, d) *MAPK1*, and e) *mTOR1* in Fe$_3$O$_4@$Glu-coumarin treated and control cells