

## Evaluation of the effect of PLGA-PAA nano-encapsulated Hydroxytyrosol on inhibiting the colorectal cancer cell line HT-29 and underlying mechanism of action

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### Abstract

**Introduction:** Chemotherapy was known as a potential approach for colon cancer therapy. Polymer-based nanocarriers prolong the circulation time of chemotherapeutic drugs, therefore anti-tumor drugs can passively accumulate in the malignant tumor position through the improved permeability and retention effect. The aim of the present study was to investigate anticancer potency of biodegradable and pH-sensitive nano-encapsulated Hydroxytyrosol in HT-29 cancer cell line and the potential molecular mechanism of action of Hydroxytyrosol.

**Materials and Methods:** The poly lactide-co-glycolide-co-polyacrylic acid (PLGA-co-PAA) nano-encapsulated Hydroxytyrosol was synthesized, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to evaluate the anti-proliferative and anti-tumor effects of both free and nano-encapsulated Hydroxytyrosol. The relative expression of colorectal cancer associated-1 (COCA1) gene was investigated by quantitative Real-Time PCR (qRT-PCR).

**Results:** We observed that free and nano-encapsulated Hydroxytyrosol significantly decreased the viability of HT-29 cancer cells. Moreover, the cytotoxic effect of nano-encapsulated Hydroxytyrosol on HT-29 cancer cells was significantly more than that of free Hydroxytyrosol. Also, the *COLCA1* gene expression was up-regulated significantly in HT-29 cancer cells treated with either free or nano-encapsulated Hydroxytyrosol.

**Conclusion:** Generally, we showed that the anticancer potency of Hydroxytyrosol was significantly increased by a biodegradable and pH-sensitive nanoparticle. However, further studies on animal models seem necessary.

**Keywords:** Colorectal cancer, PLGA-PAA copolymer, Hydroxytrylosol, *COLCA1* gene

### Introductio

Colorectal cancer (CRC) is one of the most prevalent and lethal malignancies throughout the world. Similar to other cancers, it appears

that both environmental factors and genetics play important roles in the initiation and development of CRC (1). Chemotherapy is the most common offered post-operatively treatment to patients with CRC to reduce the

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risk of a recurrence. In the other hand, the efficacy of chemotherapy for CRC is limited due to the relative insensitivity of CRC to chemotherapeutic agents and the development of multidrug resistance (2). Therefore, interest in the use of other therapeutic methods and naturally occurring compounds in the treatment of malignancies has increased. The most recent developments that make novel insights into the molecular mechanisms suggest the anti-cancer effect of nutritional and herbal compounds (3, 4).

Evidence shows that consumption of Olive oil significantly reduces the risk of CRC (5). Olive Oil contains phenolic compounds, such as Hydroxytyrosol, which can inhibit proliferation of cancer cells, induce apoptosis, regulation of cell cycle, and anti-angiogenic effects in colorectal tumorous cells (6). The phenolic compounds can significantly prevent tumor progression, regulate various signaling pathways, and modify chemotherapeutics targets through changing the gene expression contributed to tumorigenesis (7). Recently, researchers reported that Hydroxytyrosol can inhibit the proliferation of HT-29 colorectal cancerous cell line, and subsequently results in a reduced tumor-mass in a mouse HT-29 xenograft model (7). Also, in a study by Llor et al., it was reported that Hydroxytyrosol suppressed the expression of BCL-2 and COX-2 genes, which these two genes are associated with CRC, by promoting survival, growth, invasion, angiogenesis, and migration of cancer cells (5).

The therapeutic effect of chemotherapeutic drugs is decreased because of their short half-life and systemic toxicity. Polymer-based nanocarriers prolong the circulation time of chemotherapeutic drugs, therefore anti-tumor drugs can passively accumulate in the malignant tumor position through the improved permeability and retention effect (8). The phenolic compounds have low absorption and bio-distribution. One of the

known strategies to enhance the effectiveness of phenols as anti-tumor agents uses phenolic compounds combined with polymeric nanoparticles (9). Thus, along with the need for efficient tumor treatment, great cellular uptake of conjugating nanocarriers containing a specific phenolic compound or antitumor drugs may increase the internalization of natural agents into the cancerous cells and subsequently results in the enhancement of antitumor activity (10).

The C11orf92 gene (Colorectal Cancer-Associated 1 or *COLCA1*) is a locus on human chromosome 11q23, which encoded a transmembrane protein in many human tissues. The correlation between risk alleles and *COLCA1* protein levels in CRC tissues, correlation between risk alleles and *COLCA1* RNA levels in normal colon and CRC tissues, participation of *COLCA1* in many mucosal immune cells of the colon implicated in tumor immunity, provided that this gene involved in colorectal cancer progress (11).

In the present study, a biodegradable and pH-sensitive PLGA-co-PAA-nanoparticle was used for co-delivery of Hydroxytyrosol to evaluation of growth inhibition and *COLCA1* gene expression in HT-29 colorectal cancer cell line.

## Materials and Methods

### Preparation of Hydroxytyrosol Loaded PLGA-co-PAA Nanoparticles

Radical telomerization of acrylic acid monomers (AAc) was used to synthetization of Hydrogen terminated poly (acrylic acid) (PAA-OH) polymer using ME as a chain transfer agent and AIBN as a radical initiator. Ring-opening polymerization of glycolide (GA) and L-lactide (LA) with (PAA-OH) was used to synthesization of Poly (lactide-co-glycolide-co-polyacrylic acid) (PLGA-co-PAA). PLGA-co-PAA nanocarrier was dissolved in Dimethylsulfoxide (DMSO), and then Hydroxytyrosol was added and

dropped gradually into polymer contained Hydroxytyrosol solution to make dual drug nanocarriers. The nanocarriers were collected and centrifuged. The obtained supernatant was collected as Hydroxytyrosol loaded PLGA-co-PAA nanoparticles (9).

#### HT-29 Cell Culture

Roswell Park Memorial Institute (RPMI) 1640 medium in combination with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) were used for culturing the HT-29 cancer cell line at 37°C incubator containing 5% CO<sub>2</sub> and 95% humidity.

#### Cytotoxicity Assay

The HT-29 cancer cell line was plated at a seeding density of 15×10<sup>3</sup> cells/well of a 96 well plate and allowed to attach overnight. Then, culture media was replaced with medium containing different concentrations of either free or nano-encapsulated Hydroxytyrosol (1.5, 3, 6, 12, 24 µg/ml). The drug-free nano-carrier (1000 µg/ml) and normal culture medium (without both free and nano-encapsulated Hydroxytyrosol) were considered as control. The viability of HT-29 cells was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, according to the manufacturer's instructions. Briefly, culture media were replaced with 150µL culture medium containing 50µL MTT solution and incubated in standard culture conditions for 4 hours. Then, the previous culture medium was replaced with 200µL DMSO as solubilization reagent. Finally, absorbance was obtained at 570nm using a microplate ELISA reader.

#### Gene Expression Analysis

Extraction of total RNA from both treated and control cells was performed by TRIzol reagent, according to the manufacturer's instructions. Electrophoresis on 1% agarose

gel and Nanodrop instrument were used to evaluation of quantity and quality of the extracted RNA. The random hexamers were used to synthesis of complementary DNA (cDNA). The qRT-PCR reactions were performed using a Master SYBR Green on a Mic Cycler Quantitative Real-Time PCR instrument. The used primer sequences were forward:

5'-GTGGAGATGGACAGGGATGGC-3' and

reverse: 5'-GGTTGGGACAAAGAGATCCTTGC-3'.

The PCR reaction for *COLCA1* was carried out in a 14µl total volume containing 0.5µl forward primer (5pmol), 0.5µl reverse primer (5pmol), 1µg cDNA, and 7µl master mix in following conditions: initial denaturation (1 cycle in 94°C for 1 minutes), denaturation (40 cycles in 94°C for 10 seconds), annealing (40 cycles in 59°C for 30 seconds), and extension (40 cycles in 72°C for 20 seconds) β-actin gene was considered as endogenous control. The analysis of obtained data was performed by the comparative 2<sup>-ΔΔC<sub>t</sub></sup> threshold cycle method. The obtained data were presented as mean ± standard deviation (SD). The statistical analysis of obtained data was done using Tukey (post hoc), one-way analysis of variance (ANOVA) and Student's t-test by Graph Pad Prism software. P-value<0.05 was considered a significant difference.

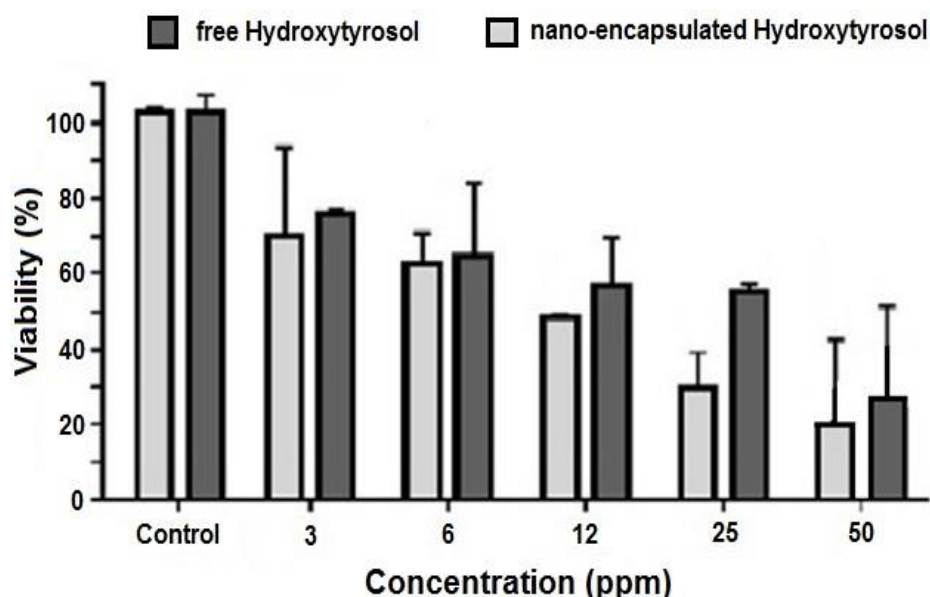
## Results

#### Cytotoxicity

According to analyzing the obtained data free and nano-encapsulated Hydroxytyrosol significantly decreased the viability of HT-29 cancer cells. The nano-encapsulated Hydroxytyrosol showed significantly more cytotoxicity against HT-29 cells compared with free Hydroxytyrosol (p<0.001). The half-maximal inhibition concentration (IC<sub>50</sub>) of free Hydroxytyrosol on HT-29 cells after 72 hours was 31µg/mL; whereas the IC<sub>50</sub> of

nano-encapsulated Hydroxytyrosol was 12 $\mu$ g/mL at the same time (Fig.1). The drug-free nanocarrier (1000 $\mu$ g/ml) showed no noticeable cytotoxicity on HT-29 cells ( $p>0.1$ ). Also, our study indicated that the free and nano-encapsulated Hydroxytyrosol

significantly cause morphological alterations, which cause cell death. However, the observed morphological alterations in treated cells with nano-encapsulated Hydroxytyrosol were significantly more than treated cells with free Hydroxytyrosol.



**Figure 1.** Evaluation of half-maximal inhibition concentration ( $IC_{50}$ ) in HT-29 cell groups treated with either pure Hydroxytyrosol drug or nano-capsulated Hydroxytyrosol. The free and nano-encapsulated Hydroxytyrosol decreased viability of cancer cells. However, nano-encapsulated Hydroxytyrosol showed significantly more cytotoxicity as compared free Hydroxytyrosol. The  $IC_{50}$  of free and nano-capsulated Hydroxytyrosol in HT-29 cells after 72 hours were 31 $\mu$ g/mL and 12 $\mu$ g/mL, respectively.

### COLCA1 Gene Expression

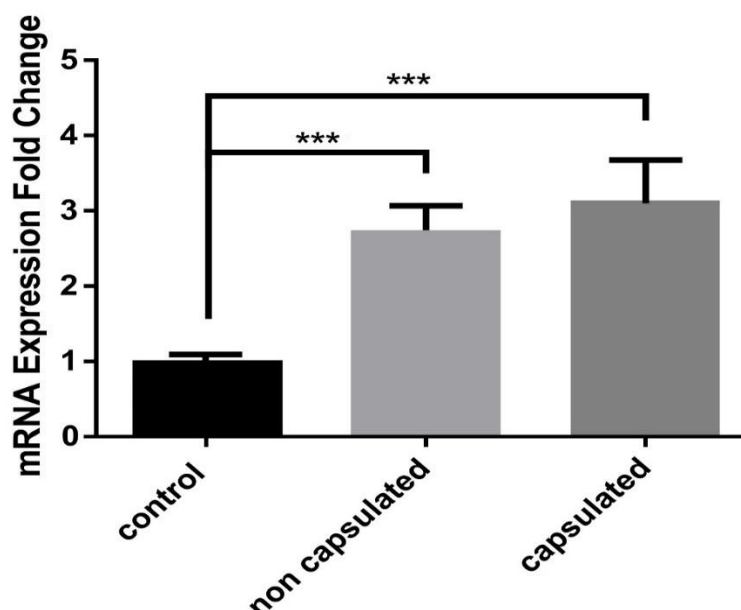
Obtained results showed that the mRNA expression of *COLCA1* gene in HT-29 cells treated with free Hydroxytyrosol increased significantly (2.7 fold) compared to the control group ( $P<0.001$ ). Also, mRNA expression of *COLCA1* gene in the cells treated with nano-encapsulated Hydroxytyrosol increased significantly (3.1 fold) compared to the control group ( $P<0.0001$ ). The expression of *COLCA1* gene in HT-29 cancer cells treated with nano-encapsulated Hydroxytyrosol was significantly more than the expression of *COLCA1* gene in the cells treated with free form of Hydroxytyrosol ( $P<0.001$ ) (Figure 2).

### Discussion

In the past years, the natural compounds including plants and fruits have been used to treatment of various diseases and infections (12-14). In addition, recent studies on pharmaceutical sciences have attracted considerations toward medicinal herbs and bioactive compounds over the past two decades (15-17). Because of the effectiveness, fewer side effects, low costs, and especially the ability of bioactive compounds to target different signaling pathways, they have been highlighted in cancer treatment studies (18, 19). It has been demonstrated that Olive oil-based Hydroxytyrosol prevents cell proliferation, induces apoptosis and modulates cell cycle

pathways in human cancer cell lines (9). However, short half-life and low solubility limited the use of Hydroxytyrosol. The use of

nanoparticles is one of the most effective methods to increase the efficiency of Hydroxytyrosol as an anti-cancer drug (6).



**Figure 2.** Expression of COLCA1 gene in HT-29 cell line exposed to pure and nano-encapsulated Hydroxytyrosol drug and non-drug-treated colon cancer cells were used as control group. The mRNA expression of COLCA1 gene significantly increased 2.7 fold and 3.1 fold in HT-29 cells treated with free Hydroxytyrosol and nano-encapsulated Hydroxytyrosol, respectively.

Therefore, we used a biodegradable and amphiphilic PLGA-co-PAA copolymer to co-delivery of Hydroxytyrosol as an anti-cancer drug. We showed a high anti-cancer and anti-proliferative activity of Hydroxytyrosol in colorectal cancer cells HT-29, which confirms previously performed studies by Lopez et al. and Ahmadi et al. (9, 20). Our study showed that nano-encapsulation increased cytotoxicity and stability of Hydroxytyrosol. We revealed a significant decrease in viability of HT-29 cancer cells in the presence of nano-encapsulated Hydroxytyrosol, which represents Hydroxytyrosol in nano-formulation form as a new chemotherapeutic agent. In another study by Ahmadi et al., it has been reported that PLGA-co-PAA copolymer increased the anti-cancer activity of Hydroxytyrosol in colorectal cancer cells (9).

In the present study, we aimed to realize the potential mechanism of action of Hydroxytyrosol and subsequently evaluated the expression pattern of *COLCA1* gene in HT-29 cancer cells treated with either free or nano-encapsulated Hydroxytyrosol, and compared with the expression of *COLCA1* gene in untreated HT-29 cancer cells. The obtained results showed significantly increased expression of *COLCA1* gene in HT-29 cancer cells treated with either free (2.7 fold) or nano-encapsulated (3.1 fold) Hydroxytyrosol compared to untreated cells. We observed that expression of *COLCA1* gene in HT-29 cells treated with nano-encapsulated Hydroxytyrosol was significantly more than that in the cells treated with free form of Hydroxytyrosol. The *COLCA1* gene encodes a transmembrane protein in many human tissues, such as the stomach, prostate, and bladder. Previous studies showed that the expression of



*COLCA1* gene was higher in normal colon tissues than in colon tumors. Increased expression of *COLCA1* gene is associated with reduced tumorigenesis and increased survival rate and life quality in patients with CRC (11, 21). Therefore, increased expression of the *COLCA1* gene may be one of the causes of HT-29 cancer cell inhibition in the consequence of Hydroxytyrosol treatment in the present study. However, further investigations are necessary to exactly identify the molecular mechanism of the anti-cancer activity of Hydroxytyrosol.

## Conclusion

In conclusion, our study showed that the synthesized PLGA-PAA polymer has a good ability to Hydroxytyrosol load, and increase the efficacy of Hydroxytyrosol on HT-29 cancer cells. We observed that PLGA-PAA polymer increases the anti-cancer and cytotoxic activity of Hydroxytyrosol,

significantly. Also, nano-encapsulated Hydroxytyrosol significantly increases the relative expression of *COLCA1* gene compared to free Hydroxytyrosol. Therefore, a combination of Hydroxytyrosol with biodegradable and pH-sensitive PLGA-PAA nanoparticles can be a promising therapeutic strategy for the treatment of CRC. However, studies on animal models as well as clinical studies seem to be necessary.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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