

Preparation of resveratrol containing poly (lactic-co-glycolic acid) nanoparticles and investigation of its cytotoxic effect in C6 glioma cells

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ABSTRACT: Cancer is a complex disease involving several important changes in cell morphology that ultimately lead to tumor formulation. The biological endpoint of cancer is the uncontrolled growth and proliferation of cells. Resveratrol (RES) is a polyphenolic compound from the stilbene family, which has an important place in the treatment of cancer, which is used as a pharmaceutical and nutraceutical. Studies have shown that RES has anti-tumor effects in addition to its antioxidant, antimicrobial and anti-inflammatory effects. RES protects the body against free radicals and inhibits metabolic events that induce the proliferation of cancer cells. RES is a chemically unstable compound that has problems crossing the blood-brain barrier. Therefore, it is concluded that carrier systems containing RES should be developed in order to increase the water dispersibility of resveratrol and to increase its bioavailability when taken orally. In this way, the RES will be successfully targeted to the tumor site and the undesirable properties of the unstable, heat-sensitive RES will be improved. For this purpose, drug delivery systems prepared using synthetic polymers such as Polylactic-co-glycolic acid (PLGA) have been developed. PLGA is a biodegradable and biocompatible polymer used in areas related to human health. In this study, cell culture study of C6 glioma cells obtained from ATCC was performed and for this purpose, biocompatible nanoparticles of RES with PLGA were prepared. With the preparation of these nanoparticles, the effects of RES on cell viability and the differences in the anticancer effect on cells were evaluated with the XTT cytotoxicity study.

KEYWORDS: Resveratrol; PLGA; polyvinyl alcohol; nanoparticles; cytotoxicity

1. INTRODUCTION

Cancer is a complicated disease containing various crucial changes in cell morphology which finally leads to tumor formulation. The biological endpoint of cancer is the uncontrolled growth of cells and proliferation [1]. In normal cells, when DNA is damaged, the cell can repair itself but in cancer cells, these injured cells do not die. Instead, it causes abnormal cell growth that all have the same DNA deficiency [2]. Cancer can affect only one organ or spread to different organs [3]. Three gene groups have a crucial role in the formation of cancer. These are oncogenes, tumor suppressor genes, and DNA repair genes [4,5]. Recently, natural compounds of plant origin, called phytochemicals, have attracted the attention of researchers in the field of pharmacy and medicine worldwide due to their very important bioactive properties. [6]. Polyphenols, which constitute an important group of secondary metabolites of plants, are intensively studied for disease prevention and treatment, especially due to their antioxidant properties. The most useful polyphenol for human use in pharmaceutical and nutraceutical use is RES [7,8]. RES was first isolated from the roots of *Veratrum grandiflorum*. The most important edible source in nature is grapes and peanuts. RES is synthesized using the enzyme stilbene synthase as phytoalexin (plant antibiotic) when exposed to environmental effects such as microbial infection, injury, UV radiation, and ozone in plants [9,10]. These substances are obtained by a mechanism of resistance in plants to other adverse conditions such as parasite and fungal infection, UV radiation, chemicals, and stress factors for the plant in general, and are not normally produced in large quantities.

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In addition, RES is found in high levels in the roots of *Polygonum cuspidatum*, which is used in Japanese medicine. This phytoestrogen draws attention with its effects in the treatment of fungal diseases in humans, hyperlipidemia, atherosclerosis, and inflammations [11]. The RES level reaches its maximum levels within 24 hours from the stress situation and starts to decrease after 40-70 hours as a result of the activation of stilbene oxidase. Because the two phenolic rings of RES are linked by a styrene double bond, it is called a stilbene-based polyphenol. RES is known to have both cis and trans forms chemically. Also, the glycosylated form was found might flour. It is mostly in the glycosylated (3-O- β -D- glucoside) form in plants. Glycosylation protects resveratrol from oxidative degradation [12]. Glycoside resveratrol maintains biological activity, is stable and water-soluble, and is easily absorbed from the gastrointestinal area [13]. The most stable and functionally active of these forms is the trans form. In UV, with high pH, the trans isomer transforms into the cis isomer, while in visible light and low pH, the cis isomer transforms into the trans isomer [14]. The cis and trans forms of RES are given in Figure 1 [15]. The positive activities of the trans form on health can be listed as reducing lipid peroxidation, showing antioxidant properties, lowering blood pressure, and anti-inflammatory activity [15,16].

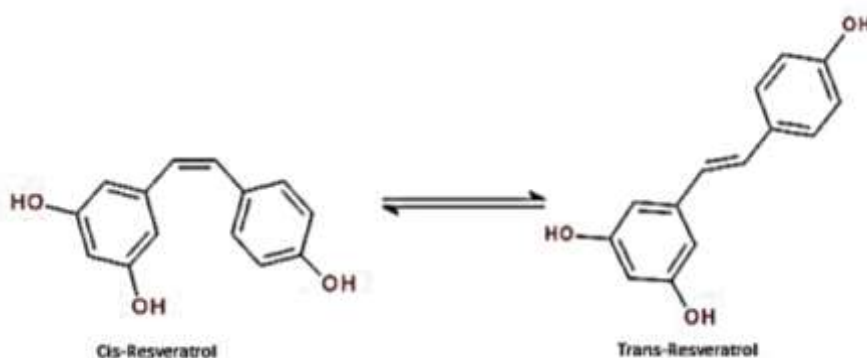


Figure 1. Cis and trans forms of resveratrol [14].

Meishiang Jang et al first demonstrated the protective effects of RES in inhibiting multistage carcinogenesis in 1997. This polyphenolic compound has been indicated to inhibit the proliferation of different human tumor cells in vitro, resulting in a variety of preclinical animal studies [17]. By inhibiting the RES Cyclooxygenase 1 (COX-1) enzyme, it shows anticancer activity and reduces the progression activity of the tumor. COX-1 is an enzyme that stimulates tumor growth. It greatly inhibits the RES COX-1 enzyme [18]. The effects of RES on various cancers such as bone, brain, prostate, lung, skin, colon, breast, and gastric have been shown in various studies [19]. For example, its inhibitory effect on cancer initiation was determined in an animal model of skin cancer given twice-weekly doses of resveratrol [20]. Despite all these beneficial effects, the factors that limit the applications of resveratrol can be listed as follows; low stability against oxidation, high photosensitivity, insoluble or low solubility in water, short biological half-life, and low bioavailability. In addition, resveratrol also undergoes chemical degradation at elevated temperatures, pH changes, ultraviolet light, and in the presence of certain types of enzymes [21]. RES is a chemically unstable compound that faces several problems when crossing the blood-brain barrier. Therefore, it was concluded that drug delivery systems containing RES should be developed to increase the water dispersibility, chemical stability, bioavailability, stability against pH changes, and UV effects of resveratrol when administered orally. For this purpose, drug delivery systems prepared using synthetic polymers such as Polylactic-co-glycolic acid (PLGA) have been developed [22, 28]. In this way, the RES will be successfully targeted to the tumor site, and the adverse properties of unstable, heat-affected, and fast-catalyzed RES will be ameliorated [23,24]. PLGA is approved by the FDA and EMA. In addition, this synthetic polymer, which is widely used in areas related to human health, is nontoxic. PLGA is formed by the polymerization of polylactic acid (PLA) and polyglycolic acid (PGA) monomers at different rates [25].

PLGA has attracted significant attention owing to some of its features. These; formulations and production methods are well adapted to different types of drugs such as biodegradability, biocompatibility, hydrophilic/hydrophobic small or large molecules, the possibility of drug remaining intact, sustained release, the possibility of modifying surface properties for better interaction with biological materials, and targeting nanoparticles to specific organs or cells to be provided. While PLGA is stable in dry environments, it begins

to undergo hydrolysis in water-containing areas such as the body. They form biodegradable monomers such as lactic acid and glycolic acid by hydrolysis in the body. These monomers participate in the Krebs cycle and are easily metabolized and removed as carbon dioxide and water. Excess glycolic acid is excreted by the kidneys [26]. Drug molecules encapsulated with PLGA are not only protected from undesired degradation but also increased in bioavailability. Thus, they create minimal systemic toxicity. With minimal systemic toxicity, it is used in drug transport or biomaterial applications [27].

PLGA is used to formulate a wide variety of biodegradable drug delivery systems such as microparticles, nanoparticles, and implants [28,29]. In addition, biodistribution studies of PLGA nanoparticle carriers increased the permeability and retention effect. Thus, it has been shown that it increases the accumulation of diagnostic and therapeutic agents in a certain region of the body. For example, healthy mice were given indocyanine green via nanoparticles using the fluorimetric analysis method. The nanoparticles ensured a much higher concentration of indocyanine green compared to the free solution in the organs as well as in the blood. PLGA nanoparticles are very advantageous in tumor diagnosis and photodynamic therapy as a drug delivery system for indocyanine green [30]. The structure of PGA, PLA, and PLGA is shown in Figure 2.

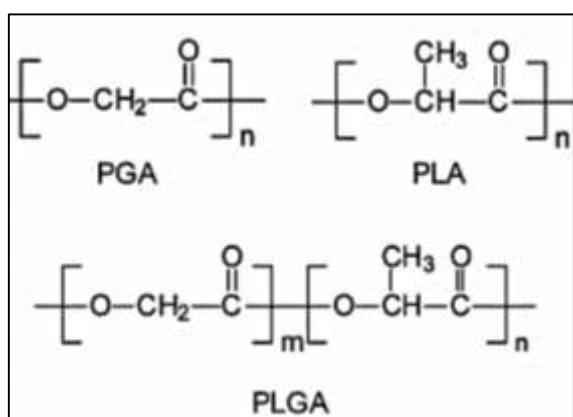


Figure 2. The structure of PLGA (the parts denoted by m and n indicate the number of lactic and glycolic acid) [30].

The present study, it is aimed to bring RES, which has an anticancer effect, to a more stable and effective form, and improve its biological activity. For this purpose, biocompatible nanoparticles of RES with PLGA will be prepared. With the XTT cytotoxicity study of the prepared nanoparticles and RES, the differences in cell viability and anticancer activity will be evaluated within the scope of the study. In the proposed hypothesis, nanoparticle formulations were applied C6 to glioma cells as compared to cells treated with only RES higher anti-cancer activity is expected. This hypothesis was created by experiencing the preliminary data of the literature and the effects of different models were performed in our laboratory before.

2. RESULTS and DISCUSSION

2.1 Results of characterization studies of nanoparticles

The particle sizes, zeta potentials and polydisperse index measurements were performed in the characterization studies of nanoparticles. The zeta potential describes the electrostatic interactions between cells and particles in a fluid medium. The liquid layer surrounding the particle exists in two parts; an inner region (Stern layer) where ions are strongly bound and an outer (diffuse) region where they are less tightly bound. Within the diffuse layer there is a conceptual boundary where ions and particles form a stable entity. When a particle moves (for example, due to gravity), the ions within the boundary move it. Ions beyond the boundary remain with the bulk dispersant. The potential at this boundary (hydrodynamic slip surface) is the zeta potential.

In a study performed by Demir et al. zeta potential values of docetaxel-loaded nanoparticles ranged from -7.44 ± 0.48 mV to -24.6 ± 5.30 mV. When the empty nanoparticles and drug-loaded nanoparticles are compared, a negative increase in the zeta potential value was observed. The reason for this is that docetaxel has a negative charge [31]. In our study, the zeta potential values of nanoparticles are between 3.14 ± 0.03 mV and 3.22 ± 0.04 mV. Thanks to the positive charge of the nanoparticle, it adheres to the negatively charged cell

membrane through the receptor and facilitates entry into the cell. Obtained zeta potential values are suitable for application. Particle size is very important for passage through the cell membrane. It is very difficult for nanoparticles above 450 nm to pass and act from the cell membrane to the cytoplasm. This adversely affects the bioactivity of the nanoparticle.

In a study carried out by Fonseca et al., empty and paclitaxel-loaded nanoparticles prepared using different PLGA resomers were compared. In the study, the particle size of drug-loaded nanoparticles prepared with RG502H was found to be 122 ± 3 nm, while the size of drug-loaded nanoparticles prepared with RG502 was found to be 133 ± 2 nm. When RG502H, which was determined as the optimum formulation in the thesis study, was compared with RG502, it was observed that the formulation prepared with carboxylic acid end-groups containing resomer had a smaller particle size [32]. In our study, nanoparticle sizes were observed to be between 266.25 ± 3.1 nm and 284.12 ± 3.3 nm (Table 1). It can be said that the nanoparticle sizes are suitable for cell culture studies, so their bioactivity may be within the expected values. The polydisperse index value is a value used to determine whether the particle size of the prepared particle shows a homogeneous distribution. It is undesirable for this value to be greater than 0.4. Looking at the results, it can be said that the zeta potential, particle size and polydisperse index values of the nanoparticles are suitable for the application. It can be said that the NP3 formulation with the lowest particle size is the most suitable for mechanical characterization.

Table 1. Particle size, zeta potential and polydispersity index values of PLGA nanoparticles

Formulations (PLGA nanoparticles)	Zeta potential(mV) \pm SD	Size (nm) \pm SD	Polydispersity index \pm SD
*NP1	3.62 ± 0.05	294.22 ± 3.3	0.296 ± 0.03
*NP2	3.49 ± 0.05	302.21 ± 3.5	0.283 ± 0.04
*NP3	3.78 ± 0.04	279.32 ± 2.1	0.252 ± 0.03

* All nanoparticles (NP1, NP2, NP3) including PLGA and RES. Each experiment was repeated three times.

2.2 Results of EE and LC from resveratrol loaded nanoparticles

EE rate represents how much resveratrol coated in PLGA nanoparticles. The linear equation was calculated as $y=0.2681 x \pm 0.0197$ based on the concentration and absorbance values. The rate of the encapsulated resveratrol into nanoparticles were calculated and the results were shown in Table 2. According to the Table 2 results, EE value of resveratrol loading NP was found 76.22 ± 0.05 %. This showed that resveratrol was successfully encapsulated by PLGA nanoparticles. In addition, LC in nanoparticles was found as 9.02 ± 0.01 %. According to the results, it can be concluded both EE and LC values of resveratrol loading nanoparticles were suitable for the in vitro cell culture studies.

Table 2. EE and LC of resveratrol loading nanoparticles.

Parameter	Resveratrol
Linear equation	$y=0.2681 x \pm 0.0197$
Slope \pm SD	0.2681 ± 0.04
Intercept	0.0197 ± 0.0003
r	0.9957
EE %	76.22 ± 0.05
LC %	9.02 ± 0.01

2.3 In vitro release kinetics study result of resveratrol loaded NP

The release results of resveratrol showed a controlled release characterized by a fast initial release (50 %) during the first 24 h, followed by a continuous and slower release (80 %) till 120 h. This type of continuous and slow-release has been experienced for diclofenac-loaded nanoparticles [25]. Diffusion and molecular

matrix degradation of the resveratrol play an important role in the release of the resveratrol from the nanoparticles [21, 23]. The release study was continued for 288 h and in this period 99.48 % of the resveratrol was released (Figure 3).

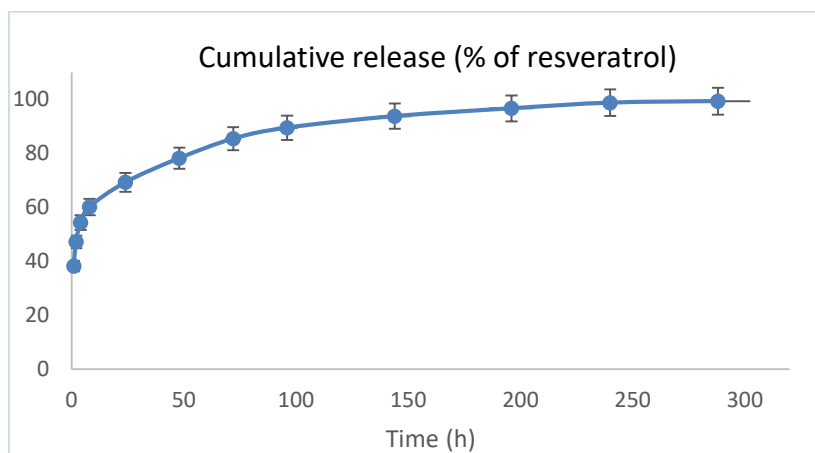


Figure 3. In vitro release kinetics of resveratrol loading nanoparticles. The error bars indicate the standard deviations with n=3 for each one. (PBS buffer, pH 7.4, 37°C)

2.4 Results of the cytotoxicity study of nanoparticles

Nanoparticles The cytotoxicity results of nanoparticles containing RES and RES only in the C6 cell line are given in Figure 1. Each nanoparticle formulation used in the cell culture study contains 30 µg of RES. The low rate of C6 cell viability in this study indicates that the cytotoxic effect of RES is high. As a result of the literature review, it has been seen that resveratrol has a cytotoxic effect depending on time and dose in many cell lines and inhibits cell proliferation.

In a study conducted by Yurdakul et al., the cytotoxic effects of resveratrol were investigated on H1299 and MRC-5 cells, depending on time and dose. The effects of resveratrol were investigated against oxidative damage induced by hydrogen peroxide, which is a strong oxidant in cells, to reveal the antioxidant property of the component. In this study, it was observed that the cytotoxic effect of resveratrol increased with time and dose [33]. In another study, C6 astroglial cells were used. A cytotoxic effect was created by applying buthionine sulfoximine to these cells and decreased GSH amount, GPx, and GRx antioxidant enzyme activities. After resveratrol administration, an increase was observed in the amount of GSH, GPx, and GRx enzyme activities. In addition, it was stated that when heme oxygenase 1 was pharmacologically inhibited, the protection of resveratrol against oxidative damage and inflammatory injury was abolished. This showed that resveratrol regulates the GSH system through the heme oxygenase pathway in C6 astroglial cells [34]. In our cell culture study, cell viability was measured as 66.46 %, 61.26 %, 63.57 %, and 73.16 % after NP1, NP2, NP3, and RES application, respectively. According to the results, the NP2 formulation showed the highest cytotoxic effect in the C6 cell line. It was observed that all three nanoparticles showed a significantly higher cytotoxic effect compared to RES alone. The results of this study showed that nanoparticle preparation increased the biological activity of RES and we obtained more effective data in the C6 glioma cell line.

Table 3. Concentration dependent C6 cell viability results of resveratrol and nanoparticles including resveratrol.

Samples	Concentrations of RES				
	(2,5 µg/mL)	(5 µg/mL)	(10 µg/mL)	(25 µg/mL)	(50 µg/mL)
RES	84.45 %	78.89 %	73.16 %	60.36 %	46.12 %
NP1	76.62 %	71.23 %	66.46 %	50.28 %	41.16 %
NP2	73.28 %	67.78 %	61.26 %	49.78 %	40.35 %
NP3	71.12 %	66.23 %	63.57 %	48.36 %	38.25 %

3. CONCLUSION

This study performed a cell culture study of C6 cells obtained from ATCC, and biocompatible nanoparticles of RES with PLGA were prepared. With the preparation of these nanoparticles, it was observed that the antiproliferative effect increased in cell culture studies.

4. MATERIALS AND METHODS

4.1 Chemicals and consumables

C6 Glioma (ATCC CRL-107) cell line, penicillin/streptomycin (Sigma-Aldrich, Germany, TMS-AB2-C, 10,000U/mL), DMEM/Nutrient Mixture (Merck Millipore, United States, D5796), Fetal Bovine Serum (FBS, Merck Millipore, United States, ES-009-B), Trypsin-EDTA solution (Sigma-Aldrich, Germany), and various consumables required for cell culture were used. PLGA, dichloromethane, and polyethylene glycol (Sigma-Aldrich, Germany) were used for the preparation of nanoparticles. XTT reagent was purchased from Roche Diagnostic. RES in lyophilized powder form with all analytical calibrations was obtained from Merck Millipore, United States. All materials used are in analytical grade.

4.2 Preparation and characterization of PLGA nanoparticles

PLGA nanoparticles containing RES were prepared by a dual emulsion (water/oil/water) solvent evaporation technique [35]. 40 mg of PLGA was dissolved in 2 ml of dichloromethane. It was homogenized by adding 50 µg of RES dissolved in 200 µl of polyethylene glycol. Then the mixture was added to 50 ml of PVA (1%) solution and homogenized to form a water/oil/water emulsion. Excess DCM in the mixture was removed from the solution with the help of a magnetic stirrer. The nanoparticles were centrifuged at 12,000 rpm for 20 minutes. In this way, separation was achieved. It was then washed three times with deionized water to obtain purer nanoparticles. It was lyophilized for drying. Mechanical characterization studies were performed. In these assays, deionized water solution at 25 °C and a zeta and size measuring device (Malvern Instrument, UK) were used. Measurements were made three times for each nanoparticle.

4.3 Evaluation of encapsulation efficiency (EE) and loading capacity (LC) of resveratrol loaded nanoparticles

Ultraviolet visible spectrophotometer was used to measure the EE % and LC % of resveratrol in nanoparticles. By reading the absorbance of the resveratrol at different concentrations at a wavelength of 285 nm, the standard calibration curve of resveratrol was established and spectral line equation was obtained. The amount of resveratrol in the supernatant was calculated from line equation. The following equations were used to determine the encapsulation efficiency and loading capacity of the nanoparticles.

$$EE (\%) = ((m_o - m_s)/m_o) \times 100$$

$$LC (\%) = ((m_o - m_s)/w_{np}) \times 100$$

where, m_o is the initial mass of resveratrol and m_s mass of resveratrol in the supernatant and w_{np} = total weight of the resveratrol of nanoparticles [21]. All measurements were performed in triplicate and were reported as mean \pm SEM ($n = 3$)

4.4 In vitro release study of resveratrol loaded nanoparticles

In vitro release of resveratrol from PLGA nanoparticles was performed for 12 days in a 37 °C water bath. PLGA nanoparticles including 10 mg resveratrol were weighed and incubated in 1 ml PBS (pH: 7.4) buffer. At the determined times the samples were centrifuged at 12000 rpm for 10 minutes, the supernatant was maintained at -20 °C and fresh PBS was added the nanoparticles. The amount of released resveratrol at a specific time was determined using ultraviolet visible spectrophotometer [17, 21].

4.5 Cell culture

C6 cells obtained from ATCC were grown in a DMEM cell culture medium containing 1% penicillin-streptomycin, 1% L-glutamine, and 10% fetal bovine serum in 25 cm² flasks in an incubator at 37°C and 5% carbon dioxide. Since there are many studies on the effects of resveratrol on the central nervous system, the use of the C6 cell line was deemed appropriate in this study. When the cells reached a certain density (80%), the cells were passaged and studies were started after a certain passage.

4.6 XTT cell viability assay

The effect of resveratrol and resveratrol-loaded nanoparticles on the viability of C6 cells was investigated by XTT cell viability test. In this method, metabolically active cells reduce XTT, a tetrazolium salt, to orange-colored formazan crystals. Although the dye formed is water-soluble, the dye density can be read at certain wavelengths (450 nm) with the help of a spectrophotometer. The dye intensity in orange is proportional to the number of metabolically active cells. For cytotoxicity experiments, first of all, 10×10^3 cells were taken from the medium containing 100 μ L DMEM + 10% FBS + 1% antibiotic, and the cells were planted in a sterile 96-well microplate and incubated overnight for the cells to adhere. The next day, the medium on the cells was removed, the wells were washed with PBS, fresh medium was added to the cells, and resveratrol at different concentrations was applied to the cells and incubated for 24 hours. At the end of this period, the medium was removed and the cells were washed three times with PBS. Then, 100 μ L of transparent DMEM and 50 μ L of XTT solution were added to each well and incubated for 4 hours in a carbon dioxide incubator. After incubation, the optical density value was read at 450 nm in the microplate reader, and the cell viability rate of the control group was accepted as 100%.

4.7 Statistical analysis

Statistical evaluation of the data to be obtained was performed within the SPSS package program, by applying the one-way ANOVA Analysis of Variance Test for data with normal distribution and the Kruskal-Wallis and Mann-Whitney U test for data that did not show normal distribution. Values of $P < 0.05$ were determined significant.

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