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Research Article

Preparation and characterization of chitosan nanoparticles with extracts of *Rheum ribes*, evaluation of biological activities of extracts and extract loaded nanoparticles

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Abstract: The biological activities of different parts of the *Rheum ribes* plant were evaluated comparatively. Extracts showing strong biological activity were identified and it was determined which of the extract-loaded nanoparticles showed stronger activity. Cytotoxic activity of *R. ribes* extracts was calculated on glial (C6) and fibroblast (NIH 3T3) cells using XTT assay. Spectrophotometry was used to evaluate the impact of these compounds on the enzyme activities of human carbonic anhydrase I and II (hCA I and hCA II). The findings showed that chitosan NPs with extracts loaded on them have a lower IC_{50} value and more cytotoxic activity in C6 cells than chitosan NPs with only extracts. *R. ribes* young shoots ultrasonic methanol extract (RYU) was shown to have the strongest antiproliferative efficacy against C6 cells. Results showed that RYU and ultrasonic methanol extract of *R. ribes* radix (RRU) were determined as the best carbonic anhydrase inhibitors. According to results of particle size, encapsulation efficiency, and release studies of chitosan NPs, it has been observed that they are suitable for application. At a concentration of 10 µg/mL, it was found that none of the *R. ribes* extracts exhibited cytotoxic action toward the NIH 3T3 cell line. According to results of particle size, encapsulation efficiency, and release studies of chitosan NPs, it has been observed that they are suitable for application. It was observed that none of the extracts of *R. ribes* at a concentration of 10 µg/mL showed cytotoxic activity in the NIH 3T3 cell line.

1. INTRODUCTION

Gliomas are complex malignancies with unknown biochemical and molecular detrimental effects. (Mamelak & Jacoby, 2007). In long-term chemotherapeutic drug treatment, chemical resistance occurs against the drug, and this causes the recurrence of glioma (Devasagayam & Sainis, 2002). Studies on natural substances with immunomodulatory and neuroprotective effects have become more important to remove or minimize these drawbacks associated with chemotherapy and long-term treatment. Phytochemicals with neuroprotective effects have gained value in studies by preventing or reducing the recurrence and growth of glioma. Extracts

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made from phytochemicals have been found to improve therapeutic effectiveness and lower the side-effect profile (Choudhari *et al*., 2020). Especially in cancer treatment, phytochemicals, which are natural herbal sources, are administered together with drug delivery systems and controlled release systems. Natural plant extracts can benefit from co-administration with a carrier system because it makes them more bioactive and makes them easier to apply, dose, and target. Applications of nanotechnology make it significantly easier to target, diagnose, and treat tumors in an effective and accurate manner. Important benefits of nanoparticular systems include optimal pharmacokinetic features, simple specific targeting, reduced adverse effects, and reduced drug resistance in the treatment of cancer (Dadwal *et al*., 2018; Palazzolo *et al*., 2018). The metalloenzyme CA (Carbonic anhydrase carbonate hydro-lyase, EC 4.2.1.1) works as a catalyst in the reversible hydration of carbon dioxide to bicarbonate and is present in many different tissues. It contains zinc (Zn2+) ions in its active site (Tutar *et al*., 2019; Koçyiğit *et al*., 2020; Huseynova *et al*., 2022). Based on where they are found in the body, carbonic anhydrases are divided into four groups. There are at least 16 different isoforms of these enzymes, spanning from CA I to CA XVI. In addition to being used as active ingredients in painkillers and diuretics to treat cancer, osteoporosis, and epilepsy, CA isoenzyme inhibitors (hCA I and hCA II) are also used to treat eye problems and hypertension (Koçyigit *et al*., 2017; Tutar *et al*., 2019; Baltacı *et al*., 2021; Gezegen *et al*., 2021).

Rheum ribes is a species of Polygonaceae family used by the public as food and medicine. The dried stem and root of this plant are used in traditional medicine to cure anemia, fatigue, anxiety, depression, diabetes, and to prevent cancer (Amiri *et al*., 2015). Different parts of the *R. ribes* contain bioactive substances such as tannin, anthracene derivatives, flavonoids quercetin, 5-desoxyquercetin, quercetin 3-0 rhamnoside, quercetin 3-0 galactose and auercetin 3-O-rutinoside (Amiri *et al*., 2015; Noori *et al*., 2022). Additionally, plants include minerals like Calcium, Potassium, Magnesium, and certain vitamins like A, B1, and C, as well as organic acids like citric and malic acids. In addition, it is known that the plant has a therapeutic effect against various ailments such as stomach upset, vomiting, hemorrhoids, diabetes, measles and smallpox symptoms and loss of appetite due to these bioactive compounds it contains (Amiri *et al*., 2015; Taskin & Bulut, 2019).

In this investigation, we sought to assess the effects of *R. ribes* extracts on the C6 and NIH 3T3 cell lines in terms of their enzyme, antibacterial, and cytotoxic properties. Additionally, mechanical, and in vitro characterization investigations were carried out, and the NPs of the most efficient extracts were synthesized. We investigated whether the antiproliferative activity of NPs containing the extracts increased compared to the extracts alone, while also examining their cytotoxic effects on the C6 and NIH 3T3 cell lines.

2. MATERIAL and METHODS

2.1. Plant Material And Preparation of Plant Extracts

The *R. ribes* was collected from Van-Gürpınar, Turkey. The voucher specimen was deposited in the Pharmacy Faculty of Marmara University Herbarium and the voucher specimen number was MARE 18817. The dried parts of the plant (young shoots, leaves, roots and flowers) were cut into small pieces and 10 g of each were weighed. Maceration of each part of the plant was carried out by adding methanol and keeping it in a dry and shaded place until the solvent became colorless. The solvent was processed in methanol using a Soxhlet apparatus until it became colorless, and in the ultrasonic bath extraction, the specified parts of the plant were kept in an ultrasonic bath with methanol solvent for a certain hour and prepared. Following extraction, the samples were filtered using filter paper, the solvents were vaporized using a rotary evaporator, and the unprocessed extracts were kept chilled at 4 $^{\circ}$C. The extracts used in the study are listed in Table 1.

Table 1. The extracts used in this study.

Rheum ribes parts	Methods of extraction	Extract name
Young shoots	Soxhlet	RYS
	Ultrasonic bath	RYU
	Maceration	RYM
Leaves	Soxhlet	RLS
	Ultrasonic bath	RLU
	Maceration	RLM
Flowers	Soxhlet	RFS
	Ultrasonic bath	RFU
	Maceration	RFM
Radix	Soxhlet	RRS
	Ultrasonic bath	RRU
	Maceration	RRM

2.2. Enzyme Studies

The enzyme activity of carbonic anhydrase was measured using the esterase activity technique. The technique is based on the esterase activity of CA. The method's underlying tenet is that the carbonic anhydrase enzyme's substrate, p-nitrophenyl acetate, is utilized. According to Baltac *et al*. (2021) and Verpoorte *et al*. (1967), p-nitrophenol or p-nitrophenol is hydrolyzed to produce absorbance at 348 nm. Armstrong, 1966) also support this theory. This method gives the same absorbance at 348 nm for p-nitrophenol and p-nitrophenolate. As a result, the measurement during the process is unaffected by phenol or phenolate generation (Armstrong, 1966; Verpoorte *et al*., 1967; Koçyigit *et al*., 2017; Baltac *et al*., 2021). The use of 348 nm p-nitrophenyl acetate is blind because of its extremely low absorption. By combining the reaction mixture in 3 mL quartz cuvettes, an activity determination approach was used in the measurements.

2.3. Cell Culture Studies

C6 cell line (ATCC CCL 107) and NIH 3T3 (ATCC CRL-1658) cell line were obtained from ATCC for the cytotoxic activity research. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were procured from Merck Millipore. Phosphate buffer saline (PBS), tripolyphosphate (TPP), and chitosan (400 kDa, DD 92) were purchased from Sigma-Aldrich. The Sigma-Aldrich penicillin-streptomycin-L-glutamine solution was bought. Studies on cytotoxic action used the XTT reagent from Roche Diagnostic. In DMEM containing FBS (10%), penicillin (100 IU/mL), L-glutamine (1%), and streptomycin (10 mg/mL), C6 and NIH 3T3 cells were planted. Well plates with cells were cultured at 37 °C and 5% CO2 in an incubator. Cells have to be at least 80% confluent for the cytotoxic activity assays to be completed (Wolf *et al*., 2009).

2.4. Cytotoxicity Assay

On the C6 and NIH 3T3 cells, the XTT assay was used to determine the cytotoxic activity of *Rheum ribes* extracts. DMEM (100 L, 10% FBS) was first used to seed cells in two 96-well plates, where they were then left to incubate for the night (Wolf *et al*., 2009; Taskin *et al*., 2020). Twelve distinct *R. ribes* extracts, including those made using soxhlet methanol, ultrasonic methanol, and methanol maceration, were dissolved in DMSO (20% v/v) to test their ability to inhibit the proliferation of cell lines. The control group received an equivalent amount of DMSO while the extracts were added to each well after being pipetted and vortexed in DMEM at a concentration of 10 µg/mL. Incubation of plates containing cells and extracts for 24 hours followed. After this time, wells were cleaned with PBS (200 µL). Each well received 100 µL of colorless DMEM and 50 µL of the XTT reagent before the cells were given a 4-hour incubation period. The absorbance of XTT-formazan at 450 nm was measured using a micro plate ELISA reader. Calculations of *R. ribes'* cell viability in comparison to controls were made.

After reviewing the findings, the *R. ribes* extract with the best antiproliferative activity against the C6 cell line was made into nanoparticles (NPs). To determine the IC_{50} values on the C6 and NIH 3T3 cell lines, XTT experiments were conducted with and without NP.

2.5. Preparation of Chitosan NPs

NPs containing *Rheum ribes* were prepared using the ionic gelation technique. Based on information from the literature, the concentration of chitosan was dissolved in acetic acid (0.5% v/v) at 1000 rpm while being magnetically stirred. The pH of the chitosan solution should be between 4 and 5 to achieve good encapsulation and loading capacity of NPs. Using 5M sodium hydroxide, the pH of the chitosan solution was brought down to 4.3 (Calvo *et al*., 1997; Taskin *et al*., 2020). TPP was dissolved at a predetermined concentration (0.25% w/v) in sterile deionized water. Chitosan solution (0.5% w/v) with the extract dripped into the TPP solution. Centrifuging the NP expansion was done for 30 minutes at 10.000 rpm. To determine the encapsulation effectiveness of the NPs, the supernatant was taken out and a 1 mL sample was isolated from it. The pellet was afterwards cleaned with deionized water. The pellet received 30 mL of deionized water before being centrifuged for 15 minutes at 10.000 rpm. This procedure was carried out twice. NPs were then lyophilized and kept at +4 °C.

2.6. Encapsulation Efficiency (EE) and Loading Capacity (LC) Studies of NPs

The EE% and LC% of the *R. ribes* extract in NPs were determined using an ultraviolet visible (UV vis) spectrophotometer (Taskin *et al*., 2020). The extract's standard calibration curve and spectral line equation were established by measuring the extract's absorbance at various concentrations at a wavelength of 350 nm. The line equation was used to determine how much extract was present in the supernatant. The loading capacity and encapsulation effectiveness of the NPs were calculated using the following formulae.

$$EE\ (\%) = ((mo - ms)/mo) \times 100$$

$$LC\ (\%) = ((mo - ms)/wnp) \times 100$$

where, mo stands for the initial mass of natural extracts and ms stands for mass of natural the extracts in the supernatant and wnp = total weight of the naturally obtained extract of NPs (Purbowatiningrum and Ismiyarto, 2017) Each measurement was made three times, and the results were presented as mean SD (n = 3).

2.7. Measurement of Particle Size and Zeta (ζ) Potential

A Zetasizer Nano ZS equipment was used to assess the measurements of NPs' size and ζ potential. In this work, NPs were assessed while suspended in PBS (pH 7.4).

2.8. In vitro Release Study of Extract Loaded Nps

R. ribes young shoots ultrasonic methanol extract (RYU) and ultrasonic methanol extract of *R. ribes* radix (RRU) were released in vitro from chitosan nanoparticles in PBS (pH 7.4) using the same procedures as described in Keawchaoon & Yoksan (2011) and Mohammadi *et al*. (2015), with a few minor modifications. Initially, 2 mL of buffer solution was mixed with a certain quantity of *R. ribes* extract-loaded NPs, and then vortexed at room temperature. Samples were centrifuged at 10000 rpm for 10 min at 25 °C at preset intervals. To keep the total volume constant, 400 µL of the supernatant was removed for analysis and replaced with an equivalent volume of new buffer. Utilizing UV vis spectrophotometer, the amount of liberated *R. ribes* extract at a certain moment was calculated.

3. FINDINGS

3.1. Carbonic Anhydrases Inhibition Activity Results

The study also explored the potential of using these antiepileptic substances as safer alternatives to the currently available drugs, which may have various potentially harmful side effects. Spectrophotometric analysis was used to determine how these chemicals affected the enzyme

activity of carbonic anhydrase I and II isoenzymes. The inhibitory potentials of these medicines against two physiologically significant CA isoforms, the slower cytosolic isoform (hCA I) and the quicker cytosolic isoenzyme (hCA II), were investigated using an esterase assay approach. The inhibitory results of medicines against CA I and II isoforms are shown in Table 2 and Figure 1 (IC_{50} values expressed as µg/mL).

Table 2. The extracts that inhibit the enzymes carbonic anhydrase I and II.

Extracts	IC_{50} (µg/mL)			
	hCA I	r^2	hCA II	r^2
RYS	7.72	0.9551	6.73	0.9634
RYU	3.21	0.9966	4.76	0.9940
RYU NP	3.83	0.9407	2.70	0.9780
RYM	5.71	0.9538	5.31	0.9232
RLS	12.93	0.9027	3.66	0.8357
RLU	6.04	0.9396	8.13	0.9604
RLM	7.42	0.9170	10.69	0.8481
RFS	7.39	0.9429	7.30	0.9985
RFU	7.55	0.9552	12.96	0.9851
RFM	10.82	0.8372	6.52	0.9840
RRS	4.25	0.9515	5.07	0.9567
RRU	4.43	0.9787	3.44	0.9315
RRU NP	2.97	0.8049	5.37	0.9061
RRM	6.82	0.8773	4.57	0.9821

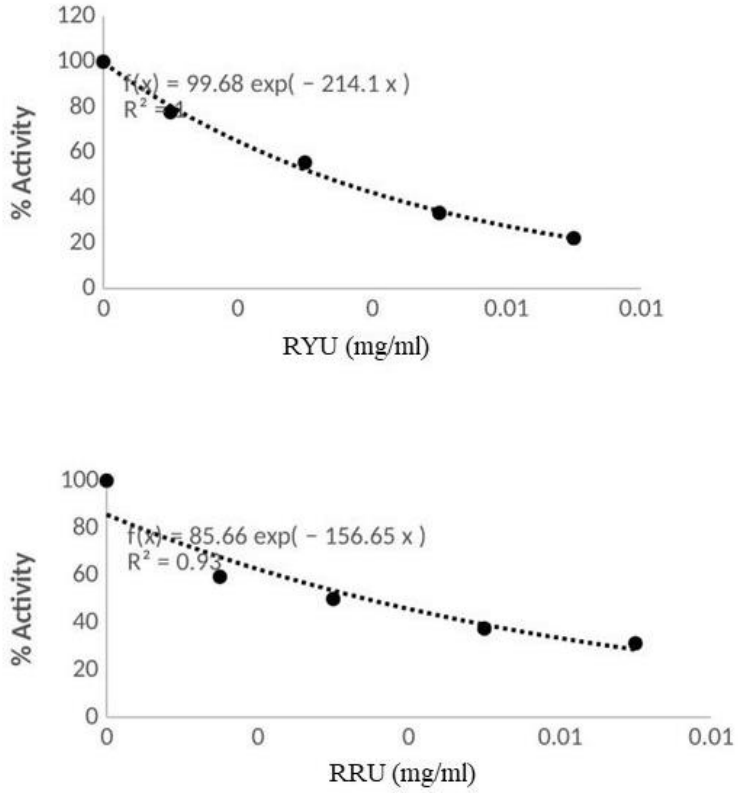

Figure 1. IC_{50} values for the hCA I and hCA II isoenzymes, with RYU (Rheum ribes young shoots ultrasonic bath extract) and RRU (*Rheum ribes* radix ultrasonic bath extract) respectively serving as the best inhibitors

All the compounds were remarkably inhibited both the cytosolic isoforms hCA I (IC_{50} ranging between 3.21 and 12.93 µg/mL) and hCA II (IC_{50} ranging between 3.44 and 12.96

µg/mL). We found that the most effective inhibitors for these isoforms were RYU and RRU (hCA I and hCA II) with IC_{50} values of 3.21 and 3.44 µg/mL, respectively. In addition, IC_{50} values on hCA I enzyme of NP samples including RYU and RRU were calculated as 3.83 µg/mL and 2.97 µg/mL. IC_{50} values on hCA II enzyme of NP samples including RYU and RRU were calculated as 2.70 µg/mL and 5.37 µg/mL (Table 2, Figure 1-2). These results indicated that NP containing RYU has more effective hCAII enzyme inhibitory activity compared to the extract. In addition, it can be said that RRU loaded NP has a higher hCAI enzyme inhibitory effect compared to only the extract.

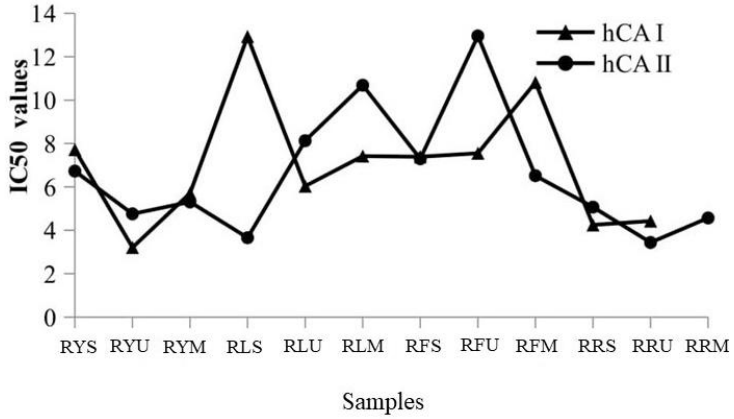

Figure 2. IC_{50} values for the isoenzymes hCA I and hCA II.

3.1. Assessment of Cytotoxic Activity Results

Rheum ribes extracts' cytotoxic activity was evaluated on C6 and NIH 3T3 cell lines using XTT assay. This method is based on the reduction of XTT, a tetrazolium salt, to orange formazan crystals by metabolically active cells (Taşkın *et al*., 2020). At the beginning of the study, DMEM + fetal bovine serum (FBS) + antibiotic-containing medium will be seeded into a 96-well microplate and incubated overnight for the cells to adhere. Then, the medium will be removed, the wells containing cells will be washed with PBS, and immediately after that, fresh medium and extracts will be seeded into the cells and incubated for 24 hours. Then, the medium will be removed and the cells will be washed three times with PBS. 100 µl of transparent DMEM and 50 µl of XTT solution will be added to each well and incubated for 4 hours in a CO2 oven. After incubation, the cell viability rate of the control group was calculated as % by reading at 450 nm in a microplate reader. Antiproliferative activity results of twelve extracts of *R. ribes* against C6 cells were indicated in Figure 3. When the extracts were given to C6 cells at a concentration of 10 g/mL, the soxhlet *R. ribes* rhizome methanol extract's cell viability was assessed to be 65.27±0.23% in the RYS, 59.13±0.25% in the RYU, 67.89±0.18% in the RYM, 69.05±0.32 % in the RLS, 71.92±0.24 % in the RLU, 76.86±0.22 % in the RLM, 73.02±0.24 % in the RFS, 64.99±0.22% in the RFU, 67.16±0.27% in the RFM, 72.23±0.19% in the RRS, 68.52±0.23 % in the RRU, and 78.96±0.32% in the RRM. The antiproliferative activity of R. ribes extracts RYU and RFU against the C6 cell line was found to be the most potent, as shown by the results of Figure 3. The best carbonic anhydrase inhibitors were found to be *R. ribes* extracts RYU and RRU. To do this, chitosan nanoparticle (NP) was prepared, and RYU and the NP containing the extract were applied to C6 and NIH 3T3 cells at varied concentrations.

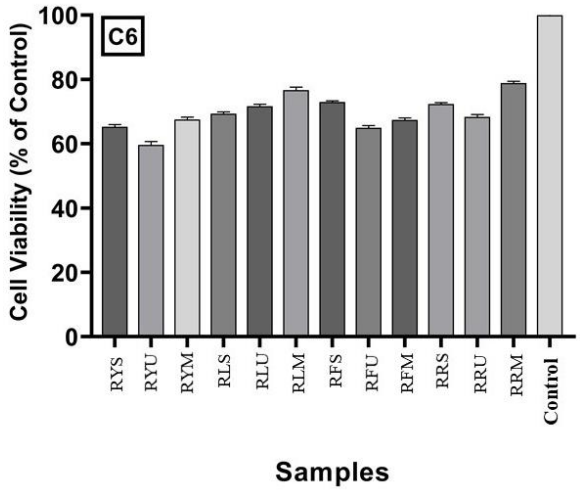

Figure 3. Cytotoxic effects of several *Rheum ribes* extracts on the C6 cell line at doses of 10 µg/mL.

Figure 4 displays the results of *R. ribes* extracts' cytotoxic efficacy against NIH 3T3 cells. The RYS was treated with NIH 3T3 cells at a preset concentration (10 µg/mL), and the cell viability was estimated to be 75.99±0.22%, 82.51±0.25% in the RYU, 85.16±0.19% in the RYM, 80.05±0.26 % in the RLS, 82.66±0.22 % in the RLU, 76.66±0.24% in the RLM, 85.06±0.19 % in the RFS, 78.66±0.28% in the RFU, 74.52±0.25% in the RFM, 84.10±0.22% in the RRS, 75.02±0.23% in the RRU and 80.86±0.27% in the RRM. According to the findings of Figure 4, none of the *R. ribes* extracts at a concentration of 10 µg/mL significantly reduced the vitality of NIH 3T3 cells, and they were also not cytotoxic because the viability of the cells reached 70%.

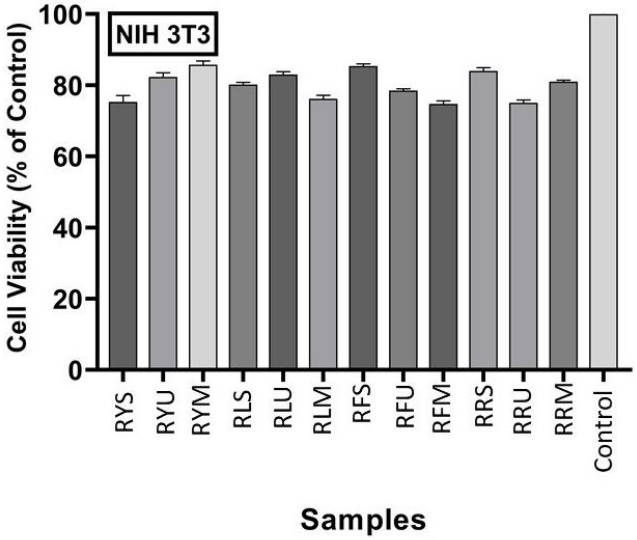

Figure 4. Different *Rheum ribes* extracts treated with doses at 10 µg/mL had anti-proliferative effects on the NIH 3T3 cell line.

To evaluate the IC_{50} values, C6 and NIH 3T3 cell lines were treated with NP containing RYU of *R. ribes* at predetermined doses. The C6 cell viability was severely decreased by both the RYU and the RYU loading NPs, according to the findings of the XTT investigation (Table 3), depending on the concentration. The cell viability rates were calculated as 66.16±0.24% and 68.28±0.31%, respectively, when the sole extract and the extract loading NP were fed to C6 cells at a concentration of 2.5 µg/mL. When C6 cells were exposed to extract and extract loading NP at a concentration of 5 µg/mL, the cell viability was estimated to be 63.28±0.29% and

63.44±0.21%, respectively. Additionally, the extract and NP containing extract were supplied to cells at a concentration of 10 g/ml, and the results showed that the cell viability was, respectively, 59.23 ±0.24% and 55.28 ±0.26%. Additionally, cells were treated with extract and extract loading NP at a concentration of 25 µg/mL, and the cell viability was estimated to be 50.16±0.22 and 47.24±0.32, respectively. The cell viability rates were calculated as 39.24±0.25% and 36.16±0.23%, respectively, when the extract and the extract loading NP were treated with C6 cells at the 50 µg/mL concentration. The greatest concentration (100 µg/mL) of the extract and extract loading NP on C6 cells resulted in cell viability rates of 28.22±0.23% and 25.16±0.21%, respectively. These results were used to calculate the IC_{50} values for RYU and RYU loading NP of *R. ribes*. On the C6 cell line, the IC_{50} values for RYU and NP containing RYU were 25.36±0.18 µg/mL and 18.23±0.21 µg/mL, respectively. The findings demonstrate that compared to RYU alone, RYU loaded NP has a lower IC_{50} value and greater cytotoxic effect in C6 cells. When C6 cells were exposed to simply RRU or RRU loading NP at a concentration of 2.5 µg/mL, the cell viability rates were calculated to be 78.12±0.27 % and 70.24±0.25 %, respectively. When C6 cells were treated with extract and extract loading NP at a concentration of 5 µg/mL, the cell viability was estimated to be 74.29±0.25 % and 67.75±0.17 %, respectively. Additionally, the extract and NP containing extract were fed to cells at a concentration of 10 µg/mL, and the estimated percentages of cell viability were 68.52±0.23 %, and 61.28±0.18 %, respectively. Additionally, cells were treated with extract and extract loading NP at a concentration of 25 µg/mL, and the cell viability was estimated to be 58.26±0.22 %, and 52.16±0.26 %, respectively. The cell viability rates were calculated as 44.52±0.11 % and 41.21±0.19 %, respectively, when the extract and the extract loading NP were treated with C6 cells at the 50 µg/mL concentration. The greatest concentration of the extract and extract loading NP (100 µg/mL) used to treat C6 cells resulted in cell viability rates of 30.16±0.13% and 24.98±0.14 %, respectively. These results were used to calculate the IC_{50} values for RRU and RRU loading NP of *R. ribes*. On the C6 cell line, the IC_{50} values for RRU and RRU loaded NPs were respectively 39.66±0.22 µg/mL and 29.53±0.14 µg/mL. According to the findings, compared to RRU, the RRU loading NP has a lower IC_{50} value and greater antiproliferative action in C6 cells. The solo RYU and the RYU loading NP were treated with NIH 3T3 cells at a concentration of 2.5 g/ml, and the cell survival rates were determined as 89.98±0.26 % and 87.48±0.33 %, respectively, according to the concentration-dependent XTT research results in the NIH 3T3 cell line (Table 4). NIH 3T3 cells were treated with the extract and extract loading NP at a concentration of 5 µg/mL, and the cell viability was estimated to be 86.420.27% and 83.240.27%, respectively. Additionally, cells were given the extract and NP containing the extract at a concentration of 10 µg/mL, and the cell viability was estimated to be 82.51±0.22 %, and 81.28±0.20 %, respectively. Additionally, cells were treated with extract and extract loading NP at a concentration of 25 µg/mL, and the estimated percentages of cell viability were 75.44±0.26 %, and 76.16±0.27 %, respectively.

Table 3. Results of extracts and NPs samples on the C6 cell line in terms of concentration-dependent cell viability.

	C6 cell viability (% of the control)			
Samples	RYU	NP loading RYU	RRU	NP loading RRU
2.5 µg/mL	66.16±0.24	68.28±0.31	78.12±0.27	70.24±0.25
5 µg/mL	63.28±0.29	63.44±0.21	74.29±0.25	67.75±0.17
10 µg/mL	59.23±0.24	55.28±0.26	68.52±0.23	61.28±0.18
25 µg/mL	50.16±0.22	47.24±0.32	58.26±0.22	52.16±0.26
50 µg/mL	39.24±0.25	36.16±0.23	44.52±0.11	41.21±0.19
100 µg/mL	28.22±0.23	25.16±0.21	30.16±0.13	24.98±0.14

Table 4. Results of extracts and NPs samples on the NIH 3T3 cell line in relation to concentration-dependent cell viability.

NIH 3T3 cell viability (% of the control)				
Samples	RYU	NP loading RYU	RRU	NP loading RRU
2.5 µg/mL	89.98±0.26	87.48±0.33	82.25±0.31	79.56±0.22
5 µg/mL	86.42±0.27	83.24±0.27	79.98±0.27	77.65±0.27
10 µg/mL	82.51±0.22	81.28±0.20	75.02±0.23	75.28±0.26
25 µg/mL	75.44±0.26	76.16±0.27	66.27±0.16	68.98±0.21
50 µg/mL	60.16±0.28	64.38±0.29	56.14±0.13	58.27±0.19
100 µg/mL	48.24±0.28	55.44±0.17	44.21±0.13	51.26±0.22

The cell viability rates were calculated as 60.16±0.28% and 64.38±0.29% respectively when the extract and the extract loading NP were administered to NIH 3T3 cells at the 50 µg/mL concentration. The cell viability rates were calculated as 48.24±0.28% and 55.44±0.17%, respectively, when the extract and the extract loading NP were treated with NIH 3T3 cells at the 100 µg/mL concentration. The IC_{50} value of the NP containing RYU at the indicated concentrations could not be calculated, and the IC_{50} value of RYU was determined to be 88.26±0.23 µg/mL. According to the findings, RYU loading NP has less antiproliferative action in NIH 3T3 cells than RYU alone. When NIH 3T3 cells were exposed to the single RRU and the NP containing RRU at a concentration of at least 2.5 µg/mL, the cell viability rates were calculated to be 82.25±0.31% and 79.56±0.22%, respectively. When NIH 3T3 cells were treated with RRU and RRU loading NP at the highest concentration (100 µg/mL), the cell viability was estimated to be 44.21±0.13% and 51.26±0.22%, respectively. The IC_{50} value of the NP containing RRU at the determined concentrations could not be estimated, and the IC_{50} value of RRU was calculated to be 73.28±0.18 µg/mL.

3.2. EE and LC of NP Including *Rheum ribes* Extract

The amount of RRU and RYU extract coated in chitosan nanoparticles is shown by the EE rate. The findings of the calculation used to determine the rate at which the encapsulated extract turned into chitosan NP are shown in Table 5. EE value of NPs containing RYU was determined at 78.27±0.03%, according to the results. Furthermore, the LC of NPs was determined to be 9.02±0.02%. The computed EE value of NP incorporating RRU was 69.36±0.04%. These findings demonstrated that chitosan nanoparticles effectively contained RYU and RRU. Furthermore, LC of NPs, comprising RRU, was discovered to be 8.62±0.01 %. The findings indicate that NPs containing *R. ribes* extracts were appropriate for cell culture investigations in terms of both EE and LC values.

Table 5. EE and LC of RYU and RRU loading NPs,

Parameter	RYU	RRU
Linear equation	y=0.2908 x ± 0.0197	y=0.3016 x ± 0.0221
Slope ± SD	0.2908 ± 0.027	0.3016 ± 0.022
Intercept	0.0197 ± 0.0012	0.0221 ± 0.0015
r	0.9903	0.9926
EE %	78.27 ± 0.03	69.36 ± 0.04
LC %	9.02 ± 0.02	8.62 ± 0.01

3.3. Characterization of Chitosan NPs

The findings of the evaluation of the NPs' particle size, ζ potential, and polydispersity index (PDI) are shown in Table 6. The dimensions of the NP1 and NP2 were determined to be respectively 384.28±2.40nm and 342.36±2.20 nm. The NP1 and NP2's respective potentials were measured at 2.65±0.03 and 2.42±0.02 mV. PDI values NP1 and NP2 were 0.278±0.06

and 0.306±0.03, respectively. The findings support the notion that NPs were homogeneous features devoid of any aggregation.

Table 6. Extract loading NPs' ζ potential, particle size, and PDI values.

Samples	ζ potential (mV) ± SD	Size (nm) ± SD	PDI ± SD
*NP1	2.65±0.03	384.28±2.40	0.278±0.06
*NP2	2.42±0.02	342.36±2.20	0.306±0.03

* NP1 and *NP2 include chitosan (MW: 400 kD, DD: 92 %), TPP, RYU and RRU respectively.

3.4. *In vitro* Release Kinetics Study Result of Extract Loading NP

At 37 ºC for 300 hours, the release profile of RYU and RRU from NPs was examined (Figure 5). 0.1 M PBS was utilized in this study to mimic physiological circumstances. The results of the controlled release of *R. ribes* extracts showed a quick initial release (50%) during the first 24 hours, followed by a constant and gradual release (75%) until 144 hours. The release characteristics of RYU and RRU containing NPs were comparable. The NPs released almost all the extracts (99.26% and 99.96%) in 288 hours. In a study for acetylsalicylic acid, this kind of continuous and slow release was seen (Ajun *et al*., 2009). The release of the extract from the NP is significantly influenced by diffusion and the breakdown of the extract's molecular matrix. Since the extract's size is less than that of the particle, it can easily diffuse from the NP's surface or pores (Zhou *et al*., 2001; Hu *et al*., 2008).

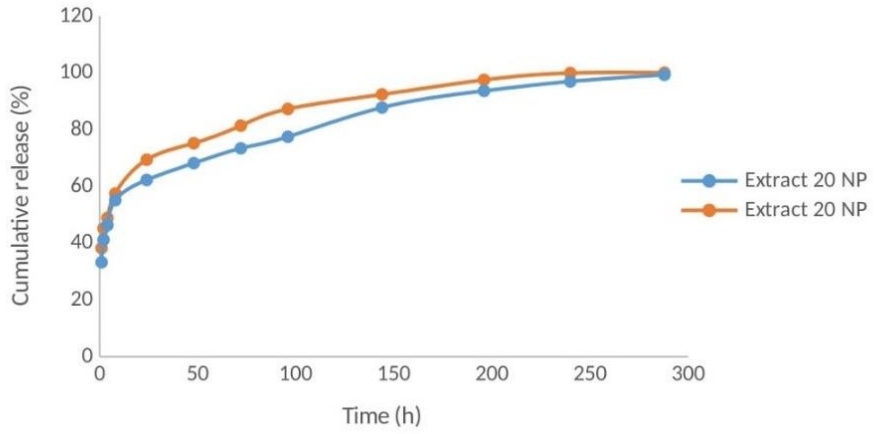

Figure 5. Release kinetics of NPs in 0.1 M PBS (pH 7.4), containing extracts RYU and RRU.

4. DISCUSSION and CONCLUSION

In Turkey, locals also use the *R. ribes* plant as an anti-cancer remedy. Furthermore, the abundant amounts of vitamins C, A, B1, B2, E, and K present in *R. ribes* are also significant. Moreover, antioxidant qualities are provided by the polyphenolic compounds. Additionally, it has been shown to be beneficial for conditions like diabetes, hypertension, asthma, colds, diarrhea, hemorrhoids, ulcers, influenza, and kidney disease (Aygun *et al*., 2020). Researchers investigated *Rheum emodi* root extracts from the *Rheum* genus. These investigations showed that *Rheum emodi* rhizome extracts, both methanolic and aqueous, demonstrated concentration-dependent cytotoxicity against cell lines that represent liver cancer (Hep3B) and breast cancer (MDA-MB-435S) (Rajkumar *et al*., 2011). Furthermore, extracts from various *Rheum* species, such as *R. ribes*, exhibited anti-proliferative properties against cell lines that were specifically related to breast, liver, and cervical cancer (Keser *et al*., 2011). The findings of this study support earlier findings in the literature and demonstrate that *R. ribes* extract nanoparticles at low concentrations can have encouraging effects on cancer cell death.

In conclusion, each *R. ribes* extract showed significant cytotoxic activity in the C6 cell line. The extracts decreased cell viability but did not show cytotoxic activity (cell viability up to 70

%) in the NIH 3T3 cell line. Both NP containing RYU and NP loaded RRU showed significant carbonic anhydrase enzyme inhibitory activity. The particle sizes of the NPs containing RYU and RRU were below 400 nm, making them applicable in both enzyme activity and cell culture studies. The high encapsulation rate of NPs ensured the suitability of the method used in the preparation phase and the minimum loss of extract. The fact that the extracts from the NPs are completely released in a period of more than 240 h showed that the NPs have the controlled release feature. While the extract-loaded NPs showed better cytotoxic activity in C6 cells, they did not show cytotoxic effects in healthy NIH 3T3 cells, while the high cell viability rates indicate that the study achieved the desired aim.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Murat Doğan: Investigation, Resources, Methodology, Experiment, Visualization, Formal Analysis, Writing. **Ümit Muhammet Koçyiğit:** Investigation, Resources, Experiment, Visualization, Formal Analysis, Writing. **Duygu Taşkın:** Investigation, Resources, Writing, Supervision. **Beyza Nur Yılmaz:** Investigation, Methodology, Experiment, Resources, Writing. **Turgut Taşkın:** Investigation, Methodology, Experiment, Resources, Supervision.

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