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ORIGINAL ARTICLE

Royal Jelly Nanoparticle Alleviates Experimental Model of Breast Cancer Through Suppressing Regulatory T Cells and Upregulating TH1 Cells

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SUMMARY

Background: **Royal jelly, a natural product from bees' hypopharyngeal glands, is commonly used in biomedicine due to its antioxidant and anti-tumor activities. The aim of this study was to compare royal jelly in free form and loaded in layered double hydroxide (LDH) nanoparticle for the treatment of breast cancer with a focus on Th1 and T regulatory parameters in an animal model.**

Methods: **Nanoparticles were produced using the coprecipitation method and characterized using DLS, FTIR, and SEM techniques. Forty female BALB/c mice were inoculated with 7.5 x 10^5 4T1 cells and treated with royal jelly in free and nanoparticle form. Clinical signs and tumor volume were assessed weekly. The effect of royal jelly products on the serum level of IFN-γ and TGF-β was measured by ELISA. In addition, the mRNA expression of these cytokines and Th1 and regulatory T cells' transcription factors (T-bet and FoxP3) was assessed by real-time PCR in the splenocytes of tumor-bearing mice.**

Results: **The physicochemical analysis of nanoparticles confirmed the synthesis of LDH nanoparticles and loading of royal jelly into the LDH structures (RJ-LDH). Animal studies showed that royal jelly and RJ-LDH significantly reduced the size of tumor in BALB/c mice. Additionally, treatment with RJ-LDH significantly inhibited TGF-β and increased IFN-γ production. The data also revealed that RJ-LDH inhibited the differentiation of regulatory T cells, while promoting Th1 cell differentiation via regulating their master transcription factors.**

Conclusions: **These results indicated that royal jelly and RJ-LDH could inhibit breast cancer progression by inhibiting regulatory T cells and expansion of Th1 cell. Furthermore, the current study demonstrated the therapeutic efficacy of royal jelly is enhanced by LDH nanoparticles; hence, RJ-LDH is significantly more efficient than Free-RJ in the treatment of breast cancer.**

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KEYWORDS

breast cancer, royal jelly, layered double hydroxide nanoparticle (LDH), royal jelly-LDH conjugate (RJ-LDH)

INTRODUCTION

Breast cancer is the most common cancer in women, affecting 2.3 million individuals worldwide. In addition, breast cancer has led to the death of over 685,000 women in 2020 [1].

The acquired immune system, especially T lymphocytes, play a critical role in the immune system's response to malignancies [2].

TH1 cells play an essential role in immunity against tumors via interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) production [3]. Research on several tumors, such as colon cancer, melanoma, and multiple myeloma, demonstrated that the presence of TH1 and CTL cells in the tumor microenvironment is associated with a better prognosis [4].

The studies demonstrated that differentiation of naive CD4+ T cells into TH1 cells depends on cytokines such as IFN-γ, IL-12 and transcription factor (T-bet) [5]. Various research on breast cancer have found that the presence of T-bet+ lymphocytes is associated with a better prognosis, in contrast the absence of T-bet+ lymphocytes is related to increased tumor size and aggravation of the disease [6].

In addition to TH1 cells, regulatory T cells (Treg) are crucial in immune responses in the tumor microenvironment. These cells suppress effector cells by releasing TGF-β and IL-10 or cell-cell interaction [7]. Studies have shown that Treg cells could inhibit anti-cancer responses, thus resulting in tumor growth and a poor prognosis [8].

FoxP3 is a crucial transcription factor for Treg cells, as well as a pivotal regulator of their development and function [9]. Further evidence demonstrates that patients with a high level of FoxP3 have a worse prognosis than those with a low level. FoxP3 expression has also been proven to be correlated with patient survival in the disease [10].

Despite a wide range of research, current treatment approaches, including surgery, chemotherapy, and radiation therapy, are invasive, and in addition to eliminating cancer cells, they lead to damage normal cells [11].

Consequently, scientists are currently searching for novel and low-risk medications derived from natural products to treat breast cancer.

Royal jelly is a material generated by the hypopharynxgeal glands of the young worker bee (nurse bee) from the Apis mellifera L species. It has been noted that the effect of royal jelly depends on physiological conditions, dose, and application method [12].

Research has shown that royal jelly exerts its anti-oxidant and anti-tumor activities through reducing the proliferation and invasion of tumor cells [13].

Although the exact function of royal jelly compounds in immune responses is not yet clear, royal jelly's potency in eliminating malignant cells is widely known.

Treatment of tumors by current drugs or natural products requires high doses and long-term pharmacotherapy, which results in side effects and low efficacy of drugs. Therefore, in this study, we used a new formulation of royal jelly loaded with layered double hydroxide (LDH) nanostructure (RJ-LDH) that serves as a drug delivery system.

Nanotechnology researchers have demonstrated that LDH nanoparticles enhance drug bioavailability and stability and increase drug delivery to the targeted cells or tissues, while minimizing adverse effects through changing the pharmacokinetic parameters of the drug [14].

According to the properties of royal jelly and LDH nanostructures, the main aim of this study was to evaluate the effects of royal jelly in free form and conjugated with LDH on clinical and pathological symptoms of breast cancer in an animal model.

MATERIALS AND METHODS

Synthesis and characterization of LDH nanoparticle products

Synthesis of LDH nanoparticle

The co-precipitation method was used to for the synthesis of layered double hydroxide nanoparticles (LDH). First, 0.05 g of aluminum powder, 0.05 g of magnesium powder, and 3 mg of carbomer biopolymer were weighed and then were placed in a 100 mL round bottom balloon, followed by 10 cc of distilled water at a pH of 8. After connecting the balloon to the reflux system, 2 cc of Triethylamine and 5 mg of Acetyltrimethylammonium Bromide were added, and it was clamped to the magnetic stirrer heater. The heater was set to 400 rpm at 30℃ for 15 minutes, then kept at 50℃ for another 15 minutes. At the end of this period, the contents of the balloon were transferred to a 50 mL beaker and placed in the microwave at 180 watts for 10 minutes. Laminated polymer solutions of various concentrations were prepared and ultrasonically processed at 60 watts.

Finally, the resulting solution was sent for loading with royal jelly and characterization of LDH products.

Synthesis of royal jelly-LDH conjugate (RJ-LDH)

A co-precipitation method was used to load the royal jelly into the LDH nanoparticles. To do this, 10 mL of the synthesized LDH nanoparticles was placed on a magnetic heater at 50℃ and connected to the reflux system. The reflux system cools the vapors of the solution and leads to a phenomenon called nucleation. Nucleation is a phenomenon in which layers of LDH nanoparticles open and close, sequentially. At the same time, the desired amount of royal jelly was added to the solution. During the nucleation phenomena, royal jelly was

loaded between the nanoparticle layers.

Calculation of drug loading

To determine the loading efficacy of royal jelly into LDH nanoparticles, the above solution containing royal jelly-LDH conjugate was centrifuged at 14,000 rpm for 30 minutes. The supernatant was separated and unloaded royal jelly was quantified by measuring the amount of royal jelly in the supernatant solution at 570 nm using UV-spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). Encapsulation efficiency (EE) was calculated according to the below equation:

(EE%) = (Wt/Wi) x 100%

where Wt was the total amount of drug in the nanostructure suspension and Wi was the total quantity of drug added initially during preparation.

The amount of loading efficiency was calculated from the difference between the initial and unloaded royal jelly concentrations. The amount of royal jelly adsorbed onto LDH after 2 hours incubation was calculated to be 67.5%.

Particle size analysis

The size and zeta potential of nanoparticles were estimated using dynamic light scattering (DLS) with a laser beam at 633.8 nm (Malvern Zetasizer, UK). One milligram of each sample was dispersed in 1 cc distilled water, then the size and zeta-potential were measured by DLS. Data on zeta potentials were acquired in triplicate in pure water employing electrophoretic light scattering at 20°C and 150 V. The Malvern 50 V standard was used to calibrate the instrument.

Scanning Electron Microscopy (SEM)

The morphology and particle size of LDH and RJ-LDH products were observed with scanning electron microscopy (Philips Company, Germany, EM208S model). In this regard, LDH nanoparticles samples were centrifuged and redispersed in deionized water. Five milliliters of the synthesized nanoparticle products was filtered through a 0.22 μm filter, and an aliquot of the reaction mixture was dried using a grade III hood for 2 hours. Finally, the samples were assessed using SEM.

Fourier transform infrared spectroscopy (FTIR)

To determine the functional groups and possible interactions between LDH and royal jelly, Fourier transform infrared spectroscopy (FTIR) was used.

FT-IR spectra were recorded on a spectrometer (Perkin-Elmer Spectrum 100 FT-IR spectrometer) at room temperature. The sample was pushed onto a disc with KBr. The spectrum of each sample was obtained within 4,000 - 400 cm^{-1} wavenumber range.

Animals

Forty female BALB/c mice (Pasteur Institute, Tehran, Iran), aged 6 - 8 weeks, were utilized in this study. The animals were purchased from the Pasteur Institute of Tehran (Iran) and kept in standard conditions in terms of water, food and environment.

Experiments were carried out according to the NIH Guide for the Care and Use of Laboratory Animals, with approval of the Animal Ethics Committee of Kerman University of Medical Sciences (IR.KMU.REC. 1399.354).

The animals were randomly separated into five groups (n = 8/group) as follows: Group 1 (control) was breast cancer induced, and the mice were treated with a vehicle.

Group 2: Was breast cancer induced and the mice were intraperitoneally treated with royal jelly at a dose of 100 mg/kg and were named Free-RJ.

Group 3: In this group, the tumor was induced in mice and the intervention was performed by treatment with unloaded LDH nanostructures at a dose of 100 mg/kg via intraperitoneal injection and was named Free-LDH.

For the treatment groups, RJ-LDH were intraperitoneally administered at 50 and 100 mg/kg every other day to mice in groups 4 and 5, respectively. These groups were named RJ-LDH-50 and RJ-LDH-100. The doses were selected based on the findings of Dalfardi, et al. [15]. All animals in the study were treated from day 7 post-challenge until day 28.

Cell culture

Breast cancer cell line, 4T1, (obtained from Pasteur Institute, Tehran, Iran) was cultured in RPMI 1,640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. All components of the cell culture medium were purchased from Gibco Life Technologies, USA.

Breast cancer induction and treatment protocol

For breast cancer induction, 5 x 10^5 4T1 cells suspended in serum-free medium were inoculated into the second mammary fat pad of BALB/c mice.

Mice were assessed for tumor development and then the tumor size was measured using a digital caliper once a week. The volume of tumors was calculated using the formula [$V = (L \times W^2)/2$], where L was tumor length and W was tumor width.

Treatment was started when tumors were palpable at the injection site. Then royal jelly in free or nanoparticle form was administered every other day intraperitoneally. At the end of the experimental period (28 days), the mice were slaughtered as per animal ethics.

Cytokine enzyme-linked immunosorbent assay (ELISA)

To determine the effect of royal jelly in free or nanoparticle form on the cytokine level, the mice were sacrificed at the end of the experimental period. Serum from peripheral blood was collected and the level of TGF-β and IFN-γ in the serum was measured using ELISA kits (mice TGF-β and IFN-γ platinum ELISA®, Bender Med systems, Austria) according to the manufacturer's in-

structions.

Quantitative real-time PCR (qPCR)
qPCR was used to evaluate the effects of royal jelly and its nanoparticle products on the expression of Th1 and Treg transcription factors (T-bet and FoxP3) and their cytokines (IFN-γ and TGF-β). Splenocytes were isolated and total RNA was extracted using Trizol reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The PrimeScript TMRT reagent kit (Takara Biotechnology, Otsu-Shiga, Japan) was used to synthesize cDNA according to the manufacturer's guidelines. The Rotor Gene Q (Qiagen Hilden, Germany) was used for quantitative real-time PCR using Syber Green Master Mix (Prime mix Syber Green™, Takara Biotechnology, Otsu-Shiga, Japan) and appropriate primers (Table 1). For sample normalization, mouse beta-actin was utilized as an endogenous control. Delta-delta Ct was used for relative quantification based on target gene expression normalized to beta-actin, and the findings are given as fold change compared to control.

Statistical analysis
All data were reported as mean ± SEM. The differences between the groups were measured using a one-way analysis of variance (ANOVA) followed by a post hoc Tukey test. Statistical analysis was performed using SPSS 19 (IBM SPSS, Statistics for Windows; IBM Corp., Armonk, NY, USA). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Characterization of LDH nanoparticle products
Dynamic light scattering measurements (DLS)
DLS results show that the average diameters of Free-LDH and RJ-LDH products were found to be 102.81 and 118.11 nm, which is closely related to the data obtained from the particle size calculated by the Debay-Scherer equation and the SEM image (Figure 1A and B).
The results also show that the particles are very uniform, with a suitable size distribution.

SEM results
The structural properties of free and royal jelly-coated LDHs were analyzed using SEM microscopy. The results of the scanning electron microscopy show that the layered structures are well formed. Figures 2A and B show a typical SEM image of Free-LDH (LDH nanostructures) and RJ-LDH (royal jelly-LDH conjugate), respectively. The results show that royal jelly is well trapped between LDH nanostructures.
The results also show that with the increase in particle size, the surface of the particles becomes more active and adhesion in the layers occurs more. The morphology of nanostructures under the influence of concentration transforms into rods with quantum particles-like on their surface. Results show which concentration has a great impact on particle size final products.

FTIR results
Figure 3 shows FTIR spectra of nanostructured LDH (Free-LDH, curve A), royal jelly (Free-RJ, curve B), and LDH loaded with royal jelly (RJ-LDH, curve C). As shown in Figure 3, the wide absorption peak at 3,500 - 3,100 cm^{-1} is related to the OH band. The adsorption peak at 12,452 cm^{-1} proves the presence of the N - H carbomer bond. In addition, the band at 2,927 cm^{-1} is attributed to the C - H tension in the royal jelly, and the band below 1,000 cm^{-1} confirms the presence of metal-to-metal connections. A few weak bands in the center 615 - 793 cm^{-1} can be attributed to the tensile vibration of the C - H bond of sodium dodecyl sulfate molecules adsorbed on the nanoparticle surface.

Effects of Free-RJ and RJ-LDH on tumor size in BALB/c mice
Tumor volume was measured after tumor induction and throughout the treatment period every week. Seven days after the injection of the 4T1 cell line, tumor growth was clearly visible and tumors were palpable. The results of tumor growth in the control group and the groups treated with Free-RJ and RJ-LDH products are presented in Figure 4. As shown in Figure 4, there is a significant difference in tumor volume between the control and treatment groups ($p < 0.05$ for Free-RJ, RJ-LDH-100, and $p < 0.05$ for RJ-LDH-50). The data showed that the decrease in tumor volume in the group treated with RJ-LDH-50 was more significant than in the other groups ($p < 0.01$).

RJ-LDH and Free-RJ increase pro-inflammatory and decrease anti-inflammatory cytokines
To address the effect of Free-RJ and RJ-LDH products on T cell responses, the serum levels of IFN-γ and TGF-β were measured. *In vitro* analysis of serum samples collected from BALB/c mice showed significantly increased secretion of Th1 cytokine, IFN-γ, from cells in RJ-LDH-50 treated mice, in comparison with control mice (Figure 5A, $p < 0.001$). In mice receiving RJ-LDH-100, higher level of IFN-γ was observed than the control mice. However, the effect was not significant. In contrast, the release of Treg cytokine, TGF-β, was highly reduced in RJ-LDH-50 treated animals (Figure 5B, $p < 0.05$). No significant differences were noted in serum TGF-β production following RJ-LDH-100 treatment when compared to the control group.
These results indicate that RJ-LDH at a dose of 50 mg/kg induces the promotion of Th1 response and suppression of Treg cells through the stimulation of IFN-γ and inhibition of TGF-β production.

Effects of Free-RJ and RJ-LDH on the polarization of Th1 and Treg responses in tumor-induced mice
To evaluate the therapeutic potential of Free-RJ and RJ-

Table 1. The sequences of primers which were used in the study.

Genes		Sequences
Mouse IFN-γ	F	5′- ATTGCCAAGTTTGAGGTCAACAA-3′
	R	5′- ATCTCTTCCCCACCCCGAAT-3′
Mouse TGF-β	F	5′- ACAATTCCTGGCGTTACCTTGG-3′
	R	5′- AGCCCTGTATTCCGTCTCCTTG-3′
Mouse T-bet	F	5′- CTTCCAACAATGTGACCCAGATGA-3′
	R	5′- AAGACGTGTGTGTTAGAAGCACT -3′
Mouse FoxP3	F	5′- GGCAATAGTTCCTTCCCAGAGTTC -3′
	R	5′- CGGATAAGGGTGGCATAGGTG -3′
Mouse beta-actin	F	5′- CACTGTCGAGTCGCGTCC-3′
	R	5′- TCATCCATGGCGAACTGGTG-3′

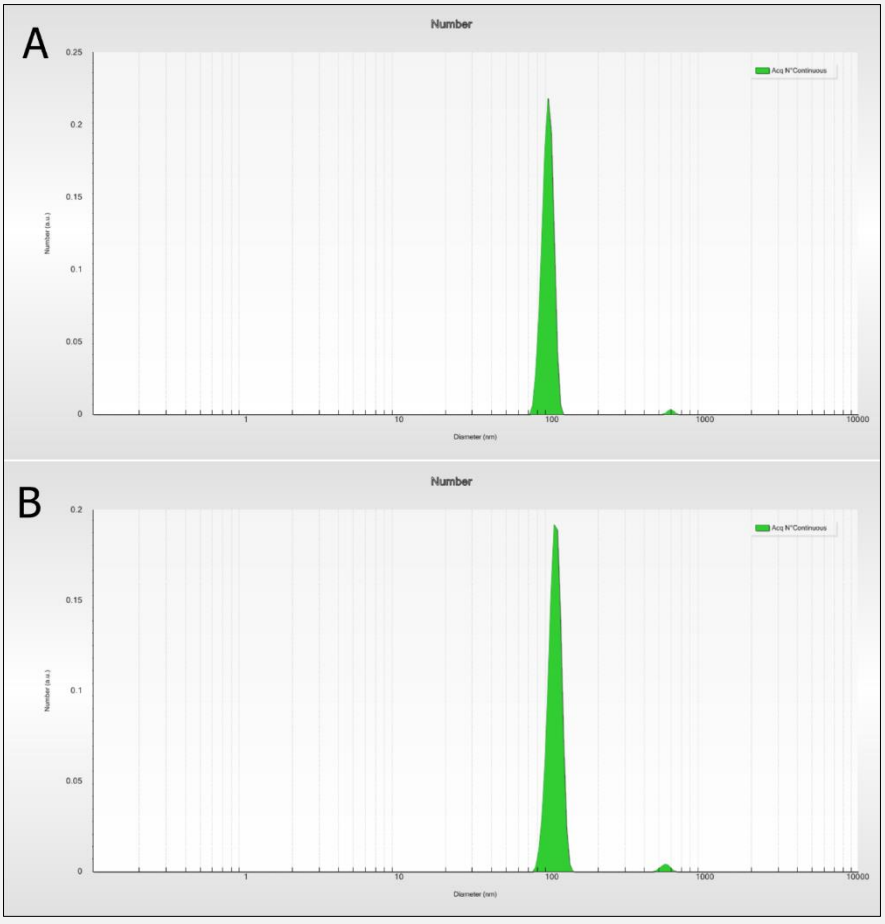

Figure 1. Particle size analyzing by DLS.

Average particle size obtained from DLS data for (A) LDH nanoparticles and (B) Royal jelly conjugated LDH (RJ-LDH). The sizes of LDH and RJ-LDH are estimated to be 102.81 and 118.11 nm, respectively.

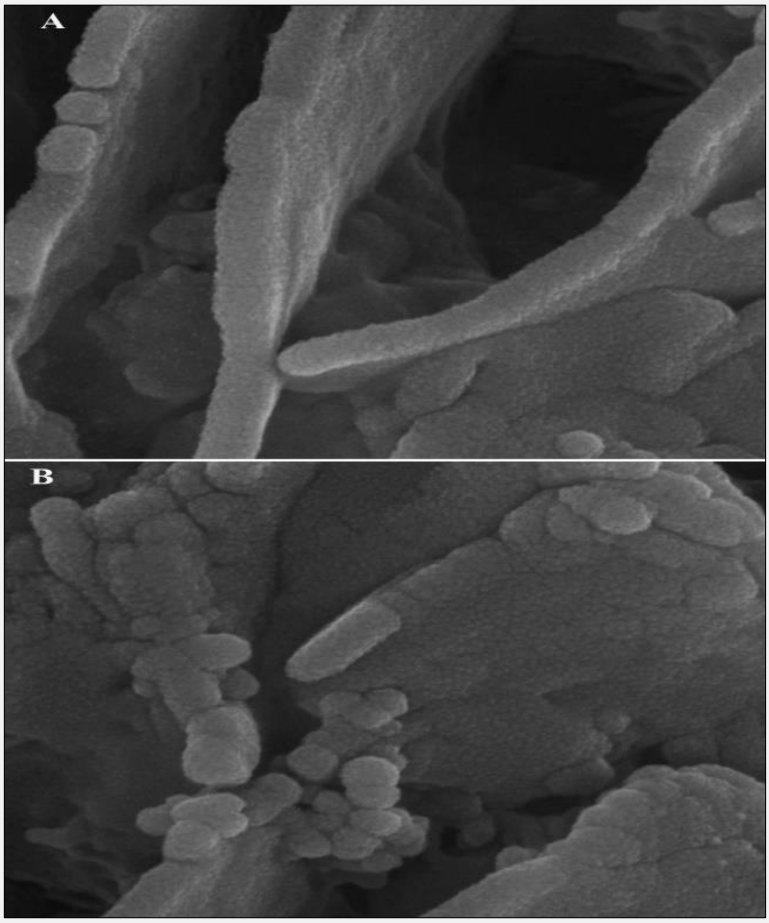

Figure 2. Scanning electron microscopy (SEM) image of LDH nanoparticle.

SEM images of LDH nanoparticles synthesized using co-precipitation method (A) LDH and (B) after royal jelly loading onto the LDH layers.

LDH on the polarization of Th1 and Treg responses, real-time PCR was performed.

Figure 6 shows, RJ-LDH treated mice displayed a significant increase in the expression of T-bet in a dose of 50 mg/kg of RJ-LDH ($p < 0.05$, Figure 6A), but the expression of T-bet was not significant in RJ-LDH-100-treated group. The results also showed that RJ-LDH could decrease the expression of Treg transcription factor (FoxP3) in the spleen of mice in doses of 50 and 100 mg/kg ($p < 0.01$ for RJ-LDH-50 and $p < 0.05$ for RJ-LDH-100, Figure 6B) compared to control mice.

Furthermore, we evaluated the mRNA expression of signature cytokines for Th1 and Treg subsets. The results revealed an increased expression of IFN-γ in RJ-LDH-50 groups than in the control group ($p < 0.05$), but the expression of IFN-γ was not significant in mice treated with RJ-LDH-100 (Figure 6C).

In contrast, the expression of TGF-β was reduced in both RJ-LDH-50 and RJ-LDH-100 treated mice ($p < 0.01$ and $p < 0.05$, respectively, Figure 6D) compared to control mice.

As a result, royal jelly in nano form has an anti-cancer effect in the treatment of breast cancer through the deviation of immune responses to Th1 responses.

DISCUSSION

Breast cancer is the *most frequent* type of *cancer* diagnosed in women in the United States.

It is well known that immune cells, especially T lymphocytes and their cytokines, as well as, the polarization of the cells towards either Th1 or Treg responses, can significantly influence the disease [16,17].

Animal models are the best way to evaluate immune responses during the disease and the development of new

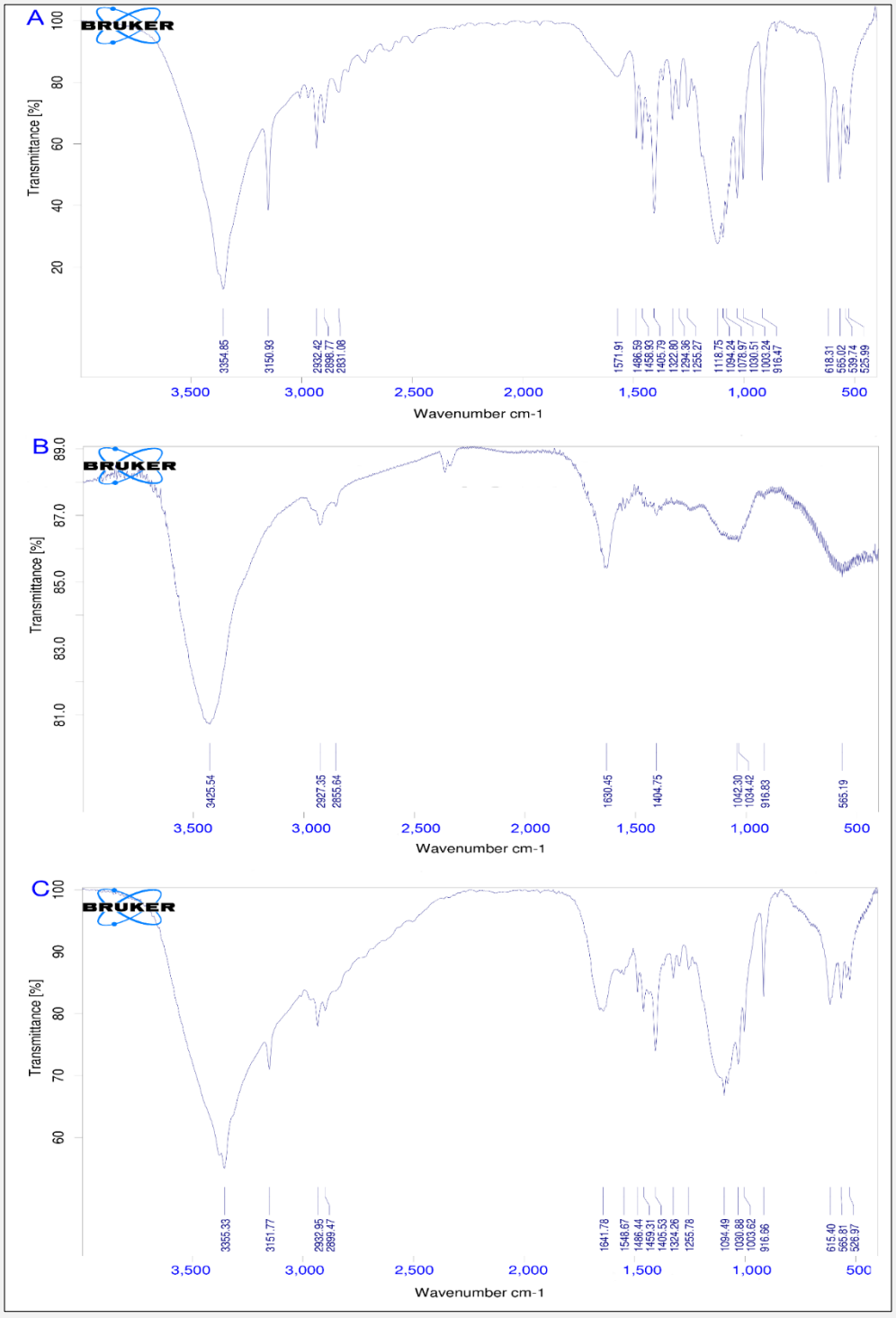

Figure 3. Fourier-transform infrared (FTIR) spectra for LDH nanoparticles.

FTIR graphics for (A) Free-LDH nanoparticle, (B) Free-royal jelly, and (C) royal jelly loaded LDH nanoparticles.

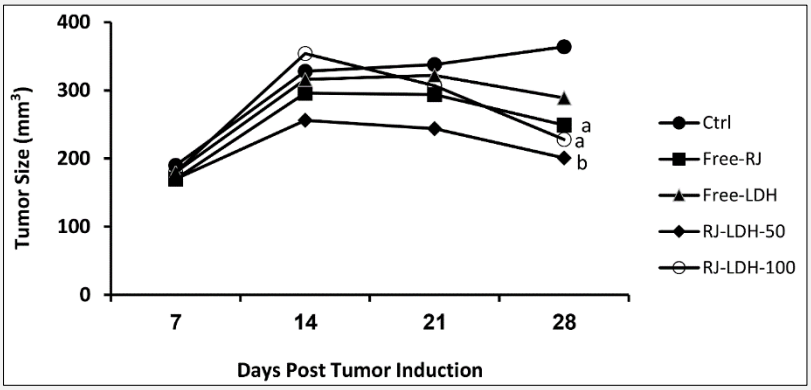

Figure 4. Free-RJ and RJ-LDH products decreased tumor size in BALB/c mice.

Mice were induced with 4T1 cell line and then tumor development was monitored. Treatment with Free-RJ or RJ-LDH products started from Day 7 post-tumor induction onward for 20 days. Tumor volume was measured at days 7, 14, 21, and 28. Data were shown as mean ± SEM.
[a] - $p < 0.05$ and [b] - $p < 0.01$ compared with control group.

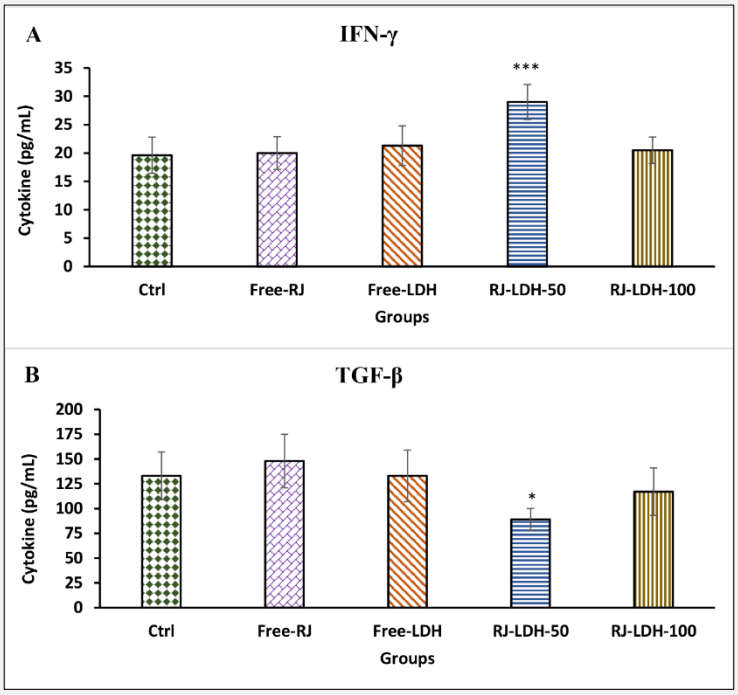

Figure 5. Effects of Free-RJ and RJ-LDH products on key cytokines of Th1 and Treg.

Serum from immunized mice treated with vehicle, Free-RJ, and RJ-LDH products in different groups were isolated at time of sacrifice (Day 28) and analyzed by ELISA technique.
The figure shows that treatment with RJ-LDH led to enhancement of IFN-γ (A) and suppression of TGF-β (B) cytokines. The values are means of triplicates at each point and the error bars represent SEM. Statistical analysis was performed by one-way ANOVA followed by post hoc Tukey's test.
*** - $p < 0.05$ and *** - $p < 0.001$ versus control group.**

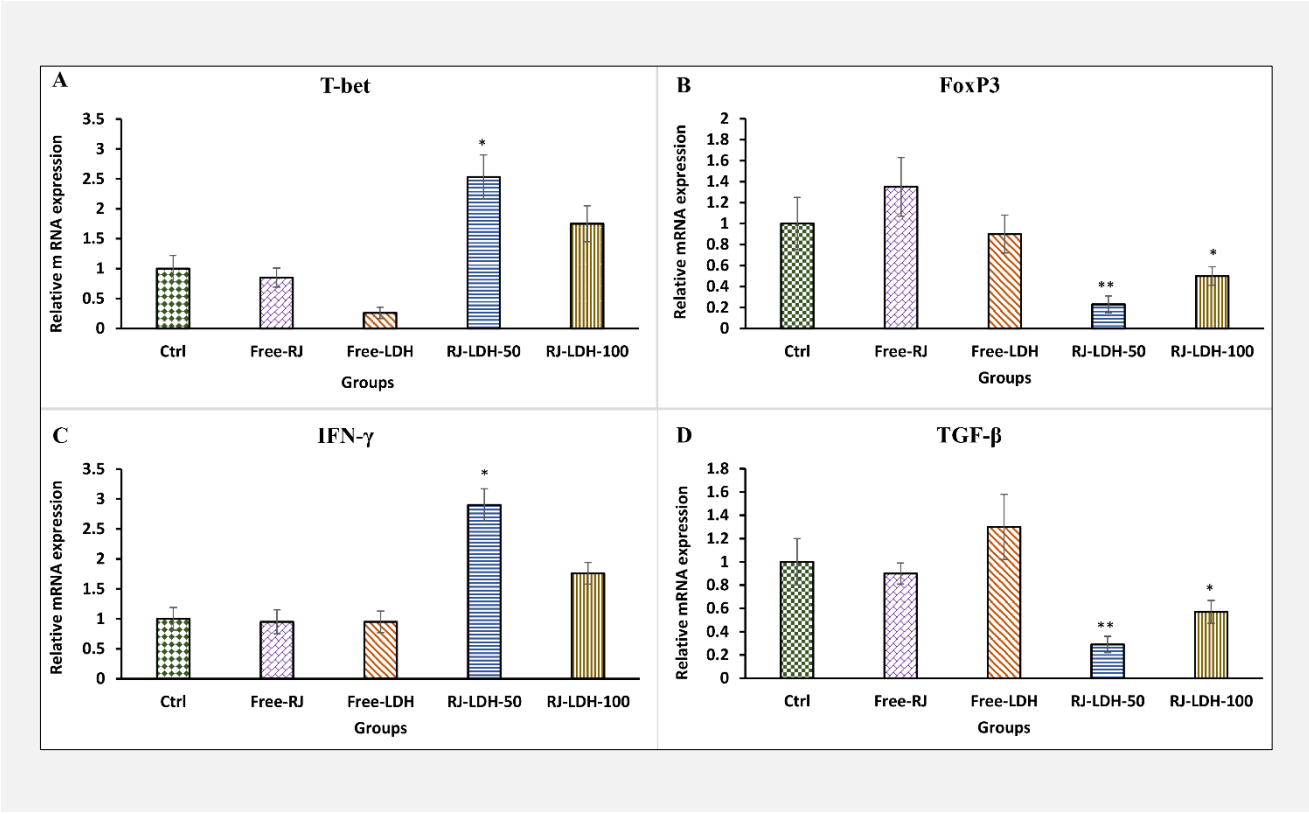

Figure 6. Effects of Free-RJ and RJ-LDH products on the expression of Th1 and Treg transcription factors and related cytokines.

Splenocytes were isolated from mice on day 28 post immunization. Total RNA was extracted and reverse transcribed into cDNA. Assay was run in triplicate and relative expression of Th1 and Treg transcription factors (T-bet and FoxP3) and related cytokines (IFN-γ and TGF-β) was determined by real time quantitative PCR compared to mouse beta-actin gene.
Results are expressed as mean ± SEM. * - $p < 0.05$ and ** - $p < 0.01$ versus control group.

drugs for treating breast cancer in humans. The model is also used in assessing the anti-tumor and anti-oxidant properties of drugs for breast cancer treatment [18].

In this research, a drug-LDH nanoparticle conjugate (RJ-LDH) was synthesized to investigate its effect in an animal model of breast cancer. The design of this model was due to the fact that LDH nanoparticles have proven properties such as suitable size, greater solubility, low cytotoxicity, better permeability of cellular and tissue barriers, as well as, increased drug delivery [19].

Today, there has been growing interest in developing new drugs, from natural products to biomedical applications. Therefore, in this study, the effectiveness of royal jelly in free form and conjugated with LDH nanoparticles in the treatment of breast cancer was investigated. To the best of our knowledge, this is the first report in which royal jelly, in combination with LDH nanoparticles, is used for the treatment of breast cancer. A similar study by Choi et al. used LDH nanostructures to deliver methotrexate in breast cancer treatment. This study showed that these nanostructures have a good ability in loading and delivery of drugs [20]. In addition, Senapati et al. also used these nanoparticles to transport raloxifene hydrochloride in an *in vivo* model of breast cancer [21].

Our study showed that in mice treated with free or nanostructures containing royal jelly, the drug led to a significant decrease in tumor volume, and this decrease was more in the groups treated with RJ-LDH. This reduction in tumor volume proved that the drugs loaded with LDH nanoparticles are more effective in reducing the severity of the disease compared to the free form of the drugs. The current results would be in line with a study by Zhang et al. that reported royal jelly led to inhibition of growth and reduction of volume and weight of tumor in breast cancer-induced mice [13].

In addition, findings of an *in vitro* study on renal cell carcinoma and human lung cancer cells also established that royal jelly or its active ingredient, 10-HDA, leads to a decrease in tumor volume and weight, as well as inhibition of tumor cell metastasis [22,23].

Further studies have also indicated the effect of royal jelly and 10-HDA on triple-negative breast cancer cells. The result of this research showed that royal jelly and

10-HDA led to the inhibition of metastasis through inhibition of the angiogenetic factor VEGF [24].

To determine the mechanisms involved in the control and improvement of disease by therapeutic compounds, we investigated the direct effect of drugs on T cell responses. T lymphocytes play an essential role in tumor inhibition and eradication, and the polarization of these cells towards Th1, or Treg responses can significantly affect the outcome of the disease. Various factors are potent drivers of T cell differentiation, notably the most important of which are cytokines (in the T cell microenvironment) and transcription factors [25,26]. IFN-γ and TGF-β are the key cytokines of Th1 and Treg cells. In addition, T-bet and FoxP3 are the key transcription factors that are essential for the differentiation of naive T cells into Th1 and Treg, respectively [25,27].

The results of this study show that royal jelly in nanoparticle form increases the serum level of IFN-γ, and this increase was higher in the RJ-LDH-50 group, which indicates a better effect of royal jelly in nano form. So far, there is paucity of information regarding the impact of royal jelly on the amount of IFN-γ in breast cancer. A study by Zhang et al. on an animal model of breast cancer showed that the treatment of mice with royal jelly led to an increase in the serum level of IFN-α, IL-2 and also antioxidant compounds [13]. However, in an *in vivo* study on the animal model of pulmonary fibrosis, Zargar et al. found out that royal jelly led to an increase in the serum level of IFN-γ in the animals [28].

Moreover, a study on allergic BALB/c mice proved that royal jelly administration led to an increase in IFN-γ and a decrease in IL-4 production from TCD4+ cells on day 14 after immunization. In addition, the results of this study showed that in mice treated with royal jelly, TH cells shifted from TH2 to TH1 cells in the groups treated with royal jelly [29].

In examining the serum level of TGF-β cytokine, this research showed that the treatment of mice with royal jelly and its nanoparticle form led to a decrease in the serum level of this cytokine, and this decrease occurred in the RJ-LDH-50 and RJ-LDH-100 groups, although it was not statistically significant in the case of RJ-LDH-100 group. This effect would be in keeping with Miyata's study on patients with renal cell carcinoma (RCC), which showed that oral administration of royal jelly led to a decrease in TGF-β level and tumor volume in patients with RCC [22].

Analysis of bronchoalveolar lavage fluid (BALF) from mice with pulmonary fibrosis showed that royal jelly led to a decrease in the level of TGF-β in the BALF of pulmonary fibrosis-induced rats [28].

An *in vivo* study on a rodent model of kidney injury also revealed that treatment of rats with honey and royal jelly resulted in a decrease in the production of TGF-β. This study also proved that royal jelly leads to a reduction in the expression of α-smooth muscle actin (α-SMA) and pathological complications of the disease [30].

According to these results, to better understand the mechanisms involved in the serum levels of cytokines, we investigated the effect of drugs on the polarization of T lymphocytes. Since Th1 and Treg lymphocytes play an important role against cancers, in this research, the effect of royal jelly in free and nano form on the key transcription factors T-bet and FoxP3 (for Th1 and Treg lymphocytes, respectively) in the spleen was investigated.

T-bet is the main transcription factor in the differentiating of TH cells into TH1 cells. A defect in this protein will reduce the production of TH1 cells, while the presence of this protein leads to the inhibition of the production of TH2 cells (cells involved in the development of cancer) and thus plays a vital role in anti-tumor immunity [27].

The results of this study showed that royal jelly in nano form increased the expression of T-bet in the spleen of RJ-LDH treated groups and this increase was associated with the increase of IFN-γ expression, as well as, the serum level of this cytokine. In several studies conducted on breast cancer, it has been determined that the presence of T-bet$^+$ cells in the microenvironment of a tumor is associated with a better prognosis, while a low number of T-bet$^+$ lymphocytes is related to the size of the tumor and causes breast cancer progression [6].

So far, no study has investigated the effect of royal jelly on TH cells in the breast cancer model, but research by Erem et al. on the lymphocytes of patients with Graves' disease showed that treatment of these cells with royal jelly led to a shift of TH1/TH2 responses towards TH1 cells [31]. They also showed that the level of IFN-γ was also increased by lymphocytes treated with royal jelly. Evidence from an animal model of allergy also revealed that treatment with royal jelly led to a shift in immune responses from TH2 cells in the control groups to TH1 cells in the groups treated with royal jelly in animals [29].

In addition to helper T cells, regulatory T cells (Treg) play an important role in the tumor microenvironment [32]. Treg cells are involved in immune homeostasis and regulation of autoimmune diseases, while promoting tumor development [33].

FoxP3 is the main transcription factor in the control of the growth and activity of Treg cells [9]. These cells have inhibitory effects on effector cells through the secretion of TGF-β and IL-10 or via cell-cell contact [7, 8]. It has also been reported that the increase in the level of TGF-β in the advanced stages of cancer is related to the increase in tumor size [34].

The results of this research showed that RJ-LDH led to a decrease in the expression of FoxP3 in the treatment groups, which was also associated with a decrease in the expression of TGF-β both at the mRNA and protein levels. Previous investigations have revealed that patients with strong FoxP3 expression have a worse prognosis than those with weak expression [10].

So far, there are no reports of the effect of royal jelly on the expression of transcription factors of T lymphocytes

such as FoxP3, but the study by Lin et al. on lung cancer cell line (A549 cells) showed that treatment of A549 cells with the main compound of royal jelly (10-HDA) led to a decrease in TGF-β expression in these cells. They also showed that this component of royal jelly leads to a reduction in the migration of cancer cells [23].

Although, in our study, royal jelly in free form did not have an effect on the level of T cells transcription factors as well as their key cytokines, it seems that the reason can be attributed to the short duration of the treatment period (20 days). In confirmation of this hypothesis, Zargar et al. reported that long-term treatment with royal jelly leads to its protective effects [28].

Another study also showed that treating mice with royal jelly for 6 weeks protected the mice from doxorubicin-induced damage to the animal's reproductive system [35]. Zhang et al. also in an animal model of breast cancer showed prophylactic-therapeutic treatment with royal jelly for 6 weeks limited the growth of breast cancer in mice [13].

Accordingly, by comparing the results of this study and other studies, it can be concluded that the reason for the lack of effect of free royal jelly is due to the short treatment period.

On the other hand, studies have shown that LDH nanostructures lead to increased stability, bioavailability, and uptake of drug by cells [36]; hence, it can be concluded that uptake of royal jelly by cells in nano form is better than in free form, and this leads to a reduction in the required dose of the drug as well as a reduction in the treatment period.

Taken together, it appears that royal jelly in nanoparticle form exerts anti-tumor activity through its effect on T cells by inhibiting their expansion into Treg cells, as well as the promotion of pro-inflammatory cytokine production.

CONCLUSION

In conclusion, the current research showed that administration of RJ-LDH inhibited the increase in the tumor volume in breast cancer-induced mice. The inhibition of tumor volume was associated with an up-regulation of T-bet expression versus a down-regulation of FoxP3 expression, demonstrating the anti-tumor activity of the drug. These effects were accompanied by an increased IFN-γ level, whereas the level of FoxP3 diminished in RJ-LDH treated mice.

In addition, the results of this study showed that the loading of royal jelly by LDH nanoparticles increases the pharmacological properties and therapeutic efficiency of royal jelly *in vivo*.

Moreover, we showed that RJ-LDH-50 was superior to RJ-LDH-100 and Free-RJ in ameliorating disease severity, which indicated that loading royal jelly with LDH increases the pharmacological properties and therapeutic efficiency of royal jelly and can be considered as new therapeutic targets for treatment of breast cancer.

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The authors declare no competing interests.

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