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Research Article

The synergistic effects of chitosan and *Ganoderma Lucidum* nanoparticles on p53 and HER3 gene expression

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ABSTRACT

Ganoderma lucidum has effective anti-cancer compounds. Chitosan has the capability of encapsulating and stabilizing Ganoderma compounds, in addition to its antioxidant properties. Separation of *Ganoderma lucidum* polysaccharides was carried out by hot-water extraction and treatment with 96% ethanol and protein dehydration. Using the ion exchange copolymerization method, the Ganoderma's polysaccharides were stabilized on the chitosan nanoparticle. The morphology and type of nanoparticle were determined by SEM and FTIR methods. After the treatment of MCF-7 cells with different concentrations of nanoparticles, as well as polysaccharide extracted from the fungus, expression of the two HER3 and p53 genes was determined using the method Real-time PCR. The phenol sulfuric acid test showed the presence of 23.77 µg/ml polysaccharide in the *Ganoderma lucidum*. Also, Real-time PCR showed that chitosan-polysaccharide nanoparticle increased the gene expression of p53 by 7.3-fold while the expression of the HER3 gene decreased by 0.36-fold change. Also, the polysaccharide grew the expression of the p53 gene by 2.5-fold change and the HER3 gene expression fell by 0.48-fold change. The combination of chitosan and Ganoderma polysaccharides nanoparticle affects the expression of p53 and HER3 genes, causing resistance to tumor cell.

1. Introduction

Cancer is one of the deadliest deaths in humans throughout the world and the most common malignant disease among women and one of the leading causes of death in breast cancer (Maryam et al., 2017). According to statistical data for 2018, 18.1 million of patients have cancer, with an estimated 9 million deaths from this malignant disease (Siegel et al., 2019). Despite significant changes in the modern age in the third century, there is still no definitive cure for most cancers. This can be due to the current pathogenicity of cancer and the lack of effective treatment methods. On the other hand, there are different treatment modalities for these cancers including breast cancer that exist, such as treatment, hormone therapy, immunotherapy,

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and surgery for untreated side effects. Therefore, the quest for new therapies continues to be perused throughout the world. Nowadays, natural remedies are becoming more and more popular due to having less side effects than chemical medicines and the therapeutic effects of these natural products have been proven for different types of cancers (Stanculeanu et al., 2016). Secondary metabolites of Ganoderma are a favorite of new therapies for the treatment of cancer which its polysaccharides have antioxidant and antibacterial properties against viruses that cause virulence (Qu et al., 2017). Polysaccharide extracts isolated from *Ganoderma Lucidum* can inhibit tumor growth by activating the immune response and stimulating the production of cytokines and by activating the interlukin-2, they can activate the immune system (N. Li et al., 2010). Targeted drug delivery can improve the efficacy of the drug at the tumor site. A system that has the capability of delivering the drug to the target, maximizing drug loading, adaptable and easy, is a desirable drug delivery system. The most common of these is chitosan, one of the most promising nanomaterials in the pharmaceutical industry (Elgadir et al., 2015).

p53 or tumor cell antigen p53 or transformation-related protein 53 (TRP53) is a regulatory protein that is frequently mutated in human cancers. The p53 protein is very important in vertebrates and prevents the formation of cancer. p53 has been described in maintaining stability by preventing genome mutations, and TP53 is classified as a tumor suppressor gene. HER3 protein, which belongs to the ErbB/HER receptor tyrosine kinase (RTK) family, is expressed in various types of tumors. HER3 may play a role in cancer treatment. HER3 activation is associated with resistance to drugs that target other receptors. HER3 is associated with resistance to some chemotherapy drugs. Therefore, in this research, the effect of Ganoderma and chitosan polysaccharide on the expression of HER3 and p53 genes has been investigated.

The nano-properties of chitosan include high biocompatibility, biodegradability, and non-toxicity (Younes & Rinaudo, 2015). This study aimed to isolate *Ganoderma lucidum* polysaccharides and load polysaccharides on nano - complete chitosan which was first synthesized and reported. This study, investigates the anti-cancer effect of a new nanoparticle on MCF-7 (breast cancer) cell line at different concentrations and time by MTT assay and also the mechanism of P53 and HER3 gene activity in breast cancer was evaluated by Real-Time PCR.

2. Materials and Methods

2.1. Specimen of fungi

The specimen of *Ganoderma lucidium* was first purchased from the Genetic and Biological Resource Center of Iran under IBRC-M ID 30306 and then grinded under a manual laminar hood after it was transferred to the laboratory.

2.2. Isolation and extraction of fungal polysaccharides

10 g of *Ganoderma lucidum* powder was treated with absolute ethanol at boiling temperature for 9 hours. This procedure was performed to remove dyes, lipids, part of the saponins, and monosaccharides. Finally, the mushroom powder was separated from ethanol and left overnight at 26°C in the incubator to dry. Extraction of polysaccharides from 10 gr of *Ganoderma lucidum* powder was carried out with 1 liter of water at 70 ° C for 3 h which was repeated two times and finally, the aqueous extract was concentrated to one-third of the initial volume by Ben Marie. The proteins were removed from the aqueous extract by the Savage method (Ya-Qin Wang et al., 2017). The polysaccharides were extracted after the addition of absolute ethanol to the aqueous extract of the protein, and maintained at 20°C refrigerated for one day, followed by centrifugation of the resulting suspension. Finally, the precipitates of the water-soluble polysaccharides were washed on filter paper with ethanol, absolute, acetone, and ether in distilled water and then dried by freeze-drying. for measuring the sugar content, the Phenol-sulfuric acid method is one of the easiest and most reliable methods among the available colorimetric methods. In this method, concentrated sulfuric acid first breaks down each polysaccharide, oligosaccharide and disaccharide into monosaccharides. Pentoses are then dehydrated to furfural and hexoses to hydroxymethylfurfural. These compounds reacted with phenol and produced a golden yellow color and their absorbance was measured

at 490 nm. The color of this reaction is stable for several hours and the accuracy of the method is in the range of ±2% under appropriate conditions (Nielsen, 2010).

2.3. Preparation of chitosan-polysaccharides nanoparticles

Polysaccharide hydrolysis was performed in acidic solution pH ~3-4 under sonication using a probe-type ultrasonic homogenizer 400 W, 60% power, and 60% frequency for about 2 hours at room temperature. Then, the supernatant was separated by centrifugation at 2500 rpm for 10 min and concentrated to 30% of the original volume with a vacuum rotary evaporator (Steroglass, Strike202, Italy) at 60°C for isolation of the insoluble materials. The solution which contains soluble polysaccharide was stored in a refrigerator for making the chitosan polysaccharide Nano gel. EDC and NHS were used to activate the carboxylic acid group of fungi's polysaccharides in a 50 mM phosphate-buffered solution, pH 5.5. Then, the chitosan solution was added drop by drop to the polysaccharide solution under vigorous stirring at room temperature. The reaction mixture was kept in this condition for an extra 2 hours. Then, the copolymer of the chitosan-polysaccharide solution was sonicated for 20 minutes at room temperature using a probe-type ultrasonic homogenizer, 400 W, 60% power, and 60% frequency. To prevent microbial contamination, all work steps were performed under a microbial hood. To analyze the structure, size, and shape of the synthesized chitosan-polysaccharide Nano gel a sample of Nano gel referred to the analysis laboratory for FTIR and SEM.

2.4. Morphological characterization of nanoparticles

FTIR method was used to investigate the chemical bonds and functional groups of chitosan-polysaccharide nanoparticles. The morphological examination of nanoparticles was carried out by scanning electron microscope (SEM).

2.5. Human cell culture

The MCF-7 (Michigan Cancer Foundation-7) cells (NCBI C135) were obtained from the National Cell Bank of Iran in Pasteur Institute.

The MCF-7 cells for experiments were plated in 25-cm2 tissue culture flasks in RPMI 1640 medium supplemented with 10% FBS then cell line was disaggregated with using 2-min incubation at 37C with a %0.05 solution of trypsin in phosphate-buffered saline (PBS). After counting by the trypan blue dye exclusion method (Bikhof Torbati et al., 2017), approximately 500 cells were transferred to flasks for doing the MTT method.

2.6. MTT assay

The chitosan-polysaccharides nanoparticles and free polysaccharides were compared on the mcf-7 cells by using the MTT method and also Tamoxifen were determined as a controlled drug. The mcf-7 cells were cultured in 96 well plate wells and incubated for 24 hours with different concentrations of the chitosan-polysaccharide nanoparticles and polysaccharides and Tamoxifen, then stained with MTT 0.5 mg/ml and after 4 hours incubation at 37°C the supernatant was taken out and 100lL volume of DMSO was added to them, after shaking the plates on an incubator for 15min, formazan and the optical density dissolve then read by ELISA-reader (TECAN-Sunrise, Mannedorf, Switzerland) at 570 nm (Mosmann, 1983). The percentage of cytotoxicity and cell viability was calculated by the following formula:

Cytotoxicity % = 1- mean absorbance of toxicant × 100

$$\text{Viability \%} = 100 - \text{Cytotoxicity \%}$$

mean absorbance of negative control

After the calculation of cell viability percentage, the concentration of chitosan-polysaccharides nanoparticle giving 50% inhibition on the MCF-7 cell line (IC50) was assigned in different incubation times.

2.7. Statistical analysis

Data are explicated as means ± SD. The difference between the means ± SD of the antioxidant between the chitosan-polysaccharides nanoparticle and polysaccharide and the control were assessed by Statistical Method (Tukey's multiple comparisons test). The difference in the lowest survival rate

obtained from the control group was considered significant at $P < 0.0001$.

2.8. RNA Extraction and Real-time PCR Analysis

The RNA from the MCF-7 cell treated with chitosan-polysaccharides nanoparticle and polysaccharides were extracted with Trizol (Sangon Biotech, Shanghai, China) following the manufacturer's instructions. 2 µg of RNA was reverse transcribed by the RiboEX (generally south Korea) RNA extraction kit according to the supplier's protocol. The reactions are included 2 µL cDNA, 10 µL Syber green, (10 pmol) 1 µL of Primer Mix (R+F), and nuclease-free water to a final volume of 7 µL which incubated at 25°C for 10 min, at 42°C for 60 min, then at 70°C for 10 min and the products were kept in the refrigerator (−20°C) before use.

Reactions were carried out under the following conditions: The first stage (Hold) is at 95 ° C for 15 minutes, which results in the enzyme being activated.

The second step (Cycle) of the gene we are synthesizing is as follows:

At 95 ° C for 15 seconds, the double strand of DNA was separated, then the primer binding step was on its specific region on the template DNA, which was placed at 60 ° C for 30 seconds, Finally, it was placed at 72 ° C for 20 seconds to expand the desired area. After completing the steps, the data were checked for the melting curve and the diagrams were created for dimerization. Finally, the CT data were obtained and the resulting diagrams were plotted. Real-TimePCR products were verified by agarose gel electrophoresis and sequencing. The *Ganoderma lucidum* constitutive gene ribosomal protein L4 (RPL4) was used as the internal reference gene. The relative expression levels of laccase gene were indicated as a percentage to the expression of an internal control gene. RPL4 and the following formula was used to calculate the relative expression level of each gene.

3. Results

3.1. Evaluation of sugar content in Ganoderma lucidium

The amount of sugar in the extract was determined by the phenol sulfuric acid method. The results showed that there were 23.77 µg of sugar per 1 ml of the sample.

3.2. chitosan-polysaccharides nanoparticle characterization

The FTIR spectrum illustrates the C–O of stretching bands at 1090 nm, the 3443 nm is related to the N-H bonds, and the stretching bands of C–H bonds are appearing at 2900nm.

In the chitosan-polysaccharide spectrum the peak of the NH2 functional group is plotted, which is less involved with OH groups than NH2 of chitosan. It can be assumed that in the final structure, the chitosan surrounds the polysaccharides, the OH groups of the chitosan being hydrogen-bonded to the OH polysaccharide groups, and on the other hand, the NH2 groups are freely oriented outward. In figure1 is showed the FTIR pattern of chitosan and polysaccharides and the chitosan-polysaccharide spectrum the peak.

The FESEM image demonstrates plates of the synthesized chitosan-polysaccharides nanoparticle. Because of the overlap of polysaccharide and chitosan, SEM imaging showed cubic shapes with an almost homogeneous structure and also the grains appear as rectangular fibers. The results are shown in figure 2.

3.3. MTT analysis

MCF-7 cells were treated with different concentrations of chitosan polysaccharide nanoparticle, polysaccharides and tamoxifen by MTT method in 3 times of 24, 48, 72 hours of incubation. Figure5 demonstrate a comparison of effects of different concentrations (10,100,500,1000) (µg/ml) of chitosan polysaccharide nanoparticle on MCF-7 cell viability at three times of 24, 48, and 72 hours compared to control. The figures show the percentage of cell viability at different concentrations. The results show that as the concentration of nanoparticles decreased, the percentage of cell viability decreased at the ($p \leq 0.0001$). Besides, increasing the incubation time of

nanoparticle from 24 to 72 hours significantly reduces the cell viability of MCF-7 at concentrations of 10 µg / ml and above. Increasing the duration of cell treatment from 24 hours to 48 hours increases the toxicity of nanoparticle on the MCF-7 cell line. The results are shown in figure 3.

Figure4 shows that with increasing drug concentration, cell viability decreased at the ($p \leq 0.0001$). Also, prolonging the incubation time of polysaccharides from 24 to 72 hours significantly reduces the cell viability of MCF-7 at concentrations of 10 µg / ml and above. Increasing the duration of cell treatment from 24 hours to 48 hours boosts the toxicity of polysaccharides on the MCF-7 cell line.

3.4. IC50s of polysaccharide-chitosan nanoparticles and polysaccharide and tamoxifen

According to the obtained IC50, chitosan-polysaccharide nanoparticles perform better than polysaccharides, whereas tamoxifen (as control) with lower concentrations than chitosan-polysaccharides and polysaccharides causes more cell death and the ratio of it works better than chitosan-polysaccharide nanoparticles. The results are shown in figure 5.

3.5. Results of Apoptotic Induction in MCF-7 Cells (Real-Time PCR)

To better compare the gene expressions of p53 and HER3, high and low concentrations of nanoparticles and polysaccharides extracted from *Ganoderma lucidum* and tamoxifen were used. the gene expressions of p53 and Her3 have been identified in figure8 and figure9. Tamoxifen as a controlled drug has shown that the gene expression of p53 is increased and according to the results, the synthesized nanoparticles have also shown increased expression of p53. Chitosan-polysaccharide nanoparticles were more effective than polysaccharides and grew the expression of p53. Expression of p53 in the chitosan-polysaccharide treatment group increased by 7.3 at high concentration and 2.9 at low drug concentration also increased 5.2 at high concentration and 2.3 in low concentration of polysaccharide. according to column control, drugs show increased gene expression by 3.4 in high concentration and 1.6 at low concentration.

As can be seen in the HER3, there was a decrease in expression in the control group (tamoxifen) and the synthesized drugs, in which chitosan-polysaccharide nanoparticles were more effective than extracted polysaccharides. And the expression level of HER3 in chitosan-polysaccharide nanoparticles showed more decline than polysaccharide treatment group in different concentrations of them. According to the data obtained, extracted polysaccharide and chitosan-polysaccharide nanoparticles can induce apoptosis of cancer cells by increasing p53 expression and decreasing HER3 gene expression. The expression of HER3 treated with polysaccharides decreased by 0.48 at high concentration while at low concentration decreased by 0.70. Expression of Her3 in chitosan-polysaccharide treatment decreased by 0.36 at high concentration and 0.42 at low concentration. The expression of HER3 treated with tamoxifen showed a decrease of 0.22 at high concentration while 0.19 at a low concentration. The results are shown in figure 6.

4. Discussion

Breast cancer is the most common malignant disease among women and one of the leading causes of death in developed and developing countries (Berghoff et al., 2014). Despite the existence of various treatments, today surgery is considered the first treatment for breast cancer. Although common treatments may reduce the size of the tumor, they are transient and do not have a positive effect on patient survival and there is a possibility of recurrence of the disease. High side effects, low specificity, and the possibility of recurrence of the disease are the limitations of surgical treatment, so the need to replace more effective, more specific treatments with fewer side effects is very much felt. Today, some compounds have immunomodulatory properties, such as *Ganoderma lucidum* and chitosan polysaccharides. (Jayakumar et al., 2007 and Cao et al., 2018).

Ganoderma lucidum is one of the most important fungi in traditional Chinese and Iranian medicine so that it is used together with other fungi or alone for medicinal purposes.

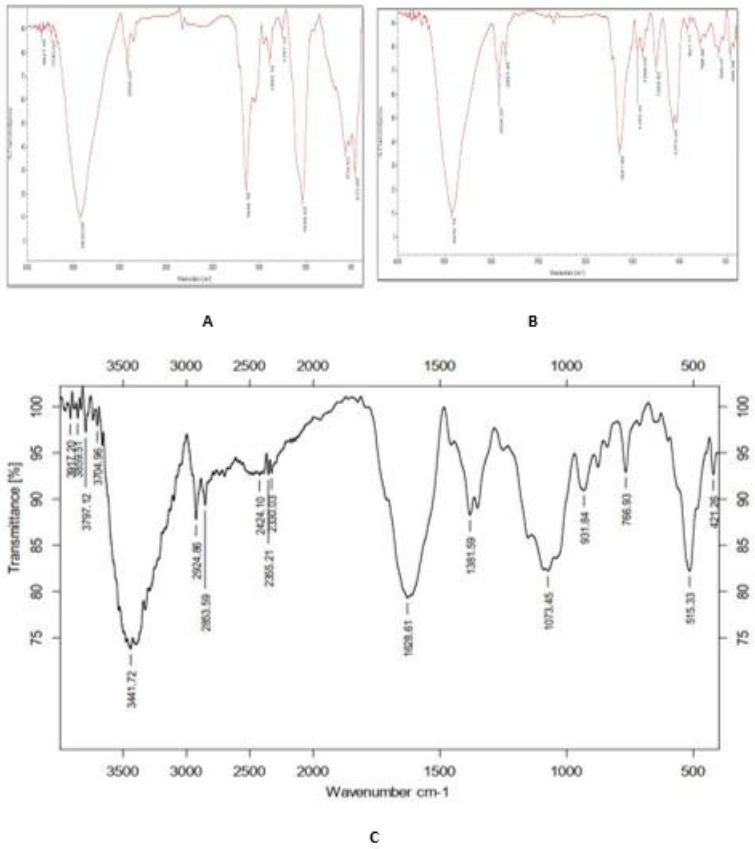

Figure1. In the shape is showed the FTIR pattern of chitosan(a), polysaccharides extract from Ganoderma lucidum (b) and the chitosan-polysaccharides nanoparticles spectrum the peak(c)

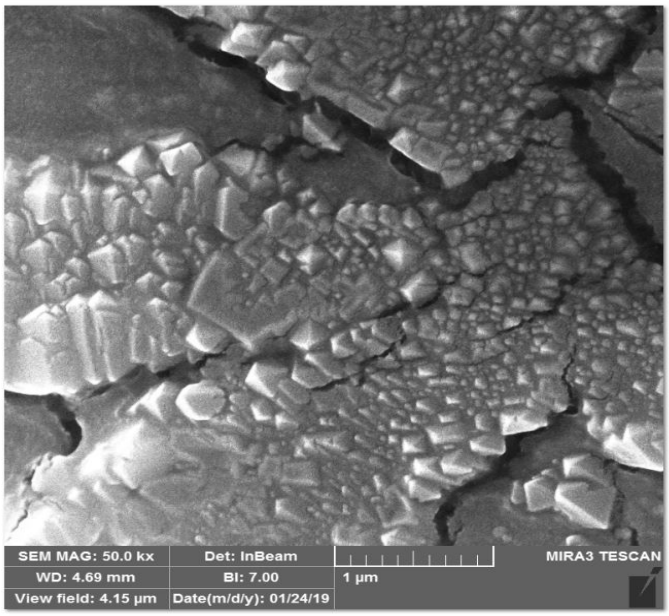

Figure2. FESEM chitosan-polysaccharides nanoparticles

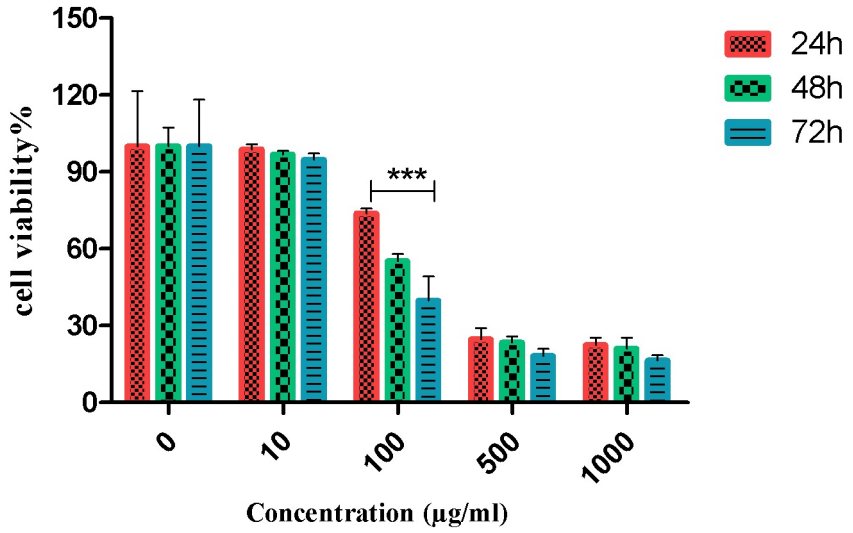

Figure3. demonstrates a comparison of effects of different concentrations (10,100,500,1000) (µg/ml) of chitosan polysaccharide nanoparticle on MCF-7 cell viability at three times of 24, 48, and 72 hours compared to control.

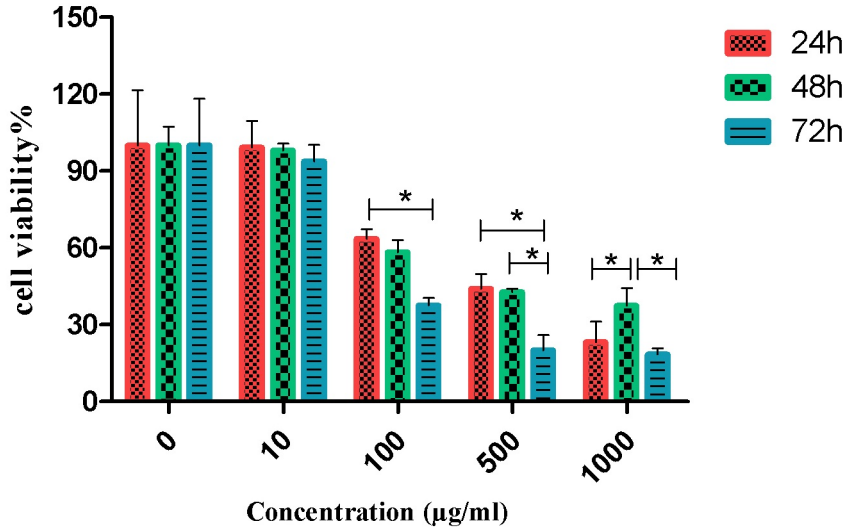

Figure4. demonstrates the Percentage of cell viability of MCF-7 cells under different polysaccharide concentrations compared to control and different concentrations (10,100,500,1000) (µg/ml) at three times 24,48 and 72 hours of impact.

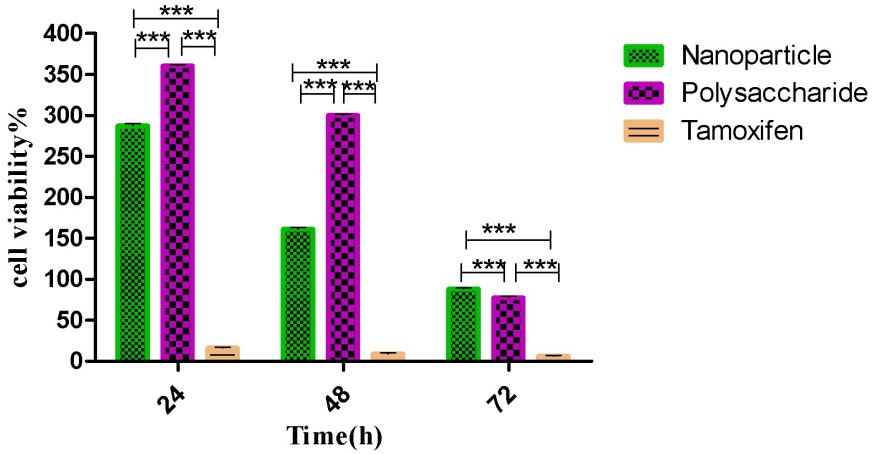

Figure 5. IC50s of polysaccharide-chitosan nanoparticles and polysaccharide extract and tamoxifen at 24, 48, 72 hours

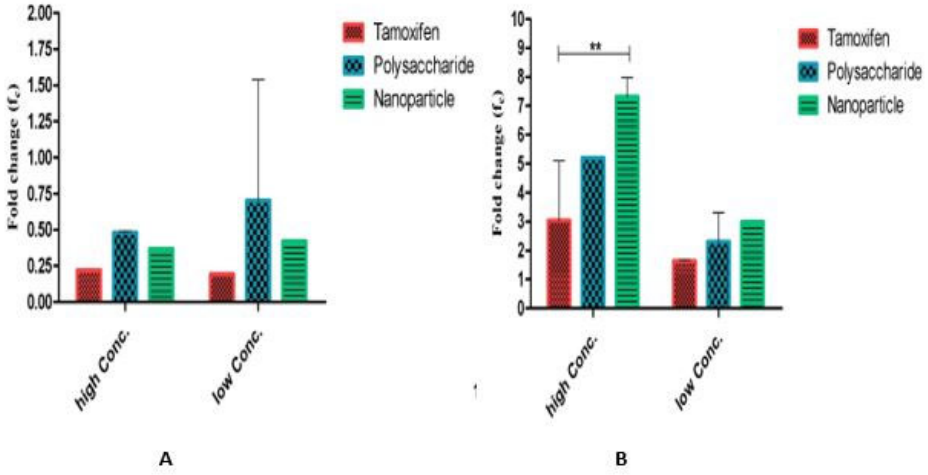

Figure 6. fold Change in p53(a) and Her3(b) gene expression in different treatment groups.

Ancient sources believe that this fungus is useful for treating shortness of breath, strengthening memory, increasing physical strength, increasing longevity, treating kidney and liver diseases, arthritis, asthma, stomach ulcers, diabetes, and anorexia. (Paterson, 2006).

Today different parts of *Ganoderma lucidum*, are used for therapeutic purposes such as headache and weakness of nerves, insomnia, dizziness, hepatitis, lowering cholesterol and blood pressure, cardiovascular problems, poisoning caused by the consumption of poisonous mushrooms and cancer. (Ghobadi et al., 2018)

Chitin and its derivatives such as carboxymethyl chitin and dihydroxy-propyl chitin have immunomodulatory effects and increase cell activity, secretion of cytokines and chemokines by affecting innate and acquired immunity (Younes & Rinaudo, 2015). Suspensions and chitosan particles can stimulate the immune system such as chemotaxis and activate macrophages. They also secrete stimulatory cytokines. This polymer enhances the antibody response and activates cytotoxic T cells as well as natural killer cells (X. Li et al., 2018). This polymer is a biocompatible and biodegradable compound. This nanoparticle is also able to increase the response of the immune system and direct and carry substances in the cell.

In the present study, chitosan was used as a drug carrier and according to the results, the polysaccharide extract was placed next to chitosan nanoparticles by ion-bonded copolymerization method. Not only chitosan nanoparticles have antioxidant properties, but also is a suitable substrate used for anti-cancer compounds such as *Ganoderma lucidum* polysaccharide extract.

Due to the increasing prevalence of cancer, this study aimed to investigate the effect of polysaccharides extracted from *Ganoderma lucidum* as well as synthesized chitosan-polysaccharide nanoparticles on MCF-7 cell line and expression of p53 and HER3 genes. The results of the MTT test showed that the percentage of cancer cells decreased with increasing concentrations of polysaccharide extract and chitosan-polysaccharide nanoparticles and as statistics showed in 100 concentrations of chitosan polysaccharide nanoparticles as well as polysaccharide extract, half Cells have been killed, in other words, as the concentration of polysaccharide extract and chitosan-polysaccharide nanoparticles increases, so does the percentage of cell viability, to the extent that at the concentration of 1000, the highest rate of cell death was observed. According to Inoue studies in 2017, the p53 gene is known as a regulatory gene in breast cancer, which is also a tumor suppressor (Inoue et al., 2017) and also in Berghoff study in 2014, stated that the HER3 gene is one of the increased oncogenes in breast cancer (Berghoff et al., 2014).

In the group treated with chitosan-polysaccharide nanoparticles, it increased by 7.3 and the expression of the HER3 gene treated by this group decreased by 0.36. However, the control drug tamoxifen showed the same expression for p53 and HER3 genes. Polysaccharide extract for the p53 gene also showed a growth in expression by 2.5 and HER3 gene expression decreased by 0.48 which compared to the control drug, tamoxifen increased p53 gene expression by 3.04. And reduced HER3 gene expression by 0.22. According to the obtained results, it can be concluded that chitosan polysaccharide nanoparticles were superior to polysaccharide extract and acted more effectively. Therefore, polysaccharide extracts and chitosan-polysaccharide nanoparticles may be associated with increased apoptosis of Mcf-7 cancer cells by increasing p53 gene expression and decreasing HER3 gene expression because P53 as a tumor inhibitor can induce apoptosis. They can therefore be a good choice for non-tumor cells.

In a similar study, Dastjerdi and colleagues in 2016 researched the active substance thymoquinone (TQ) and showed that thymoquinone (TQ) can induce apoptosis in MCF-7 cells by highly regulating P53 gene expression in cells. The results of this study were in line with the present study so that the synthesized nanoparticles of chitosan polysaccharide can also cause apoptosis in MCF-7 cells by increasing the expression of the p53 gene. (Dastjerdi et al., 2016)

In another 2017 study, Lijun Qu et al., Examined the effects of triterpene extracted from *Ganoderma lucidum* on cell viability, invasion, and apoptosis in human prostate cancer cells DU-145. The results showed that a high dose of triterpene (GLT) inhibits cell viability by regulating metal opaque matrix proteases. Also, GLT induces apoptosis of DU-145 cells. The results of the present study are also in line with the results of Lijun QU because high doses of polysaccharide extract and polysaccharide chitosan nanoparticles can be associated with apoptosis of MCF-7 cells. (Qu et al., 2017)

In 2018, Kalpana Mujoo and colleagues conducted a study on the regulation of the HER3 gene in breast cancer. The results of their study showed an increase in HER3 gene expression in breast cancer, and they also found that the HER3 mutation was activated by increasing cell production in MCF-7 cancer cells that lacked HER2 overexpression. In the present study, by reducing the expression of the HER3 gene by chitosan polysaccharide nanoparticles and polysaccharide extract can be a way to prevent overexpression of the HER3 gene in breast cancer. (Mujoo et al., 2014)

Finally, it can be concluded that Ganoderma, due to its natural, native, available, and cheap anti-cancer compounds, can be a cancer-preventing compound in humans. According to the results obtained in this research and other articles and taking into account that the use of Ganoderma as a food seasoning has been customary since ancient times and it is known as the king of medicines among the people, on the other hand, its anti-cancer, anti-microbial, anti-

aging effects It has biotics, etc., using any of its forms in the form of extract, powder can be considered as an important natural medicine-food supplement without side effects. According to the findings of the present study, chitosan is a suitable substrate for polysaccharides extracted from *Ganoderma lucidum*. As a result, chitosan polysaccharide nanoparticles are synthesized and polysaccharide extract induces programmed death in damaged cells by increasing the expression of the p53 gene and decreasing the expression of the HER3 gene and can prevent cancer cells from becoming tumorous. Production of anti-cancer agents plays an important role.

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Contribution

Study conception and design: Dr.M larypoor; Analysis, interpretation of data and laboratory activities: Y,akbarzadeh ; Cell culture steps : under the supervision of Dr.M bikhof Torbati

Conflict of interest

There is no conflict of interest in this research.

Refereces

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