RESEARCH ARTICLE

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Potential Anti-Cancer Effect of Helenalin as a Natural Bioactive Compound on the Growth and Telomerase Gene Expression in Breast Cancer Cell Line

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Abstract

Objective: The telomerase gene is overexpressed in the majority of tumors and cancers compared to normal and healthy cells, and on the other hand, this enzymatic protein is overactive, therefore, the telomerase enzyme is considered a primary target for diagnostic and therapeutic purposes in most cancers. This has been hypothesized that Helenalin has anti-telomerase activity in a wide range of cancers and Tumor tissues. In this study, we investigated the inhibitory effect of helenalin extract on telomerase gene expression in the T47D breast cancer cell line. **Methods:** We used the MTT assay to evaluate the cytotoxic effect of different concentrations of helenalin on the T47D breast cancer cell line at 24, 48, and 72 hours. Besides, the expression of the hTERT gene in T47D cell lines treated with 1.0 and 5.0 μM helenalin after 24, 48, and 72 h incubation times was investigated through real-time PCR. **Results:** According to the MTT assay, the inhibitory effect of helenalin on T47D cell proliferation is time and dose-dependent. Moreover, the results of Real-time PCR showed that exposure of T47D cell lines to helenalin led to a significant Decreasing in the expressional values of the hTERT gene as a time and dose-dependent procedure compared with the control group ($P \leq 0.05$). **Conclusion:** These preliminary results demonstrated the cytotoxic potential of helenalin through inhibition of hTERT against T47D breast cancer cells.

Keywords: Breast cancer- T47D cell line- Telomerase- Helenalin

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Introduction

Around the World, Breast cancer has the highest prevalence of cancers diagnosed in women worldwide and is one of the most prevalent cancers diagnosed in women. This cancer also is the second leading cause of death after lung cancer in all cancers (Dorling et al., 2021). It has been proven that the enzymatic protein of telomerase has also been shown to be much more active in 85% of tumor cells and 90% of breast cancer cells than in non-tumor cells and tissues, whereas in healthy tissue and cells this enzyme is inactive or has undetectable expression and activity. The protein of telomerase is an enzymatic ribonucleoprotein that by its reverse transcriptase activity replicates the ends of chromosomes that are telomeres and protects these telomeric ends in chromosomes during DNA replication (Dogan and Forsyth, 2021).

In the human tissue and cells, a telomere is one chain of DNA containing simple repeated TTAGGG sequences in the chromosome terminal. The length of chromosomes end which includes telomeric replication sequences in healthy human somatic cells varies from 5 to 15 kPa, and the length of these telomeric replications decreases during the cell aging process (Pal and Shukla, 2003; Salmani Javan et al., 2022). Telomeres at the end of chromosome supply genomic stableness (conserving and protecting the end of the chromosome from being recognized as a broken DNA and in need of repair or from being recognized by nucleases) and an important and basic factor of expendable DNA (a solution for debilitation of the replication system to replicating of the telomeric ends). A significant amount of Smalling and shortening of telomeres can happen during DNA replication but have no leading dangerous effects. In normal cells, human telomeres shorten as the cell ages until the cell has been ages and at the same time the telomere size of the cell chromosomes reaches a critical length, as a result, this factor and process eventually lead to cell proliferation problems and causes cell aging and eventually cell death (Pirmoradi et al., 2018; Nejati et al., 2022). Human telomerase is a set of a protein subunit

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and an RNA subunit, in which, in this ribonucleoprotein complex the protein subunit plays a catalytic role in reverse transcriptase and is called hTERT. As mentioned, in addition to the telomerase protein subunit, it also has a ribonucleotide subunit called hTR, which it uses as a template to replicate the telomeric ends of chromosomes in human cells (Jafari-Gharabaghlou et al., 2018; Javidfar et al., 2018).

In almost all human cancers, such as breast cancer, telomerase is over-expressed; it is probably a critical factor for progressive and continuous tumor growth (Jafari-Gharabaghlou et al., 2018). In most cancer cell lines, telomerase activity levels correlate with the proliferation of state of these cells. However, telomere length is usually shorter in tumor cells in most human cancers with comparing to contiguous non-tumor cells (Zohre et al., 2014; Nault et al., 2019). Further, normal breast cells and most deficient telomerase gene expression and telomerase activity. Telomerase gene expression and its enzyme activity are commonly involved with the acquiring of more malignity in breast cancer. Also, there is a positive association between hTERT gene expression quantity and the amount of malignancy of cancerous tumors in breast cancer. In many studies, a link between telomerase gene expression and activity and breast tumor aggression has been confirmed (Guterres and Villanueva, 2020).

Moreover, it is approved that, telomerase act as a very effective and beneficial anticancer therapeutic purpose. As a result, inhibition of telomerase as a therapeutic target for breast cancer can be prospected for the proprietary purposes for cancer cell therapeutic methods, while less toxicity for healthy cells. This therapeutic targeting telomerase includes hTR antagonists, hTERT promoter replication inhibitors, gene therapy for replacing wild hTERT gene with a mutant deficient hTERT gene, and reverse transcriptase inhibitors (Sadeghzadeh et al., 2017; Mizukoshi and Kaneko, 2019).

It is expected that inhibition of telomerase in the cancer cells is leading to the shortening of telomeric ends and at last results in stop growth, cell aging, and at last cancer cell death. Inhibition of telomerase expression or activity confirmed to growth retardant of cancer cells in cell culture (Wu et al., 2020).

Helenalin is a natural sesquiterpene lactone that has been seen in the alcoholic extraction of Arnica chamissonis and Arnica Montana flowers that have anti-cancer and anti-inflammatory therapeutics effects (Figure 1) (Drogosz and Janecka, 2019; Kriplani and Guarve, 2020).

Many scientists in their studies have stated that this herbal active ingredient electorally inhibits NF-κB inflammatory actions and has negative effects on inflammatory systems and eventually stops the cycles of inflammatory processes, as farther they approved that the Helenalin extraction has anti-inflammatory inhibitory effects on the NF-κB as long as this inflammatory factor has a centrality role as a mediator in the immune system responses in human (Kriplani et al., 2020; Alagheband et al., 2022). At last, as is well known, inhibition of NF-κB expression or activity by helenalin reduces the inflammatory responses and inhibits cancer cells growth (Chauhan et al., 2021; Fang et al., 2021).

Helenalin is a chemical substance that, due to its lactone ring in its chemical structure, has been a member of a group of chemical substances, whose main characteristic of these types of chemicals in the presence of the same lactone ring that they are called sesquiterpene lactones which as mentioned, they are known and characterized for the existence of this lactone ring. Besides this lactone ring, the chemical structure of helenalin has two reactional groups, that one of these reactional groups is called α-methylene-γ-butyrolactone and another group is cyclopentenone, as well as These two groups along with the double bond in the carbonyl group in the structure of helenalin by a thiol group allow this herbal drug to react easily in Michael addition (Karadeniz et al., 2021; Mun and Townley, 2021). As a result, helenalin can easily form covalent bonds with biological proteins and peptide chains such as glutathione, which contains the amino acid cysteine, which also contains the thiol group and thus interacts with these biological molecules. Eventually, with the happening of this interaction between helenalin and biological proteins containing thiol groups, helenalin will have disruptive effects on these biological molecules' functions and rules (Potęga, 2022). Because of these scientific facts that thiol groups are highly and strongly nucleophilic, and in addition to this property, each thiol group has a pair of electrons, these Michael addition reactions occur anyway (Worch et al., 2021).

Helenalin can also target one of the major NF-κB subunits, the P65 subunit, also known as RelA that in this inhibition way P65 subunit of NF-κB. It can react with Cys38 in RelA by Michael addition will react with cysteine38 in target proteins as long as this reaction the Both chemical reactive groups, As described them and their chemical names were mentioned, can react with cysteine 38 (Widen et al., 2018). Researchers in their findings have also proven that Helenalin reacts exactly with the thiol group in the amino acid cysteine in the protein subunit of the ribonucleoprotein telomerase complex by both of its chemical reactive groups, and ultimately inhibits the human telomerase activity of this ribonucleoprotein complex by Michael addition (Huang et al., 2005).

Herein, in this study, we are going to assay the effectiveness of the Helenalin extraction on the gene expression of the human enzymatic ribonucleoprotein complex of telomerase in human breast cancer cells. And so, the most important purpose of this research was to the assessment of the inhibitory efficacy of Helenalin extraction on gene expression of human telomerase in the T47D breast cancer cell line.

Materials and Methods

Materials

RPMI 1640, Fetal Bovine Serum (FBS), Triazole reagent (RNA extraction kit), Trypsin-EDTA, and Antibiotics were purchased from Invitrogen (Germany). Syber Green Real-Time PCR Master Mix and first strand cDNA synthesis kit were obtained from Roch (Germany). MTT powder was bought from Sigma. In last, Lyophilized and completely sterile helenalin extract also was purchased

from Enzobio life science, America.

Cell culture and cell cytotoxicity assessment

The T47D cell line was grown in RPMI1640 that evolved with 10% FBS and 2 mM L-glutamine to become a suitable culture medium for cells growth, then Finally, to prevent contamination of culture media with living environmental agents such as Mycoplasma, The mentioned antibiotics were added to the culture media in certain concentrations: The used antibiotics were 80 mg per lit penicillin G, 50 mg per lit streptomycin and eventually, 2 g Na_2HCo_3 per lit was added to culture media To maintain the pH balance. Cell lines were grown at 37 ºC in a CO_2 incubator and then this incubator was adjusted to contain 5% CO_2 and 55% moisture and humidity permanently during cultivation. After culturing Adequate amounts and quantity of cells, cytotoxicity of Helenalin extract was diagnosed 3 times with MTT assays in 24, 48, and 72 hours. Generally, the cell lines were cultured in the 96 well plates, in such a way that 2,000 high-quality cells will grow in each well. After replacing the 2,000 cells in the wells at 37°C and atmospheric conditions with a humidity of 55% and 5% CO_2, incubated and growing for a period of 24 hours, we treated them with definite different concentrations of Helenalin in 5 different concentrations (1.0-5.0 µM) for 3 periods of time that were 24, 48 and 72 h in the triplicate model and at the same condition as cells which received 0 nM/ml of the therapeutic reagent with + 200 µl, culture media with 10% DMSO assayed as control. After the cells were incubated with the intended drug, the cultivation media of the wells were replaced with new and fresh culture full media, then, the plates that contain all the control and treated cells were kept in a Co2-incubator for 24 hours. hereafter, all of the culture media in the plates were removed cautiously and l of 2 mg/ml MTT (Sigma company) solvated in the PBS buffer was accessed to all of the wells in the plate. Then this plate that is used to evaluate the cytotoxicity of helenalin was wrapped and completely covered with aluminum foil so that it is no light reaching the cells that are under evaluation for a 4.5-hour incubation time in a gloomy and dark room. After eliminating of solution in the wells, 200 µl DMSO accessed to wells. After that, 25 µl Sorensen's glycine buffer was accessed, and spontaneity and as an emergency absorbance of each well was read in 570 nm using ELx800 Microplate Absorbance Reader (Bio-Tek Instruments) by reference wavelength of 630 nm.

Total RNA extraction and cDNA synthesis

The cultured cells in special flasks were trypsinized, and after those cells accumulated by centrifugation at 1,320g for 3 minutes at 37°C. Therefore, the whole RNA was extracted with purchased kit, accordingly to the Trizol procedure for treating and controlling cell lines. Effectiveness and efficacy of this method of RNA extraction assayed with nanodrop analysis. As well as, the sincerity of the extracted RNAs were determined by using spectrophotometer (Termoscientific, Wilmington, DE, USA) at 260–280 nm and the consistency of RNA extraction sets get approved with the agarose gel electrophoresis by 0.5% concentration of ethidium bromide. At last when pure RNA get extracted, cDNA was synthesized from extracted RNA using random primers (N6) and hexamer primers with fermentas reverse transcriptase kit's instructions. This cDNA that has been syntesized immediately prepared for using that in Real time PCR and the rest of the synthesized cDNA was stored at minus 70°C.

Real time PCR

At this stage, the Real-time PCR technique was used to evaluate the amount and quantity of changes in the expression of hTERT gene expression in both treated and control samples, and the gene expression of b-actin was applied as an internal control for quantitative data normalization.

In detail, the amount of hTERT gene expression in the samples was determined with quantitative Real-time PCR (qRT-PCR) technique using the Syber Green-I (Roche, Germany) by the Rotor-Gene TM 6000 machinery (Corbett Research, Australia) in triplicate accordingly to the procedure applying with the manufacturer. For running the Real-time PCR, hTERT primers (Genbank accession: NM_198255, bp 2165-2362) and beta-actin primers (Genbank accession: NM_001101, bp 787-917) were used. For hTERT, a 198 base pair and for beta-actin a 131 base pair amplification size were generated in a 25 µl reaction mixture that contained: 5 moles of the forward and reverse PCR primers of hTERT (5'CCGCCTGAGCTGTACTTTGT3', 5' CAGGTGAGCCACGAACTGT3' respectively) or for beta-actin (5'TCCCTGGAGAAGAGCTACG3', 5'GTAGTTTCGTGGATGCCACA3' respectively), 2X PCR Master Mix Syber Green I and 2µl of the cDNA was used (Table 1). Meanwhile, the modality and state of Real-time PCR reactions were measured by running standard samples in triplicate. 5- Fold serial dilutions of cDNA gained from the T47D cell line act as samples by the perfect expression of the hTERT gene. The program for real-time PCR reaction that has been applied was; Initial denaturation at 95°C for 10 seconds, followed by 30 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 30 seconds. At last, amplicons were assayed with a melting curve analysis of 72°C to 95°C. Changes that happen in amounts of telomerase expression between the control and T47D cells that were treated with Helenalin extract, normalized to β-Actin mRNA amounts, commutated with the $2^{-\Delta\Delta CT}$ model.

Statistical *analysis*

Graph Pad Prism 8.4.3 has been applied to reach the statistical analysis amounts. The difference in the expression levels of hTERT between control and treated cells was tested with ANOVA and Tukey's analysis tests. A p-value<0.05 was regarded as substantial diversity.

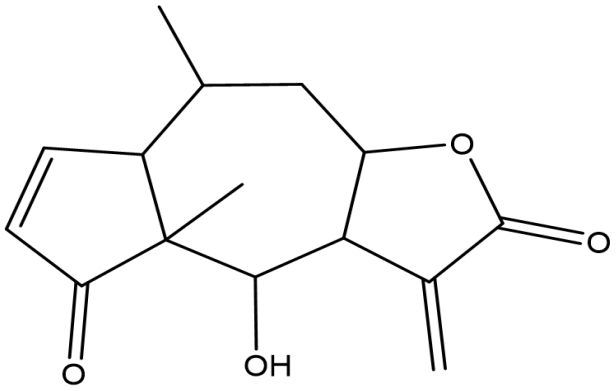
Results

In vitro cell toxicity

For assaying the cytotoxicity of different concentrations of helenalin on T47D cell lines, the MTT assay was used

Table1. Forward (F) and Reverse (R) Primer Sequences of β-actin and hTERT Used in Real-Time PCR.

Oligonucleotide	Location	Sequence	PCR product size (bp)
hTERT			
Forward primer	2165F	5′CCGCCTGAGCTGTACTTTGT3′	198
Reverse primer	2362R	5′CAGGTGAGCCACGAACTGT3′	
Beta-actin			
Forward primer	787F	5′TCCCTGGAGAAGAGCTACG 3′	131
Reverse primer	917R	5′GTAGTTTCGTGGATGCCACA 3′	



Molecular Formula: $C_{15}H_{18}O_4$

Functional groups: C=O, C-OH, C=C, C-O

Figure 1. Structure of Helenalin and Its Functional Groups.

in the manner time-dependent for 3 times 24 h, 48 h, and 72 h. Results showed helenalin inhibited the T47D cell line propagation in vitro in a time and dosage-dependent manner. Besides, analysis of MTT assay's given data has proved that IC_{50} values of Helenalin on T47D cells for 24, 48, and 72 h were 4.69, 3.67, and 2.23 μM, respectively (Figure 2) and (Tables 2,3 and 4).

hTERT gene expression

The result of Real-time PCR proven an intense and great decline and diminution in *hTERT* gene expression in the cell lines that were cured by helenalin compared with the control cells. During the measurement of hTERT gene expression by Real-Time PCR, the rate of hTERT mRNA was normalized to mRNA rates of the uniformly expressed housekeeping gene, Beta actin's gene, in the any of samples. After that, the computation of any differences in the $2^{-\Delta\Delta CT}$ scales was performed. Simultaneously with enhancing the value of $2^{-\Delta\Delta CT}$, in the rate of expression of mRNA, a significant decrease was observed (Table 5). Data statistical analysis of Real-time PCR has proved that with treatment by Helenalin extraction, a decreasing and reductional trend seemed and appeared in amounts and levels of mRNA gene expression of hTERT. Each sample was repeated 2 times and all of them in duplicate form were examined and measured. At the end of the treatment, the assessment of cell numbers showed that inhibiting the cell growth by Helenalin depended on time. The standard curves in Real-time PCR results proved a substantial diminution in mRNA gene expression of hTERT in the cells that were treated with helenalin compared with not treated cells. The same as we keep treated T47D cells with Helenalin in concentrations between 1.0 and 5.0 μM for 24, 48, and plus 72 hours, expression of the hTERT in all of the samples were considerably detracted.

At last Real-time PCR as a quantitative technique was performed in order to measure and quantify the amount of mRNA expression of hTERT gene in T47D cell lines that were treated with 1.0 and 5.0 μM helenalin after 24, 48, and 72 h incubation times. Real-time PCR data and

Table 2. Obtained ODs from 24h MTT Assay Read in 492-630nm for Helenalin

Concentration	Control 0.00	Control DMSO	0.2μM	0.4μM	0.6μM	0.8μM	1μM	1.2μM	1.4μM	1.6μM
OD	0.207	0.227	0.222	0.179	0.177	0.183	0.180	0.168	0.170	0.160

Table 3. Obtained ODs from 48h MTT Assay Read in 492-630nm for Helenalin

Concentration	Control 0.00	Control DMSO	0.2μM	0.4μM	0.6μM	0.8μM	1μM	1.2μM	1.4μM	1.6μM
OD	0.291	0.262	0.312	0.299	0.261	0.247	0.216	0.208	0.175	0.165

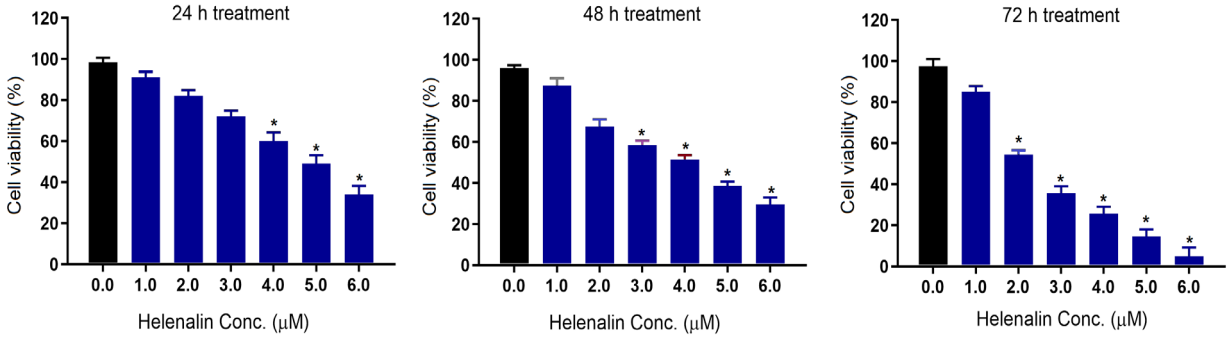


Figure 2. Graphs for the Viability Test of Helenalin on T47D Cells during 24 h (A), 48 h (B) and 72 h (C) MTT assays

Table 4. Obtained ODs from 72h MTT assay Read in 492-630nm for Helenalin

Concentration	Control 0.00	Control DMSO	0.2μM	0.4μM	0.6μM	0.8μM	1μM	1.2μM	1.4μM	1.6μM
OD	0.382	0.309	0.340	0.302	0.269	0.244	0.227	0.116	0.181	0.176

Table 5. Measurement of hTERT mRNA Level in Samples with ΔCt.

Time of treatment	24h	48h	72h	Control cell
Sample Ct	25.06	27.74	27.52	28.4
Internal control Ct	13.92	17.26	18.25	11.06
(ΔCt)	11.14	10.48	9.27	17.34
$2^{-\Delta\Delta Ct}$	0.0136	0.0086	0.0037	1.00

final results proved that the treatment of T47D cell lines by helenalin led to a significant decrease and reduction in the mRNA expression levels of hTERT in a time and dosage-dependent manner compared with control samples ($P \leq 0.05$) (Figure 3).

Cancer cells growth after treatment

Finally, after treatment of the cancer cells to the

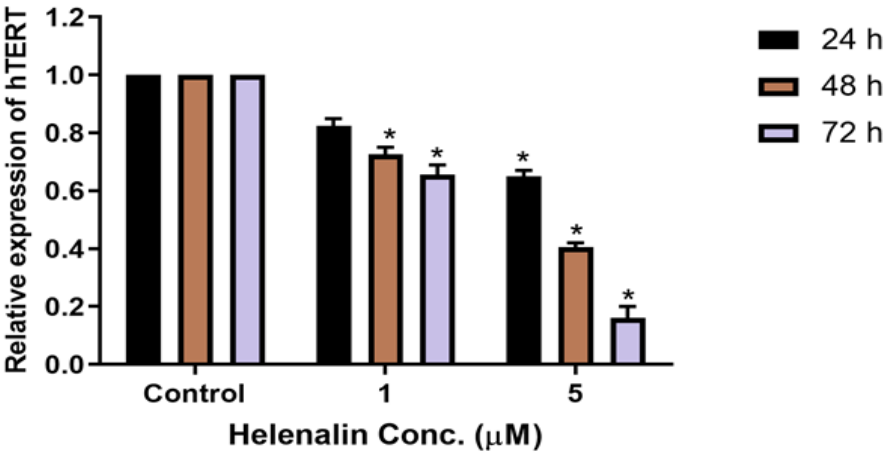


Figure 3. Inhibitory Effects of Helenalin on Expression Levels of hTERT in T47D Breast Cancer Cells. *p < 0.05 vs. control was considered significant. Data represented are from three independent experiments.

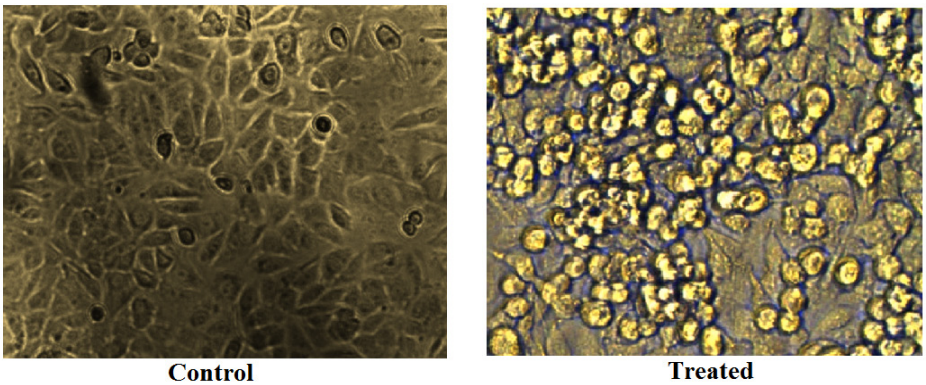


Figure 4. Changes in Appearances and Reduction of Cell Number with Increasing of Concentration after Treatment with Helenalin

therapeutic concentration of the helenalin extraction, the changes in the morphology and appearance of the cells and, most importantly, the extent of changes in the growth rate of cancer cells treated by helenalin extraction were examined by inverted microscope. The checking of Cell confidences with an inverted microscope showed that exposure of T47D cells to helenalin led to a significant change in morphology and appearance of the cells and most importantly a significant reduction in cell concentrations and numeration (Figure 4).

Discussion

Today, the serious and most considerable issue in cancer remedy and therapy is Using drugs that are most effective in treating cancer and at the same time have the least harmful effects on healthy cells and tissues. Even though today various treatments such as chemical therapeutics, hormonal therapeutics, and electrotherapy have been used for various types of cancers and tumors, however, the best and most healthy method, which has been most favorite and popular in the last decades, has been used of Natural and herbal medicines and remedies for cancer treatment (Pal and Shukla, 2003; Ji et al., 2009; Khodadadi et al., 2022; Sahabi et al., 2022).

In this study, to measure the cytotoxicity of helenalin on the T47D cell line in cancer of the breast, first, after sufficient and full cell age and growth, the T47D cell line in the RPMI 1640 culture media with 10% FBS was treated and exposed to different concentrations of free extracted helenalin for different time periods in durations of 24, 48, and 72 Hours. After all, the results of MTT assay showed that the cytotoxic effect of helenalin extraction on T47D cell lines had concentration-dependency as well as time-dependent. Our results showed that Helenalin significantly inhibits the hTERT mRNA gene expression. This finding is critical because almost all of the chemical drugs that are used for chemotherapy cause very toxicity to healthy and non-tumoral tissues in treating breast cancer. Their harmful lateral effects on health decrease their clinical succession in chemotherapy methods for cancer treatment. Hereafter, there is hope that these discoveries could have clinical valence for developing new telomerase-inhibitory chemical drugs (Javidfar et al., 2018; Kriplani et al., 2020).

Because of the increased toxicity and drug resistance to the system of the human body, the major problem of cancer chemotherapeutic agents, we have very interested to challenge in the cancer biological fields and biomedical research. To deal with this problem, many studies have been directed toward reducing toxic effects and enhancing pharmaceutical activity and efficacy in cancer cures. Because of this purpose, novel chemical therapeutic methods have received more attention because of this target that could enhance the therapeutic success in remedies for cancers. Moreover, those dietary supplements also have anticancer activity against gene expression. Drugs that act as inhibitors for telomerase and have very activity are remarked as potential anti-cancer pharmaceutical agents or reagents, with this expectancy that the reduction of mRNA expression of telomerase in healthy and natural somatic cells can eventuate to finding a so much specific cure with less and very fewer harmful side effects (Bajaj et al., 2020; Negrini et al., 2020).

Our study showed that helenalin is an agent which is nontoxic and efficient for therapeutic targets, considered a suitable, proficient, and perfect remedy for therapeutic uses and has a remarkable therapeutic activity for cancer contrary variety epithelial cancers such as breast cancer cells (Liu et al., 2019; Bajaj et al., 2020).

Helenalin also has displayed an inhibitory efficacity on expression of human telomerase reverse transcriptase gene (hTERT), decreasing telomerase activity in breast cancer cells (Drogosz and Janecka, 2019; Bajaj et al., 2020).

On the base of the mentioned discoveries, Helenalin extract would have a major use for anti-cancer therapy in breast cancer cell lines; we hypothesized that using Helenalin for inhibiting hTERT gene expression. All results of this study, showing a considerable therapeutic use of Helenalin in T47D cell lines, confirmed this hypothesis.

Recently most epidemiological studies show that many breast cancer patients are using alternative medicines that mostly have an herbal origin. Additionally, these studies suggest that a preclinical study is critical to confirm the efficacy of Helenalin chemotherapy in the human breast cancer in an in vivo model (Ghasemali et al., 2013; Kordi et al., 2016); The preclinical data presented here show significant therapeutic effect of Helenalin in these in vitro models of breast cancer (EL et al., 1995). With a favorable side effect profile and long history of human use, Helenalin represents a good candidate for treatment. This research demonstrated and proved that Helenalin is useful and effective for detraction of T47D cell lines propagation and more specific helenalin causes inhibition of hTERT mRNA production in these cells. If in the in vivo studies the effectiveness of this drug is confirmed, this drug can be regarded in a clinical trial for breast cancer patients in the future.

In conclusion, the results of this study approved that Helenalin extraction had strong inhibitory impacts on hTERT gene expression in breast cancer T47D cell lines. The therapeutic and cytotoxic effects of helenalin were not only dose-dependent but also time-dependency. Statistical data analysis approved that with accession time of the treatment, decreasing in telomerase gene expression had arisen. In summary, as our data showed that Helenalin extraction had inhibitory high efficacy on mRNA gene expression of hTERT, this chemical extraction can be used as an anticancer remedy in breast cancer therapy.

Abbreviations

cDNA: complementary DNA.

DMSO: dimethyl sulfoxide.

hTERT: Human telomerase reverse transcriptase.

mRNA: messenger RNA.

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells.

Author Contribution Statement

Writing - original draft preparation: Amin Barkhordari and Davoud Jafari-gharabaghlou; editing: Zenep Turk; Conceptualization, Supervision: Nosratollah Zarghami.

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Authorship

All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

Availability of supporting data

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Conflict of Interest

No potential competing interest was reported by the authors.

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