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The Effects of Thymoquinone on Inhibiting the Expression of *SENP1* Gene in the MCF-7 Cell Lines

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Abstract

SUMOylation, as post-translational modifications, plays essential roles in various biological functions including cell growth and migration, stress response, and tumorigenesis. In SUMOylation, *SENP1* catalyzes the SUMO protein maturation to combine with target proteins. Breast cancer is a common malignancy in women and also *SENP1* progression is high in this cancer. Thymoquinone is a biologically active substance and a secondary metabolite found in the black seed, and recent researches indicate antioxidant, anti-inflammatory, anti-cancer and other important biological activities. The effects and mechanisms of Thymoquinone on *SENP1* are not well studied. For this purpose, the first MCF-7 tumor cell line and normal MCF-10A cell line were cultured in normal conditions and then treated with specific doses of thymoquinone. The lethal activity was evaluated by MTT assay and exhibited that the toxicity of thymoquinone on MCF-7 cell line was higher than healthy cell and the intensity of its effect was different from MCF-10A cell. Then *SENP1* gene expressions were measured. Gene expression changes in tumor cells were then compared with normal cells and it was found that thymoquinone was able to reduce *SENP1* gene expression in the tumor cells. Then docking of Thymoquinone with *SENP1* protein was performed. The amount of binding energy between Thymoquinone and protein *SENP1* is -54 and with dimer of Thymoquinone is -80. Based on what researchers have concluded in this study, it is possible that the mentioned gene and *SENP1* Protease can be considered as a candidate for breast cancer treatment and drug target.

Keywords: SUMOylation • Breast Cancer • Black Seed • Cysteine Protease • SUMO

Introduction

Post Translation Modification (PTM) is widely observed in the regulation of protein activity. Proteins can be altered by small chemical groups, sugars, lipids and even by covalent binding to another polypeptide. The most well-known example is the ubiquitin polypeptide modifier. The role of ubiquitin is to target the protein to the proteasome and its proteolytic digestion. In addition to ubiquitin, there are several other ubiquitin-like proteins (UbLs) that can bind to the protein substrate and alter its function. SUMOylation, as post-translational modification, plays key role in various biological functions including cell growth and migration, stress response, and tumorigenesis. The imbalance of SUMOylation and deSUMOylation were associated with the occurrence and progression of various diseases. In Compared with ubiquitin, although SUMOylization does not promote protein digestion, it modifies proteins against some functional parameters, depending on the protein type. Some of these parameters includes intracellular protein translocation, gene expression, chromatin structure, message transfer, genome stability, DNA binding or alteration of transcription factor activity and some other activities. It is likely that the effects of ubiquitination and SUMOylation are largely due to the binding of proteins and the position of their interacting domains. Ubiquinitization and SUMOylation changes are both reversible [1]. SUMO (Small Ubiquitin-like Modifier) is a small protein with 97 amino acids that is structurally similar to ubiquitin and is also known as Smt3p, Pmt2p, PIC-1, GMP1, Ubl1 and Sentrin [2]. Sumo, like ubiquitin, has been shown to bind covalently to some of the lysine subunits in protein targets [3]. The SUMO family is a PTM protected form, in all eukaryotes. There is only one SUMO gene in germinated yeast, while there are at least 8 types of SUMO in plants. The SUMO protein in mammalian cells has four types: SUMO-1, SUMO-2, SUMO-3 and SUMO-4. SUMO-2 has 95% of homology to SUMO-3. SUMO-2 and SUMO-3 differs by only three residues at the N-terminus and have approximately 45% homology to SUMO-1. SUMO-4 has the lowest SUMO family feature. SUMO-4 is probably unbound in physiological conditions [4]. Similar to ubiquitination, SUMOyaltion involves series of enzymatic processes. The mature SUMO is activated by conjugation to the E1 enzyme (SAE1/SAE2), transferred to the E2 enzyme (Ubc9) and ligated to the specific lysine residue of the target proteins by an E3 enzyme [5]. Members of the Ulp / SENP family are the Ulp1 and Ulp2 proteins of Saccharomyces cerevisiae initially identified by Li and Hochstrasser [6]. SENPs are enzymes of the maturation and decongestion of SUMO. In addition to protected catalytic domains, SENP proteases have specific N-terminal domains; SENPs have an enzymatic dual function for immature SUMO maturation and SUMO deconjugation. In deconjugation reaction, SENP separates an isopeptide bond that transfers SUMO fragments into the ε-amino group in lysine [4]. The human protease (Sentinel-specific protease 1) has 643 amino acids and 73 kDa weighing . Its gene is located on the 12th human chromosome. It is a super family of cysteine proteases. Cysteine is at position 602, histidine at position 533 and aspartic acid at position 550. Cysteine is at the alpha-helix N terminus of the protein nucleus with two other amino acids, aspartate and histidine are at the end of beta [7]. *SENP1 is* found in both nucleus and the cytosol depending on the cell type, which is seen to be transmitted from the nucleus to the cytosol via the NES located at the C terminus. *SENP1* is predominantly located in the nucleus in mammals. *SENP1* Catalyzes the maturation of the SUMO Protein (Small Ibbokitin Reducer), which Hydrolyzes the SUMO

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peptide bond in the G-Gly- Conserved Sequence | -Ala-Thr-Tyr becomes terminal at C [8].

Thymoquinone (TQ) is a 164.2 g/mol phytochemical substance extracted from *Nigella sativa* and its different effects has been shown, such as analgesic, anticancer and antioxidation in the heart [9]. TQ is used to treat a variety of cancers such as brain, breast, colon, liver, lung, and prostate cancers [10]. Thymoquinone is an anti-neoplasic agent reported to have the therapeutic potential in the treatment of breast cancer. The anti tumour activity of thymoquinone has been reported in cells, derived from ovarian, breast and colon cancers. The apoptotic activity of thymoquinone has been reported to induce the total Bax / Bcl-2 ratio in MCF7, HCT-116 and HL-60 cancer cells. These findings were based on flow cytometry, western blot analysis and multi-color fluorescence in situ hybridization (mFISH). Therefore, further investigation is needed to determine its effects on human genome expression using cDNA microarray technology [11].

Docking of the small molecular constituents at the receptor binding site and the affinity estimation of this complex are an important part of the structure-based drug design process. For a complete understanding of the structural principles that determine the strength of a protein / ligand complex, a fast and accurate binding protocol the ability of visualize binding and interaction geometries are required [12]. There are three basic tasks any docking procedure must accomplish: (1) characterization of the binding site; (2) positioning of the ligand into the binding site (orientation); and (3) evaluating the interaction strength for a specific ligand-receptor complex ("scoring") [13].

Materials and Methods

Thymoquinone solution preparation

The 1 mM stock solution of thymoquinone (Sigma-Aldrich, Saint-Quentin-Fallavier, France) was prepared with DMSO (Sigma-Aldrich).

Cell culture and treatment

We received the breast cancer cell line *MCF7*. We also used the normal breast cell line, MCF10 as a non-cancer control. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, ThermoFisher Scientific PN: 11965118) supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals PN: S11150), grown in a monolayer at 37°C and 5% CO_2. Cells were passaged one to two times per week, or until cells reached approximately 80% confluence. The *MCF7* cells were seeded at 10000 cells/well in 96-well plates. They were cultured at 0.5% CO_2 in a humidified incubator at 37°C.

MTT assays

Determination of the thymoquinone dose and MTT implementation test: Cells were first treated with thymoquinone: 1) 200 µl of solution (DMEM + FBS +cell) was added to each plate. 2) 24-hour incubation. 3) The solution was removed from the plates. 4) Add TQ in each plate with the concentrations of 20/5/0 µM. 5)24-hour incubation. After performing the MTT test the absorbance was measured in 570 nm.

RT PCR (RT-PCR): All cell lines were seeded at 500000 cells/well in a 6-well dish and grown at 37°C and 5% CO_2 for approximately 24 hours. RNA was isolated from the *MCF7* and MCF10 cells using the RNX Plus Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The quantity of RNA was measured using a spectrophotometer (NanoDrop 7000c; Thermo Scientific). Samples with the RNA concentration (A260/A280 ≥1.8 ng/µl) and purity (A230/A260 ≥2.0 ng/µl) were selected. cDNA was generated using the cDNA Synthesis Kit (iNTRON) using 20ng of RNA, d(T)23 VN primers and following the recommended protocol (Table 1).

Bioinformatics

Setup and execution of docking runs: The human *SENP1* protein structure was downloaded from the PDB site and also TQ structure was downloaded from the pubCHEM site. The MVD software was used for performing the docking process. MVD need receptor and ligand representations with a file format called pdb which is a modified protein data bank format containing atomic charges, atom type definitions and, for ligands, topological information (rotatable bonds). These file preparations are carried out by the plugin using scripts from the MVD Tools package. Ligands for subsequent docking runs can either be prepared one by one through MVD selections. After binding site definition and the preparation of receptor and ligand, docking runs can be directly launched from MVD. In this study, the specific molecular docking software (MVD) was used to investigate the molecular interaction between drugs and protease enzyme. This software provides a three-dimensional (3D) view of the interaction between compounds and the protease enzyme virus and the amino acids participating in the interaction. In the present study, all docking conditions including the interaction frequency, the study area of interaction, the protease enzyme and the rate of docking were considered to minimize error. During this molecular docking study, the number of interaction run is 10, the diameter of interaction area 30 (angstrom) with the ability to investigate the hydrogen- electrostatic and Van der Waals interactions in the total active site of the enzyme and the results were compared.

Results

MTT assay results

Thymoquinone has been reported to suppress cell proliferation in many types of cancers, but its effect on healthy cells and target *SENP1* have not been clearly studied. To determine the anti-tumor activity of Thymoquinone on *MCF7* cells and compare with MCF10, the researcher carried out growth curve assay and cell viability assay using two cell lines. According to the repeated MTT assay, the effect of thymoquinone at various concentrations on MCF-7 cell line showed that the IC50 value of thymoquinone is 20 µM ($p<0.05$) (Table 2). The following table shows the effect of thymoquinone on different concentrations on MCF- 10A cell line, indicating that the toxicity of thymoquinone is less than cancer cells ($p<0.05$) (Table 3) (Figures 1-3).

Results of RT PCR

In order to analyze the expression of *SENP1* gene and β-actin gene, gel electrophoresis was examined by image J software and the following

Table 1. Characteristics of the primers used in the reaction.

Gene	Primer sequence (5' 3')	Tm ºC	PCR Product (bp)
SENP1 Forward Primer	ATCAGGCAGTGAAACGTTGGAC	57	164
SENP1 Reverse Primer	GCAGGCTTCATTGTTTATCCCA		
β actin Forward Primer	CCTGGGCATGGAGTCCTGT	57	152
β actin Revers Primer	ATCTCCTTCTGCATCCTGTCG		

Table 2. Results of MTT MCF7 cell lines.

Concentration of TQ (µM)	Repeat (%)	Repeat (%)	Repeat (%)	Repeat (%)	Mean (%)	SD	SE
0	100	100	100	100	100	0	0
5	88	83	98	96	91.25	6.475228	1.618807
20	60	22	50	51	45.75	15.23858	3.809645

Table 3. Results of MTT MCF10 cell lines.

Concentration of TQ (µM)	Repeat (%)	Repeat (%)	Repeat (%)	Mean (%)	SD	SE
0	100	100	100	100	0	0
5	94	96	95	95	1	0
20	82	85	84	83.66667	1.527525	0.509175

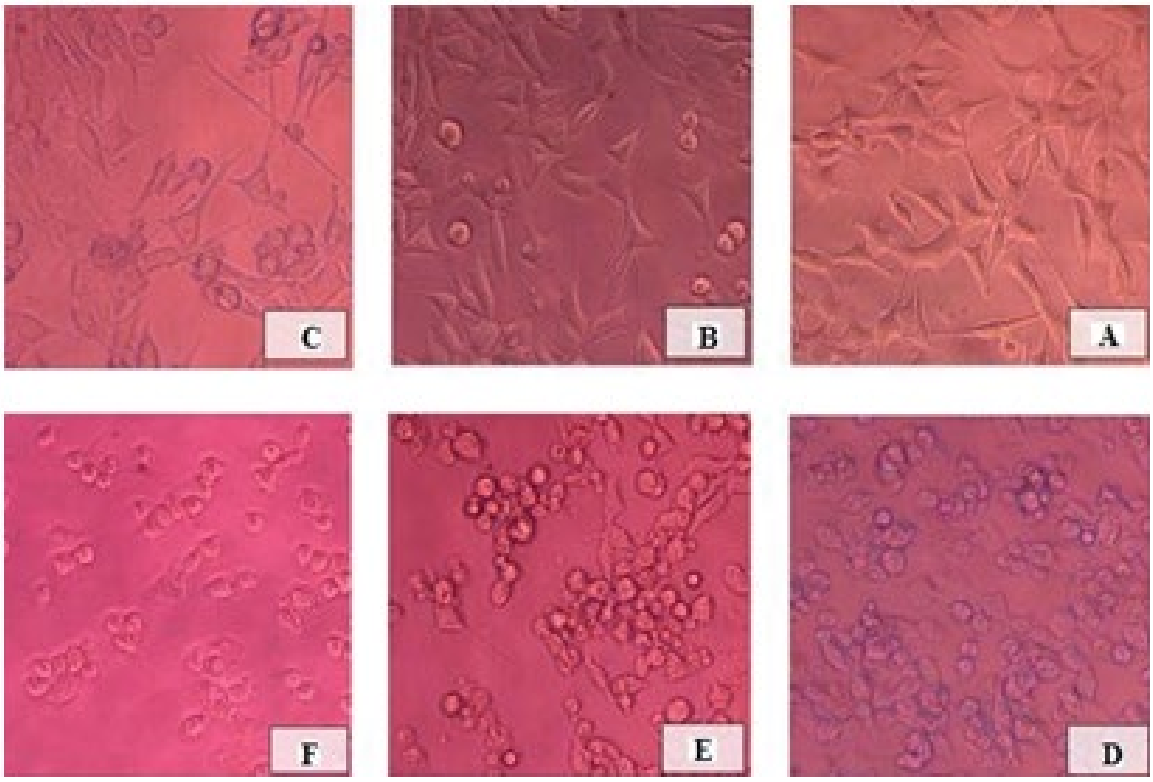

Figure 1. MCF-7 cell lines treated with thymoquinone after 24 h at 40x magnification, (a) Control, (b) 5 µM, (c) 10 µM, (d) 15 µM, (e) 20 µM and 40 µM concentration.

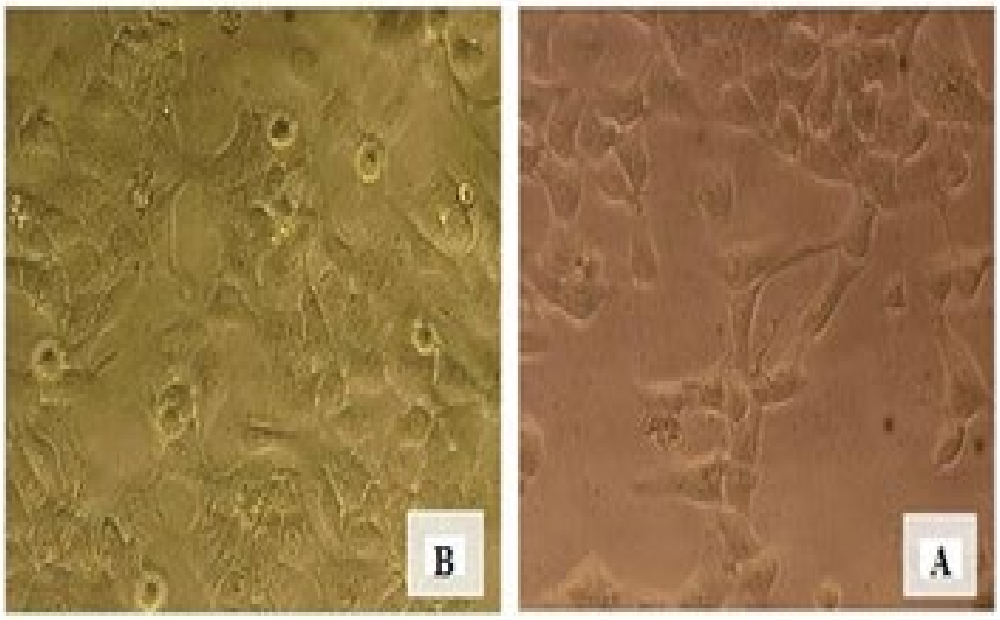

Figure 2. MCF-10 normal cell line treated with thymoquinone after 24 h with magnification of 40, (a) Concentration of 5 µM, (b) Concentration of 20 Mm.

results were obtained. The area under each peak indicates the amount of gene expression (Figure 4). According to PCR data, gene expression decreased with increasing dose of thymoquinone in *MCF7* cells (Figure 5). Also, the expression level of *SENP1* gene was significantly increased in tumor cell lines compared to the normal cells. The aforementioned results have been obtained in previous studies.

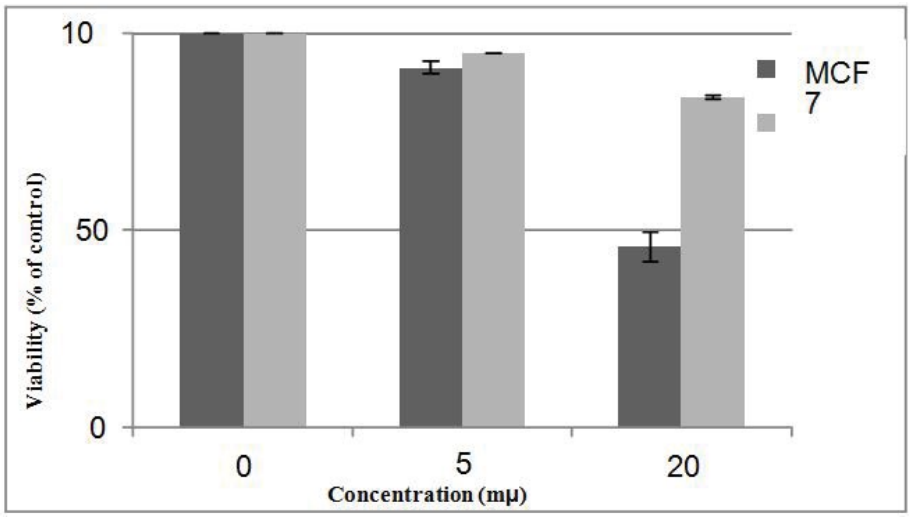

Figure 3. Thymoquinone toxicity is shown to be higher in MCF-7 cell line than in healthy cells.

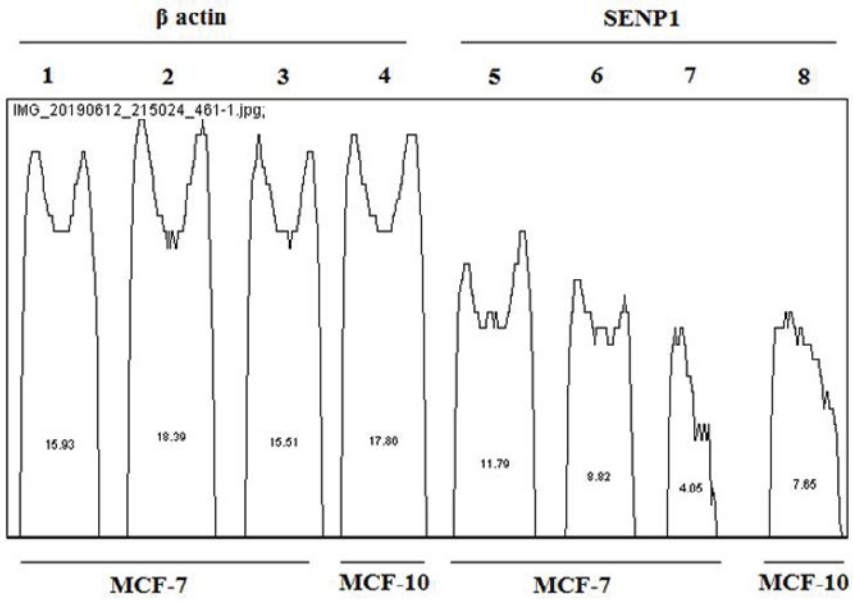

Figure 4. Results of image J software revealed that *SENP1* gene expression levels were significantly increased in tumor cell lines compared to healthy cells. Decreased expression of *SENP1* gene by thymoquinone in breast cancer cell line showed that thymoquinone has inhibitory effect on *SENP1* gene.

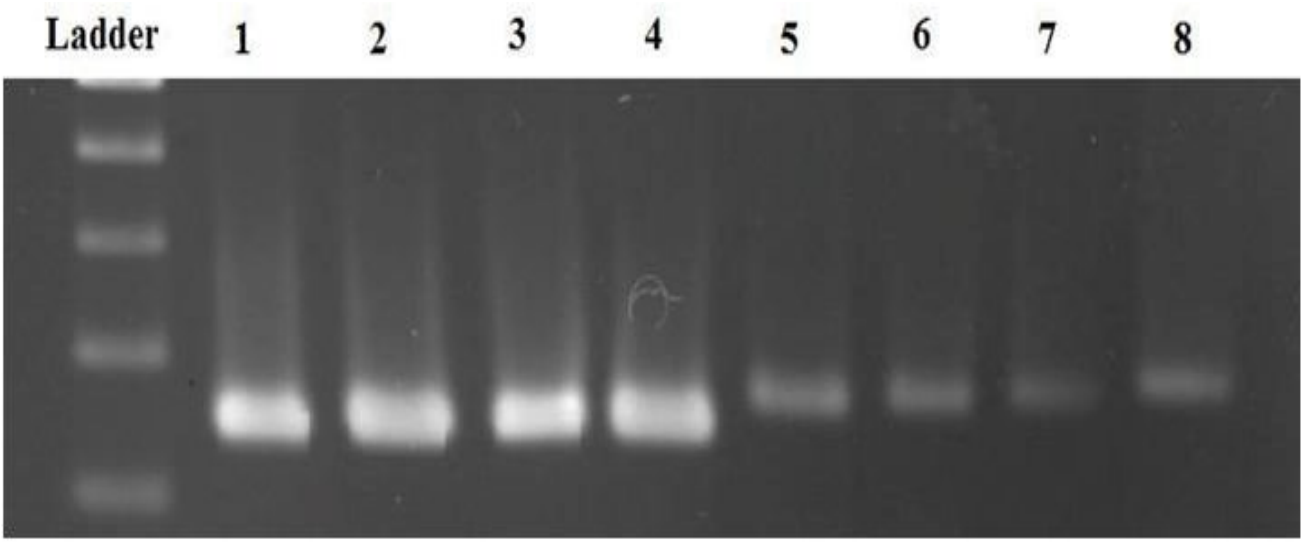

Figure 5. Results of RT_PCR: 1,2,3: β-actin tumor cDNA sample 4: β-actin normal cDNA sample 5,6,7: *SENP1* tumor cDNA sample 8: *SENP1* normal cDNA sample.

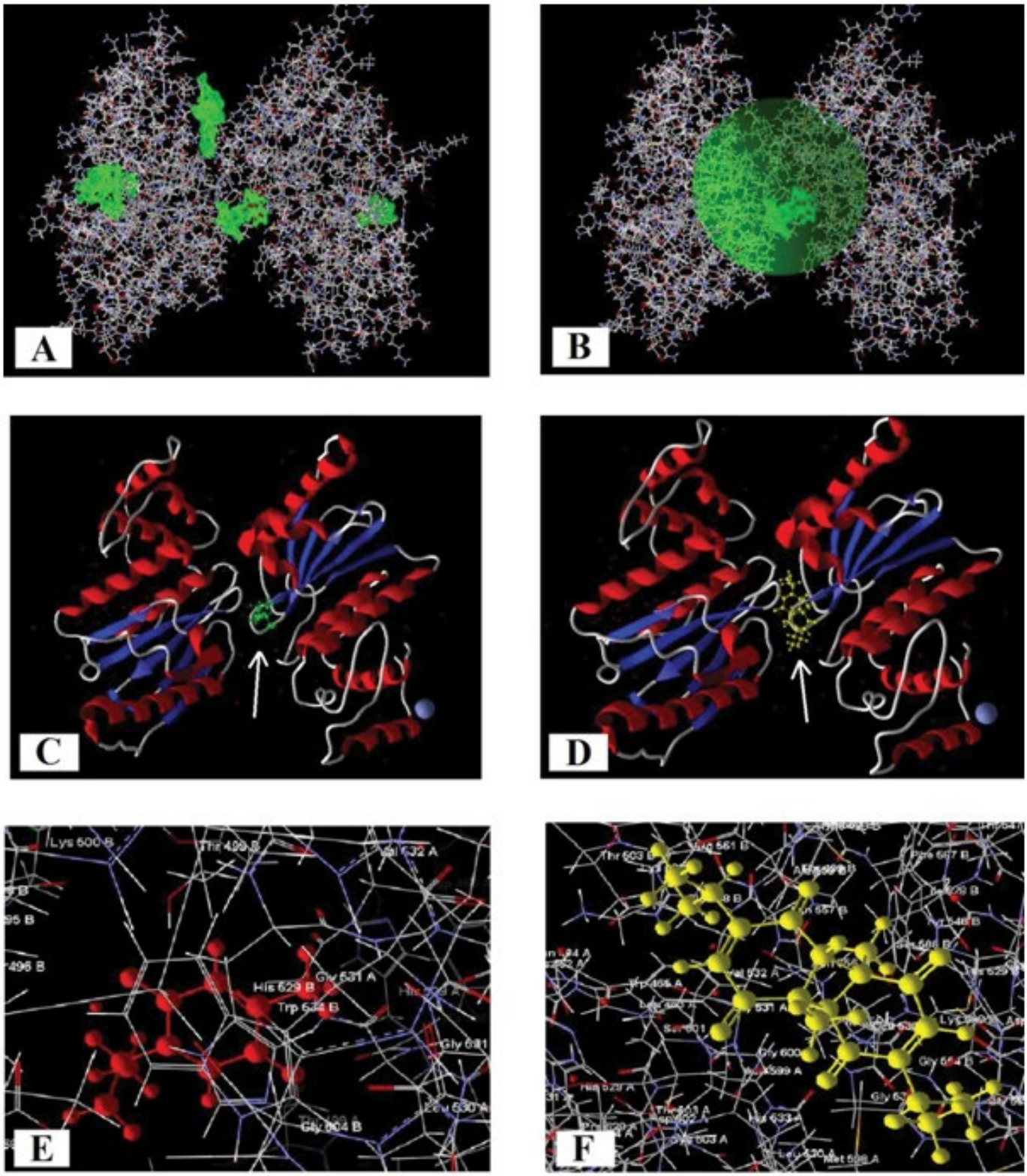

Figure 6. (A): Five cavities detected in *SENP1* enzyme (B): Thymoquinone was attached to the middle cavity (C): Attach the TQ to the protein (D): Docking TQ Dimer with *SENP1* (E): Docking composition of TQ to amino acids (F): Docking composition of TQ Dimer to amino acids.

Docking results

Docking of Thymoquinone with *SENP1* protein was done by Molegro Virtual Docker software and the result showed that Thymoquinone interacts with the *SENP1* protein and the best binding energy of the various conformations with protein was -54 and with Dimer of Thymoquinone is -80 (Figure 6). In docking of the Thymoquinone with the *SENP1* protein, two types of hydrogen bond and ester bonds are formed.

The results showed that the site of interaction of the compounds was in the conserved region of the enzymatic flap. Regarding the binding energy among these compounds the binding of 5 important amino acids due to their presence in the highly conserved region has an active site and a key role in enzymatic catalysis. These amino acids are Gly531, Leu530, His529, Phe 496, Thr 499. The results of these compounds are summarized in Tables 4-6.

Table 4. Calculate the amount of ligand binding energy.

Energy overview: Descriptors	Mol Dock Score TQ	Mol Dock Score TQ Dimer
Total Energy	-54.538	-80.121
External Ligand interactions	-61.618	-83.301
Protein - Ligand interactions	-58.079	-77.465
Steric (by PLP)	-56.102	-76.174
Hydrogen bonds	-1.977	-1.292
Electrostatic (short range)	0	0
Electrostatic (long range)	0	0
Water - Ligand interactions	-3.539	-5.835

Table 5. The amount of binding energy of protease amino acids to compounds.

Target Atoms: Amino Acid	2XPH [A]	2XPH [A]	2XPH [A]	2XPH [A]	2XPH [A]	2XPH [A]	2XPH [B]	2XPH [B]	2XPH [B]	2XPH [B]	2XPH [B]	2XPH [B]	2XPH [B]	2XPH [B]	2XPH [B]	2XPH [B]
Residue	Gly	His	Leu	Lys	Thr	Val	Asp	Gly	His	Lys	Leu	Phe	Thr	Thr	Thr	Val
ID	531	529	530	502	499	532	468	531	529	500	530	496	495	499	503	532
TQ	-6.8	-	-6.1	-	-	-1.1	-1.4	-4.2	-16.1	-	-2	-9.5	-0.7	-6.7	-	-
TQ Dimer	-14.8	-1.8	-10.1	-0.5	-0.5	-1	-2.7	-4.9	-13.3	-3.1	-	-6.9	-	-10.2	-0.7	-2.6

Table 6. The energy amount of hydrogen bind in compounds with water.

Target Atoms: Water	HOH 10	HOH 17	HOH 2023
ID	1	8	35
TQ	-3.53918	-	-
TQ Dimer	-3.5903	-0.66678	-1.33406

Discussion

Breast cancer is the most common invasive cancer in women and the second leading cause of death in women after lung cancer. Metastatic cancers, such as breast cancer, are usually resistant to chemotherapy, so researchers are finding new and effective compounds to treat these cancers. SUMOylation regulates essential cellular processes, many of which are often misregulated in human cancers. As the SUMO pathway is also misregulated in numerous cancers, it has been implicated as a contributing factor in the development and progression of these diseases [14,15]. Researchers have found that expression levels of individual SUMO pathway enzyme can be used as prognostic markers for cancers like prostate and cervical cancer [15,16].

In this study the expression of *SENP1* gene in normal and tumor cell lines was studied and the effect of Thymoquinone on *SENP1* gene expression inhibition was investigated. This gene has been shown alter expression patterns in many cancers. There have been many studies demonstrating the effective role of the *SENP1* gene in tumor promotion in all types of cancers. SENPs are often expressed in a variety of cancers, such as prostate cancer, ovarian and breast cancer, and they reduce the effects of chemotherapy. Therefore, SENPs may be effective targets for new cancer therapies. A new study showed that *SENP1*-shRNA significantly downregulates *SENP1* expression in lymphoma cell lines and induces cell apoptosis. This indicates that *SENP1* is a potential aim of targeted gene therapy [17]. Qiao and partners developed a group of small SENP-based benzodiazepine inhibitors and showed that they effectively inhibited the proliferation of prostate cancer cells [18]. In addition, Uno et al. affected a specific non-peptide *SENP1* antagonist, GN6958, on cervical cancer cell lines [19]. In 2016, Wu and colleagues identified a new *SENP1* inhibitor, Momordin IC. Momordin IC is a natural triterpenoid tropenoid, and it reduces *SENP1* expression and stability [20]. As the studies indicate many of the published data on SENP proteases are based on overexpression of SENP family members. Most of the studies of SENP deactivation was based on the use of siRNA or CRISPR, making it difficult to distinguish between direct and indirect effects of SENP deactivation [15]. Currently reported SENP inhibitors have low activity and specificity for the SENP subgroup [21]. The availability of chemical inhibitors is also a prerequisite for targeting SENPs in human diseases, including cancer, where high expression of SENPs has been observed [15].

Despite significant advances in the production of synthetic drugs, medicinal plants are still considered as one of the important strategies for the treatment of cancer. People usually choose natural antioxidants since they believe that herbal remedies have no significant side effects. Due to the growing concern about the side effects of chemical drugs and the ineffectiveness of a number of them for long-term usage, the use of natural alternatives or complementary remedies has become increasingly accepted [22]. *Nigella sativa* is a flavoring medicinal plant widely used as a supplement and also as a treatment for many disorders. Experimental studies have shown the beneficial and mechanistic effects of thymoquinone action against many diseases. Thymoquinone is an active ingredient and a phytochemical compound found in the canola plant. For the anticancer impacts of thymoquinone, various mechanisms such as effect on free radicals, affect on enzyme activity, inhibition of cell proliferation, antioxidant activity, and induction of apoptosis in cancer cells have been reported [23,24]. Several recent studies have also revealed the impresses of thymoquinone on *MCF7* cell lines in different molecular pathways:

Interpretation of the analysis of the experiments showed that the cytochrome *P450* gene and the UDP glucuronosyl transferase were significantly up-regulated in the estrogen metabolic pathway. The anionic amino acid light chain transporter gene was up-regulated 15-fold in the interferon pathway, which has been reported to be involved in the development of chemotherapy. Interferon-induced protein with tetra-tricyclic peptide repeats, G1P3 protein, interferon regulatory factor 9 (IRF9), (ISGF3, 2'-5'-oligoddenylate synthetase 1, OAS1, and signal transducer and activator of STAT1 transcription genes all after treatment Caspase-10 gene, activated by cysteine peptidase apoptosis, and tyrosine phosphatase and myocyte-factor enhancer protein were up-regulated in MAPK and p38 MAPK pathways, suggesting that thymoquinone is involved in metabolic pathways. Estrogen and interferon target specific genes [25]. In another finding, thymoquinone was used as a radiosensitizer to evaluate its ability to migrate and invade in the irradiated *MCF7* cell lines by evaluating the relevant properties. This study confirms the ability of thymoquinone to repair TGF-β in radiation migration and invasion. Moreover, the results indicated that epithelial markers of E-cadherin and cytokeratin 19 were up-regulated [26]. In another study, the ability of thymoquinone to express p53 tumor suppressor gene and induce apoptosis in MCF-7 breast cancer cell line

was reported. Thymoquinone can increase p53 expression in *MCF7* cell line by regulating time-dependent regulation and induce apoptosis in MCF-7 cells [27]. Another study was undertaken to investigate the combined effect of thymoquinone and tamoxifen on the survival and apoptosis of human breast cancer cell lines. The consequences revealed that within 24 hours of treatment, tamoxifen and all doses of thymoquinone, alone or in combination, significantly reduced the viability of both *MCF7* and MDA-MB-231 cells [28].

Conclusion

The outcomes of this assay represented that the toxicity of Thymoquinone on MCF-7 cell line was higher than those of healthy cells and had a selective effect. Evaluation of alteration and diminution of *SENP1* gene expression by Thymoquinone in breast cancer cell line exhibited that Thymoquinone has inhibitory efficacy on *SENP1* gene. Furthermore according to docking results, the amount of free energy of the bond between Thymoquinone and protein *SENP1* is -54 and with dimer of Thymoquinone is -80. Based on the results of the Docking, it can be concluded that the TQ and its dimer, can interfere with the important amino acids in the enzymatic cavity to prevent the *SENP1* protease. Therefore, the inhibition of *SENP1* gene and protein with Thymoquinone can be considered as a therapeutic target for the treatment of the breast cancer.

Acknowledgments

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