

# Expression and Purification of the Recombinant Cytochrome P450 CYP141 Protein of Mycobacterium Tuberculosis as a Diagnostic Tool and Vaccine Production

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**Background:** Tuberculosis (TB) is regarded as a health problem worldwide, particularly in developing countries. *Mycobacterium tuberculosis* (*M. tuberculosis*) is the cause of this disease. Approximately two billion people worldwide are infected by *M. tuberculosis* and annually about two million individuals die in consequence. Forty million people are estimated to die because of *M. tuberculosis* over the next 25 years if the measures for controlling this infection are not extensively developed. In the vaccination field, *Bacillus Calmette-Guérin* (BCG) is still the most effective vaccine but it shows no efficacy in adult pulmonary patients. One of the other problems regarding TB is its appropriate diagnosis.

**Objectives:** In this experimental study, the recombinant cytochrome P450 CYP141 protein of *M. tuberculosis* was expressed and purified to be used as a vaccine candidate and diagnostic purpose in subsequent investigations.

**Materials and Methods:** The optimization of the cytochrome P450 CYP141 protein expression was evaluated in different conditions. Then, this protein was purified with a resin column of nickel-nitrilotriacetic acid and investigated via Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting.

**Results:** The highest expression of the cytochrome P450 CYP141 protein was obtained by the addition of 1 mM of isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) to the bacterial culture grown to an optical density at 600 nm (OD<sub>600</sub>) of 0.6, 16 hours after induction. This protein was subsequently purified with a purification of higher than 80%. The results of Western Blotting indicated that the purified protein was specifically detected.

**Conclusions:** In this experimental study, for the first time in Iran the expression and purification of this recombinant protein was done successfully. This recombinant protein could be used as a vaccine candidate and diagnostic purpose in subsequent investigations.

**Keywords:** Gene Expression; Cytochrome P450 Enzyme System; Mycobacterium Tuberculosis

## 1. Background

*Mycobacterium tuberculosis* (*M. tuberculosis*), the pathogen of tuberculosis (TB), can co-occur in the infected humans and survive within macrophages and other phagocytic cells. This successful pathogen has an ability to escape from the immune system by several mechanisms (1, 2).

TB is an old disease and is regarded as a health problem all over the world, not least in developing countries. Two billion people worldwide are infected by *M. tuberculosis* and annually about two million individuals die as a result (3).

On the eve of the 21st century, TB turned into one of the most important health issues across the globe. The *Bacil-*

*lus Calmette-Guérin* (BCG) vaccine was discovered in 1921 to prevent TB but unfortunately, the success rate of the BCG vaccine is very variable (0 - 80%). Given the existing difficulties regarding the BCG vaccine such as developing disseminated BCG in patients with human immunodeficiency virus (HIV) and influencing the tuberculin skin test, numerous studies have assessed antigens causing strong cellular immune responses with a view to developing efficacious vaccines against *M. tuberculosis* (4-6).

On the other hand, the conventional diagnosis of active infection with *M. tuberculosis* is made through several protocols such as the microscopic examination of stained smears via the Ziehl-Neelsen method or auramine and

culture. The key aspect in the control of *M. tuberculosis* infection is a rapid and accurate diagnosis based, in most cases, on the staining of smears prepared from clinical samples to investigate the existence of acid-fast *Bacillus*. The mentioned staining technique does not have the necessary specificity, and sometimes an examination of the clinical sample yields a positive culture test, although the results of microscopic examinations are negative (7, 8).

There are currently manifold problems concerning the prevention of TB, first and foremost among which are the aforementioned defects in the BCG vaccine and the existing issues relating to the timely diagnosis of this disease. Accordingly, a considerable number of investigations are presently undertaken to detect *M. tuberculosis* antigens. The recombinant antigens of *M. tuberculosis* could be used for the development of efficacious vaccines and laboratory diagnosis of TB (4).

The cytochrome P450 (CYP) has been known as a big family of hemoproteins, the proteins containing iron. To date, 153 bacterial P450 families have been identified and 500 bacteria have been determined to contain the cytochrome P450 (9, 10). The cytochrome P450 141 (CYP141), which is not present in the BCG vaccine strains, has been recently suggested for the detection of *M. tuberculosis* in clinical samples (11). Because this protein exists in all *M. tuberculosis* strains, it could also be utilized for the formulation of effective anti-TB drugs (12).

In addition, according to preliminary investigations conducted with appropriate software, this protein, in terms of immunogenicity, contains epitopes which are well represented by the different alleles of major histocompatibility complex classes I and II. The cytochrome P450 belongs to the regions of difference 1. Since there are numerous genes in these regions which are not present in the strains of the BCG vaccine and *non-tuberculous mycobacterium*, these regions have been the focus of a large amount of research (13). Of these genes, the CYP141 gene has not been yet expressed and the recombinant protein is yet to be assessed in terms of diagnosis and candidate vaccine.

## 2. Objectives

The present experimental study aimed to express and purify the cytochrome P450 CYP141 protein to pave the way for further research.

## 3. Materials and Methods

A previous study reported the cloning of the cytochrome P450 CYP141 gene (14). After the primary expression of the recombinant cytochrome P450 CYP141 protein in a Terrific Broth Kanamycin (TBK) medium (Merck, Germany) at 37°C, the optimization of the expression and purification of the target protein was done.

The optimization of the target protein expression was evaluated in the pET26b-CYP141 recombinant plasmid with respect to the five quantities below:

- 1) Cell density at induction time (optical density at 600 nm wavelength [OD600] or OD600 of 0.4 - 0.6);
- 2) Post-induction intervals (4, 8, and 16 hours)
- 3) Growth temperature (25 and 37°C)
- 4) Type of medium (the Luria-Bertani Broth Kanamycin [LBBK] and TBK)
- 5) Concentration of isopropylβ-D-1-thiogalactopyranoside (IPTG) (0.5 and 1 mmol)

For an efficient expression of the P450 CYP141 gene, *Escherichia coli* (*E. coli*) BL21 (DE3) was transformed with the pET26b-CYP141 recombinant plasmid. Then, to study the expression of the gene in question, a single colony of *E. coli* BL21 (DE3) containing this plasmid was cultured in LBBK and TBK media and the gene expression was studied by the addition of IPTG at two different concentrations (i.e. 0.5 and 1 mmol) at 22°C and 37°C at different post-induction intervals (4, 8, and 16 hours). The expression of the P450 CYP141 gene was analyzed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with resolving gel (12.5%) and stacking gel (4%).

In the next step, the gene expression was confirmed by Western Blotting and then expression was done in a high volume. For this purpose, a colony of bacteria containing the recombinant plasmid was introduced into a 5 mL TBK medium and the solution was placed in a 37°C shaking incubator (250 rpm) overnight. Next day, 5 cc of the above medium (first-step product) was cultured in a 500 cc TBK medium, and the solution was placed in a 37°C shaking incubator (250 rpm) until an OD600 of 0.4 to 0.6 ( $2 \times 10^8$  cells/mL) was achieved.

After cell density registered the OD of interest, several volumes of the medium as uninduced cells were removed and the rest of medium was induced by IPTG at an efficient concentration. After the incubation of the medium in the optimum time and temperature derived from the previous step, the medium was centrifuged (5000 rpm for 15 minutes at 4°C) and the obtained precipitation was used for the next step (protein purification) (15).

For the lysis and solubilization of the target cells, the cell mass prepared in the previous step was well dissolved in an Erlen containing solution 1 (Tris 1M- ethylenediaminetetraacetic acid [EDTA] 0.5 M-NaCl), a complete protease inhibitor cocktail tablet, and 2.5 cc of lysis buffer (Tris 1M [pH = 8.0] lysozyme) (Roche). The obtained solution was transferred to a falcon tube and sonication was done. The falcon tube was centrifuged at 10000 rpm for one hour at 4°C. The upper solution was thereafter discarded, and the obtained precipitation was dissolved in 25cc solution 2 (Tris 1M-NaCl- imidazole 2M-Urea) (16).

For the concentration of the target protein, the solution prepared in the previous step was poured into a dialysis tube with a cut-off < 10 kDa and then concentrated using sucrose. Before purification, the solution containing the protein of interest was dialyzed against a chromatography buffer (20 mM Tris, 500 mM NaCl, 5 mM imidazole, 8M Urea). Dialysis was conducted for two purposes: removing EDTA inside the solution containing the target

protein which causes damage to the resin and replacing the chromatography buffer with water.

In the next step, the target protein was purified using a column of nickel-nitrilotriacetic acid (Ni-NTA). For this, 10 mL NTA resin was charged with nickel chloride 2%. The charged resin was rinsed using 10 mL sterile deionized water and then balanced by 5 mL chromatography buffer. Subsequently, the solution containing the protein of interest was added to a falcon tube containing agarose resin and placed on the shaker at room temperature for 3 - 4 hours.

After the completion of incubation and numerous washings, the solution was transferred to a purification column (Qiagen) and rinsed with 20 mL chromatography buffer. Afterward, the target protein was removed from the column by the addition of 20 mL elution buffer (chromatography buffer containing 500 mM imidazole).

Within this step, some fractions were obtained and the purity of the fractions extracted from the column was investigated by SDS-PAGE, Coomassie Blue Staining, and Western Blotting. After the investigation of purity, high-purity fractions were collected in a falcon tube and dialyzed against phosphate buffered saline (PBS).

## 4. Results

### 4.1. Gene Expression

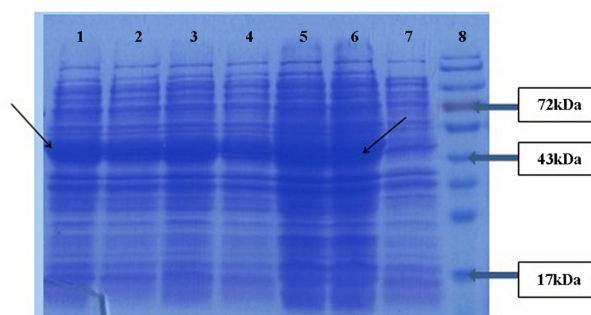
The experiments showed that *E. coli* BL21 (DE3), containing the pET26b-CYP141 recombinant plasmid in the TBK medium at 37°C, obtained the opacity of interest for initiating the induction sooner than the LBBK medium (OD = 0.6 at 600 nm wavelength). Therefore, the TBK medium was used for the investigation of gene expression. The investigations indicated that the incubation at 37°C caused an increase in the gene expression and, hence, all the experiments were done at 37°C for the better growth of the transformed *E. coli* and the more efficient expression of the gene in question.

Gene expression was investigated via the SDS-PAGE method at different intervals of post-induction and at different IPTG concentrations. The findings indicated that a band in the 44-kDa region relevant to the recombinant cytochrome P450 CYP141 protein was obviously represented by different concentrations of IPTG (0.5 and 1 mmol) at different intervals of post-induction (4, 8, and 16 hours). This band was noted for the induced (rather than uninduced) cultures. The gene in question was well expressed at the initial hours after the addition of IPTG into the culture medium, and over time the rate of expression increased. The highest expression was obtained by 1 mmol IPTG 16 hours after the initiation of the induction. Finally, the sample with the highest expression was used for the later steps and protein purification (Figure 1).

The data of the SDS-PAGE analysis were also confirmed by Western Blotting. Western Blotting has been developed based on the detection of hexahistidine (His6) tag in the C-terminal of the target protein and specific binding to the anti-His6-peroxidase antibody.

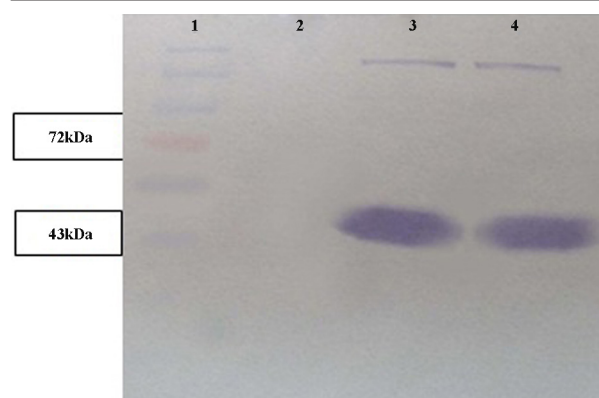
According to the findings of Western Blotting, IPTG-induced *E. coli* BL21 (DE3) containing the pET26b-CYP141 recombinant plasmid exhibited an obvious band in the region of interest, which was confirmed by the data of SDS-PAGE. However, the uninduced culture lacked this band (Figure 2).

**Figure 1.** Expression of the Target Protein at Different Intervals of post-Induction and Different Isopropyl  $\beta$ -D-Thiogalactopyranoside (IPTG) Concentrations



Column 1: Sample induced by 1 mmol IPTG, 4 hours after the initiation of induction; Column 2: Sample induced by 0.5 mmol IPTG, 4 hours after the initiation of induction; Column 3: Sample induced by 1 mmol IPTG, 8 hours after the initiation of induction; Column 4: Sample induced by 0.5 mmol IPTG, 8 hours after the initiation of induction; Column 5: Sample induced by 1 mmol IPTG, 16 hours after the initiation of induction; Column 6: Sample induced by 0.5 mmol IPTG, 16 hours after the initiation of induction; Column 7: Uninduced sample; Column 8: Molecular weight (MW) standard (Fermentas, Cat # SM0671).

**Figure 2.** Western Blotting of the Target Protein at Different Isopropyl  $\beta$ -D-Thiogalactopyranoside (IPTG)



Column 1: Molecular weight (MW) standard (Fermentas, Cat # SM0671); Column 2: Uninduced sample; Column 3: IPTG 1 mmol-induced sample; Column 4: IPTG 0.5 mmol-induced sample.

### 4.2. Purification of Protein

The purification process comprised the binding of the extract containing the recombinant protein of interest to nickel ion-charged nitrilotriacetic (NTA) resin and washing with an elution buffer.

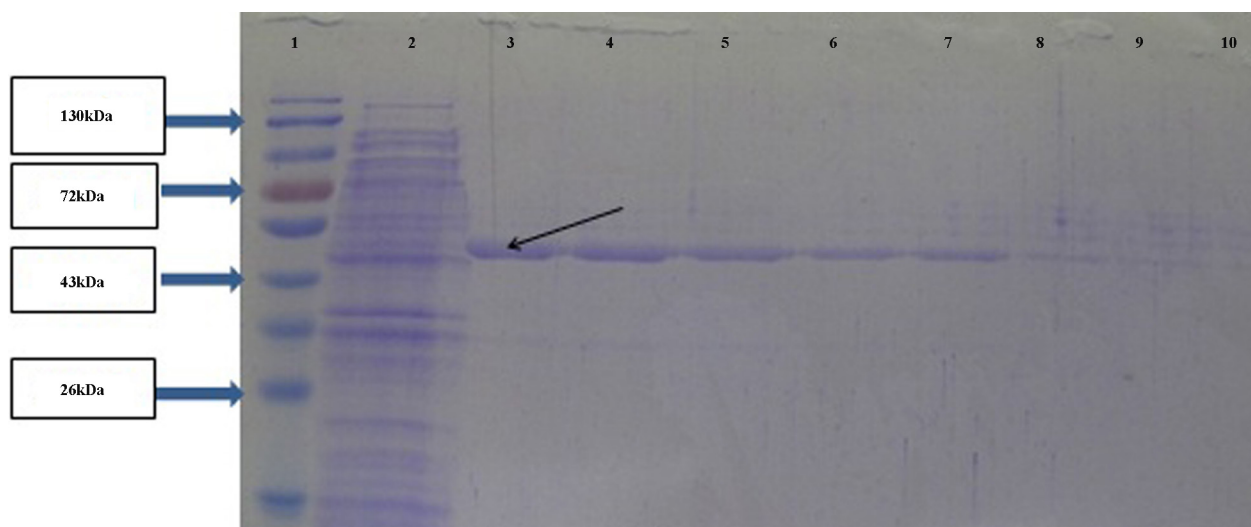
The protein in question bound to the column through the His6 tag, and the other proteins were rinsed with a chromatography buffer at a low imidazole concentration (20 mmol). Finally, the proteins binding to the His6 tag were removed from the column using a washing buffer containing a high imidazole concentration (500 mmol). After this step of chromatography, the purity of the extracted protein was approximately determined to be higher than 80%. The findings of the SDS-PAGE indicated that only one band was noted in the 44-kDa region at the different washing fractions. The size of this band was

consistent with that of the band obtained from the gene expression (Figure 3).

To sterilize the purified protein, a 0.22-micron filter was used and the concentration was approximately 300 µg/mL according to the NanoDrop spectrophotometer.

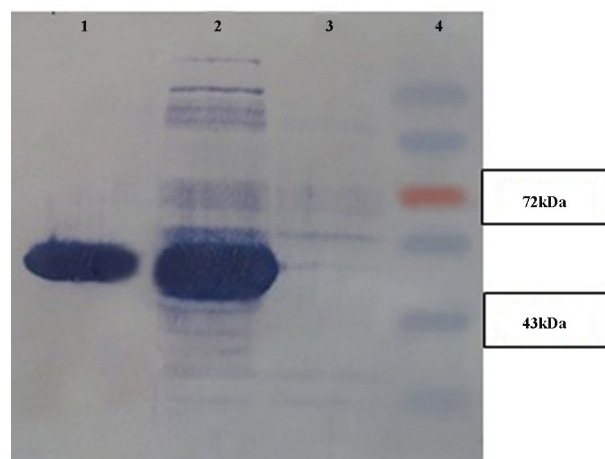
The result of the SDS-PAGE analysis of the purified recombinant protein was also confirmed by Western Blotting so that the uninduced, induced, and purified samples were treated with a specific antibody and the result was in agreement with gene expression (Figures 4 and 5).

**Figure 3.** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of the Target Protein Eluted from the Nickel-Nitrilotriacetic Acid (Ni-NTA) Column



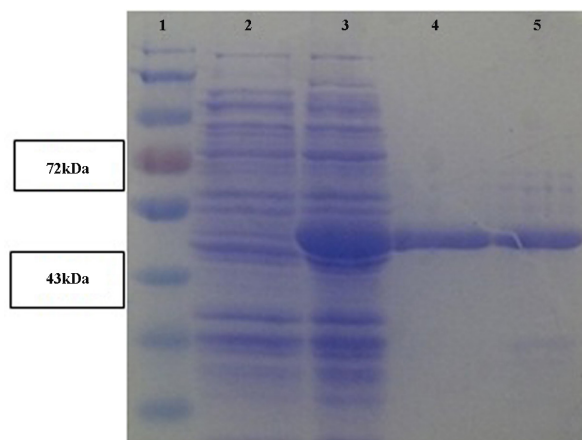
Column 1: Molecular weight (MW) standard (Fermentas, Cat # SM0671); Column 2: Uninduced sample; Columns 3 to 10: Lane 1 to 8, eluted protein fractions obtained from washing by chromatography buffer containing 500 mmol imidazole.

**Figure 4.** Western Blotting of the Target Protein at Different Intervals



Column 1: Purified sample; Column 2: Induced sample; Column 3: Uninduced sample; Column 4: Molecular weight (MW) standard (Fermentas, Cat # SM0671).

**Figure 5.** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of the Target Protein at Different Intervals



Column 1: Molecular weight (MW) standard (Fermentas, Cat # SM0671); Column 2: Uninduced sample; Column 3: Induced sample; Column 4: Purified sample; Column 5: Dialyzed sample.

## 5. Discussion

TB in conjunction with the agent causing it, *M. tuberculosis*, is one of the most complex health problems and, as such, has prompted wide-ranging research into its prevention and treatment (1, 8, 17). In TB control, prevention has priority over correct diagnosis. The immunogenicity of a large number of the cellular components of *Tubercle bacilli* has been studied as a candidate for vaccination against it, and extensive research has been conducted on DNA vaccines, recombinant BCG, and subunit vaccines including recombinant vaccines, among which the production and purification of recombinant proteins is particularly important (1, 18).

For the expression and production of recombinant proteins, the conditions of the experiments should be optimized. In other words, such different parameters as cell density at the time of induction, post-induction interval, growth temperature, concentration of the inducing substance, and the type of medium contribute fundamentally to the optimization of the expression of recombinant proteins (19). For example, for the complete expression of the target protein, induction conditions should be optimized in such a way that the induction can be commenced at the beginning of the exponential growth phase (early log phase) (OD = 0.6 at 600 nm wavelength) (20).

In the present study, the best expression of the recombinant cytochrome P450 CYP141 protein was obtained by the TBK medium: initiation of the induction at 0.6 OD with one mmol IPTG, at a growth temperature of 37°C, 16 hours post induction. The application of these changes in the expression process caused a significant difference in the expression of the target protein.

In the current study, *E. coli* was used as a host to express the gene in question thanks to its numerous advantages such as short proliferation and high capacity for the accumulation of foreign proteins. Also, *E. coli* can be cultured rapidly and easily and its genome is completely known. Relatively easy testing is one of the benefits of the protein expression in *E. coli* insofar as there are various approaches and biotechnological instruments available for its investigation. However, some defects such as a lack of post-translational modification restrict the use of this expression system (21, 22).

In addition, the host's strain contributes importantly to the expression of the gene in question. Inefficient strains for the production of the protease gene products could play a role in expressing fusion proteins through decreasing the effect of the host's proteolytic degree. In this study, *E. coli* BL21 (DE3), as an inefficient and defective strain in protease production, was used for the expression of the target protein (23).

One of the important issues concerning the increase in the recombinant protein expression is the use of a vector with promoters with high activity in the host cell. For example, bacteriophage T7 is a strong and active promoter

in *E. coli* BL21 (DE3). In this study, the pET26b expression vector was used because it contains bacteriophage T7 and the His6-tag sequence at the C-terminal region. In addition to being highly efficient and compatible with the cloned gene, this vector enjoys acceptable conditions of expression and efficiency and could largely facilitate purification since it contains the His6-tag sequence (24).

Generally, the His6-tag sequence has no significant effect on the protein structure and only facilitates the selective binding of the expressed protein to the nickel column (25). The findings of this study support the notion that *E. coli* BL21 (DE3) as a host could be a function of the vector pET26b, which is consistent with the findings of the previous studies indicating the significant effect of *E. coli* BL21 (DE3) and pET26b on the expression of recombinant proteins (26, 27).

A significant challenge in biotechnology is the purification of the recombinant protein with a suitable method whose product is a purified protein with structural integrity and biological activity and without any contamination (28).

For the purification process, immobilized-metal affinity chromatography was used. This method is based on protein isolation on the basis of the reaction of electron donor groups at the protein surface (e.g. histidine in the present study) with electron receptor ones (e.g. Ni<sup>2+</sup>) which have been immobilized within an inert phase (28). Then, the protein in question bound to the nickel ion-charged NTA resin and washing was done using an elution buffer.

Because of the above details, the target protein (i.e. P450 CYP141) was purified with a relatively high homogeneity using Ni-NTA agarose affinity chromatography.

The findings of the SDS-PAGE and Western Blotting analyses of the different fractions obtained from protein purification indicated that the His6 sequence of the target protein effectively and completely bound to the column and each one of the fractions contained highly pure protein. The findings of this study are fully consistent with those of different studies previously conducted (16, 29) on the expression and purification of recombinant proteins in terms of the optimization of the expression and purification of the studied recombinant proteins.

In view of the significance of *M. tuberculosis* infection in Iran and the need for the control of the infection through vaccination with a recombinant vaccine, the production and use of the recombinant cytochrome P450 CYP141 protein is considered one of the most significant objectives in immunization and prevention as well as differentiation of the infection from the vaccine-derived response. In the present study, the recombinant cytochrome P450 CYP141 protein was expressed and purified with high purity and concentration, which should now pave the way for subsequent, complementary studies. Such studies could investigate the immunogenicity rate of this pro-

tein *in vitro* and *in vivo* as well as the immunity responses which could contribute to TB diagnosis.

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## Authors' Contributions

Abolfazl Gholipour: designed the research and collaborated in writing the manuscript. Mohammad Rabiee Faradonbeh: analyzed the data and wrote the paper. Reza Heidari: prepared the manuscript and did the statistical analysis. Davood Darban-Sarokhalil: collected and coordinated the samples. Amirhooshang Alvandi, Narges Abdian, Ehsan Aryan, and Neda Soleimani: provided extensive intellectual contribution.

## Financial Disclosure

The authors are students, faculty members, and academic researchers at Shahrekord, Isfahan, Kermanshah, and Mashhad universities of medical sciences. They do not have any conflict of interest.

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