


The effect of biosynthesized selenium nanoparticles on the expression of *CYP51A* and *HSP90* antifungal resistance genes in *Aspergillus fumigatus* and *Aspergillus flavus*

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Abstract

The application of biological nanoparticles (NPs) can be considered as a way to overcome the problem of antifungal resistance in pathogenic fungi. This study takes a new approach to biosynthesized NPs influence on the expression of *CYP51A* and *HSP90* antifungal resistance genes in *Aspergillus fumigatus* and *A. flavus*, and comparison with antifungal agents. Selenium NPs (Se-NPs) were biosynthesized using *Aspergillus* strains and their production was proved by several methods including, UV-Vis, XRD, FTIR, FESEM, and EDX techniques. The minimum inhibitory concentrations (MICs) of *Aspergillus* strains were determined using the CLSI M38-A2 broth microdilution method. The differences in expression levels of *CYP51A* and *HSP90* genes were examined between untreated and treated of *A. fumigatus* and *A. flavus* using itraconazole and amphotericin B and biosynthesized Se-NPs through real-time PCR. After confirming the results of NPs synthesis, the MIC of itraconazole and amphotericin B against *A. fumigatus* and *A. flavus* was 4 µg/ml. Based on the real-time PCR results, the obtained $\Delta\Delta\text{CTs}$ for these strains were -0.18 , -1.46 , and -1.14 . Whereas the MIC values for treated samples with Se-NPs have decreased to 0.5 µg/ml, and the $\Delta\Delta\text{CTs}$ for these were -0.25 , -1.76 , and -1.68 . The expression of *CYP51A* and *HSP90* genes was significantly down-regulated through the use of Se-NPs against *A. fumigatus* and *A. flavus*.

KEYWORDS

Aspergillus flavus, *Aspergillus fumigatus*, *CYP51A*, *HSP90*, selenium nanoparticles

1 | INTRODUCTION

Aspergillus infections are one of the most common fungal infections that can be fatal in people with defective immune systems.¹ *A. fumigatus* and *A. flavus* are common species obtained from susceptible patients with aspergillosis worldwide.² Unfortunately, the increasing use of antifungal drugs, especially experimentally, has caused drug resistance.³ Azole antifungal drugs such as itraconazole, which are used to treat *Aspergillus* infections, inhibit ergosterol biosynthesis via

the *CYP51A* gene.^{3,4} Mutations in the *CYP51A* gene, which encodes the target enzyme for azole antifungal agents, make *Aspergillus* resistant to them.⁵ On the other hand, polyene antifungal drugs such as amphotericin B can alter *HSP90* gene expression, which enables drug resistance and survival under stressful conditions via multiple molecular mechanisms.⁶ One of the strategies to overcome the antifungal resistance development is the discovery of new putative antifungal targets, which require sophisticated screening technologies.⁷ The green synthesis approach of nanoparticles (NPs) has advantages such

as low cost and energy, availability, safety, biocompatibility, and application that does not have the disadvantages of producing toxic substances.⁸ *Aspergillus* species have good sources of enzymes for specific reactions that lead to the reduction of the desired ions and the formation of biological and inorganic NPs.⁹ These NPs have optimal stability due to their coating with secretory proteins and attach to the cell membrane and enter the cell through the plasma membrane pores.^{10,11} However, with the development of biosynthesize NPs as a therapeutic agent, this issue can be facilitated.^{12–14} For example, NPs in new intelligent drug delivery systems lead to guidance drugs to target tissue, increase effectiveness, decrease adverse drug reactions in other tissues with correction drug distribution in the body, and increase the solubility drugs.^{15,16} Selenium (Se) is one of the elements that its NPs could be effective against bacteria and fungi.^{17,18} Se is often used as a catalyst in oxidation–reduction reactions¹⁹ and has antioxidant, anti-cancer, and antimicrobial effects, and is involved in the growth and development of cells and the balance of the human immune system.^{20,21} Moreover, Se-NPs have been reported to demonstrate high biological activity and low toxicity.^{22,23} In the present study, we synthesized Se-NPs using *Aspergillus* strains and then applied these NPs against themselves. We also evaluated the effect of biosynthesized NPs based on the molecular mechanism on the expression of genes associated with antifungal resistance.

2 | MATERIAL AND METHODS

2.1 | Synthesis of nanoparticles

Se-NPs were synthesized at a concentration of 100 ppm and using the fungal culture medium's supernatant. We obtained three standard strains *A. fumigatus* TIMML-025, *A. flavus* TIMML-050, and *A. flavus* TIMML-379 from the microorganisms' standardization section of Tehran University of Medical Sciences and cultivated on Sabouraud dextrose broth (SDB) media (Merck, Germany) at 35 °C through an incubator shaker for 24–48 h. Then, 100 ml of the culture medium's supernatant was separated through Whatman No. 1 filter paper, and was added to 800 ml of 1 mM Na₂SeO₄ (Sigma Aldrich) and then mixed and stored at room temperature for 48 h away from light preferably. The color of the reaction mixture changed from yellow to orange after treatment with sodium selenate salt. Following the reduction of Se ions completely, the orange color will change to darkens colloidal solution.²⁴

2.2 | Characterization of produced nanoparticles

Biosynthesize of NPs was approved by the ultraviolet–visible (UV–Vis) spectrophotometer, X-ray diffraction (XRD) analysis, Fourier transform infra-red (FTIR) spectrum, field emission scanning electron microscopy (FESEM), and energy-dispersive X-ray (EDX) spectroscopy. The UV–Vis spectrum was recorded by the

Shimadzu (Japan) 2550 UV–visible spectrophotometer over the range of 200–700 nm against the blank. The XRD pattern was carried out by X-ray diffractometer (GNR Co., Model EXPLORER, Italy) at a voltage of 40 kV and a current of 30 mA over a 2θ range of 20–80°. FTIR analysis was performed on a Perkin-Elmer Spectrum-two in the wavelength range of 4000–450 cm⁻¹. A TESCAN (Model MIRA3, Czech Republic) field emission scanning electron microscopy and particle size analysis (PSA) was used to observe the morphological characteristics and size of biosynthesized NPs. The elemental composition of fabricated Se-NPs was determined using EDX attached to the scanning electron microscopy.

2.3 | Antifungal susceptibility testing procedure for NPs and antifungal agents

Antifungal susceptibility testing procedures were done using a modified National Committee for the Clinical and Laboratory Standards Institute (CLSI) according to the M38-A2 guideline.²⁵ The minimum inhibitory concentrations (MICs) ranges were 64–0.625 µg/ml in each 96 wells plate.

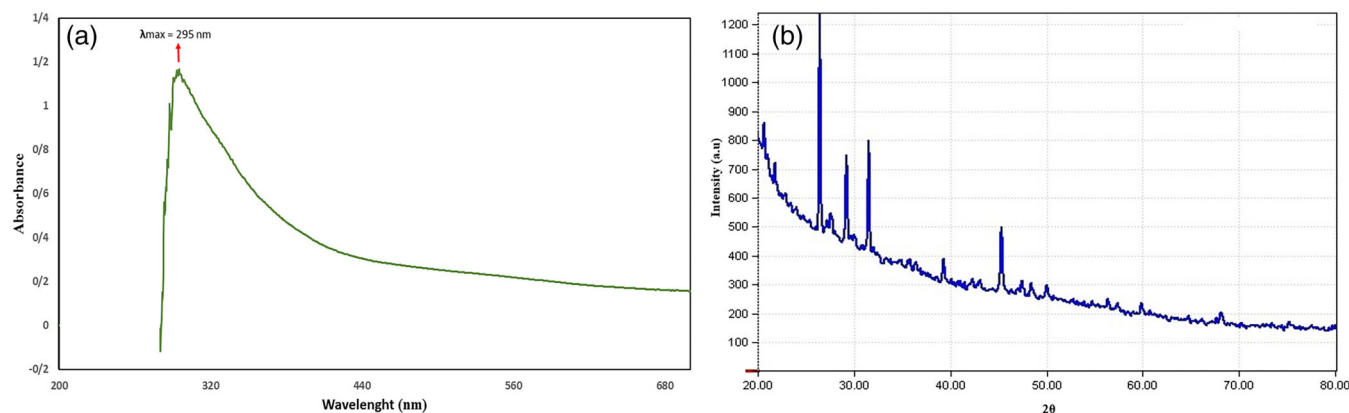
2.4 | Analysis of antifungal resistance gene expression using real-time PCR

The total RNA was extracted from the amphotericin B and itraconazole-resistant *Aspergillus* strains under drug-treated, NP-treated, and untreated conditions. RNA extraction was performed by using the total RNA column isolation kit (Dena zist, Mashhad, Iran). A Nanodrop spectrophotometer (Thermo Scientific) was used at A260/A280 nm to estimate the quality and quantity of extracted RNA. Complementary DNA (cDNA) via reverse transcription was synthesized by extracted RNA template using cDNA kit (Addbio, Korea). The relative quantitative PCR for the target *CYP51A* and *HSP90* genes was performed with the ABI Step One real-time PCR system (Applied Biosystem). Experiments under each condition were performed in duplicate, and each experiment was repeated twice on two different days to assess reproducibility. The primers for selected genes were designed using the Primer-Blast tool (NCBI) for expression study enlisted in Table 1.

The real-time PCR technique was done using SYBER-Green mater-mix with high-rox (Addbio, Korea). Each quantitative PCR reaction was performed in 20 µl total volume using 100 ng of cDNA a template. The thermo-cycling conditions for real-time PCR were comprised of initial step at 95°C for 3 min, and 39 cycles of 95°C for 10 s (denaturation), T_m (52–60)°C for 30 s, 72°C (extension) for 45 s. The housekeeping reference gene was *β-tubulin* which was considered for data analysis and melting curve analysis performed for the replicates for each gene to check the specificity of primers. To calculate the relative gene expression in the samples, the $\Delta\Delta C_T$ and $2^{-\Delta\Delta C_T}$ methods were used.²⁶ Also, the REST (2009) software was employed to

TABLE 1 The primers used for gene expression analysis by the real-time PCR

Gene name/ <i>Aspergillus</i>	Primer's sequence (5'–3')	PCR product size (bp)	GC%	References
CYP51A <i>A. fumigatus</i>	F: 5'-TTGCGTGCAGAGAAAAGTATG R: 5'-GACCTCTTCCGATTGACAT	134	42.86 50	5
TUB1 <i>A. fumigatus</i>	F: 5'-TGACCCAGCAGATGTT R: 5'-GTTGTTGGAATCCACTC	179	50 50	46
HSP90 <i>A. flavus</i>	F: 5'-CGTCAAGTCCATCACTCAGC R: 5'-GCTTGTGGATGCGCTCGGC	98	55 68.42	49
β -tubulin <i>A. flavus</i>	F: 5'-GGAATGGATCTGACGGCAAG R: 5'-GGTCAGGAGTTGCAAAGCG	122	55 57.89	49

**FIGURE 1** UV-Vis spectra of biosynthesized Se-NPs by the *Aspergillus* fungus (a); XRD spectrum of biosynthesized Se-NPs by the *Aspergillus* fungus (b)

represent the relative gene expression between the treated and untreated (control) samples.²⁷

3 | RESULTS

3.1 | Results of characterization of biosynthesized Se-NPs

3.1.1 | UV-Vis and XRD spectrum

Se-NPs were produced by observing the color change of the reaction mixture from yellow to dark orange, which is the first sign of NP formation. We used the UV-vis spectrophotometer to ensure the reduction of Se ions as the most important technique for the recognition and characterization of NPs.²⁸ The UV-vis analysis of Se-NPs (Figure 1(a)) indicated a strong absorption peak between 285 and 300 nm with maximum absorbance (λ_{max}) at 295 nm after 24 h of incubation at room temperature with an accuracy of 1 nm in the wavelength range from 200 to 700 nm.²⁹ The XRD method was applied to determine the nature of the synthesized Se-NPs in fungal culture supernatants. Figure 1(b) displays the XRD pattern of obtained Se-NPs in the microbial supernatant powder. The presence of the Bragg peaks of the elemental Se at 2θ angles shows that the reaction product was a crystalline phase.³⁰

3.1.2 | FTIR analysis

The fabricated NPs were further characterized to determine NPs interaction with fungal proteins using FTIR analysis and these proteins' roles as stabilizing agents surrounding NPs. In Figure 2(a), the graph obtained after Se-NPs biosynthesis showed index peaks in the ranges of 835.17, 1040.6, 1406.5, 1612, and 3390.1 cm^{-1} . We also measured the FTIR absorption spectra of fungal supernatant before the synthesis of NPs and put them both together and compared them (Figure 2(b)). The spectrum of Se-NPs bears similar bands corresponding to the supernatant and confirms that the fungal supernatant components are present on the surface of NPs. It can be concluded that the proteins might have formed a coating over the nanoselenium, which in turn supports their stabilization.³¹

3.1.3 | FESEM/EDX/PSA

To study the morphology and size of the synthesized Se-NPs, field emission scanning electron microscopy (FESEM) and particle size analysis (PSA) were used (Figure 3(a-c)). FESEM images (Figure 3(a,b)) distinguished the presence of NPs with almost spherical shapes and sizes of 37–46 nm. Moreover, according to the existing EDX diagram, the presence of the element Se in the current structure was also proved (Figure 3(d)).

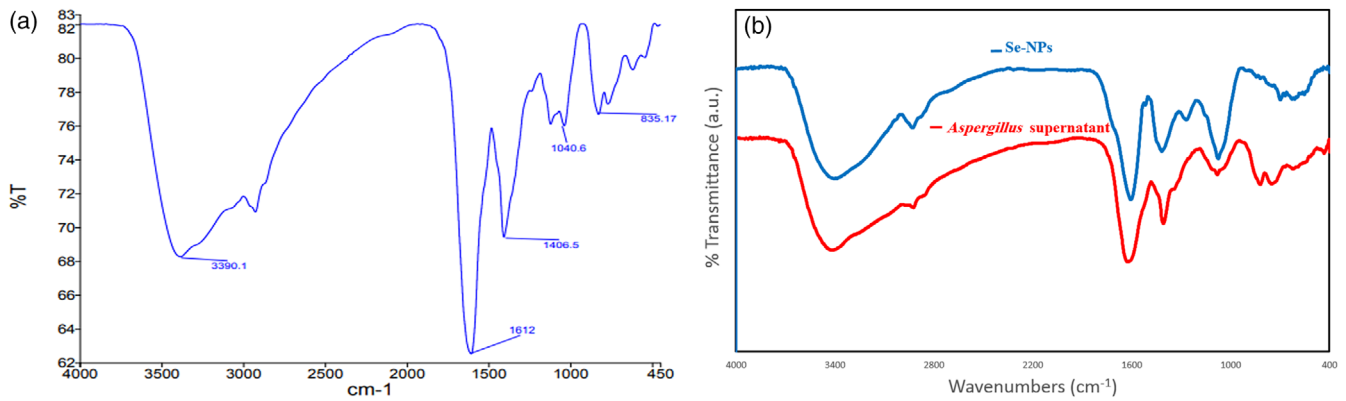


FIGURE 2 FTIR spectra from biosynthesized Se-NPs (a); FTIR absorption spectra of fungal supernatant before and after bioreduction of sodium selenate (b)

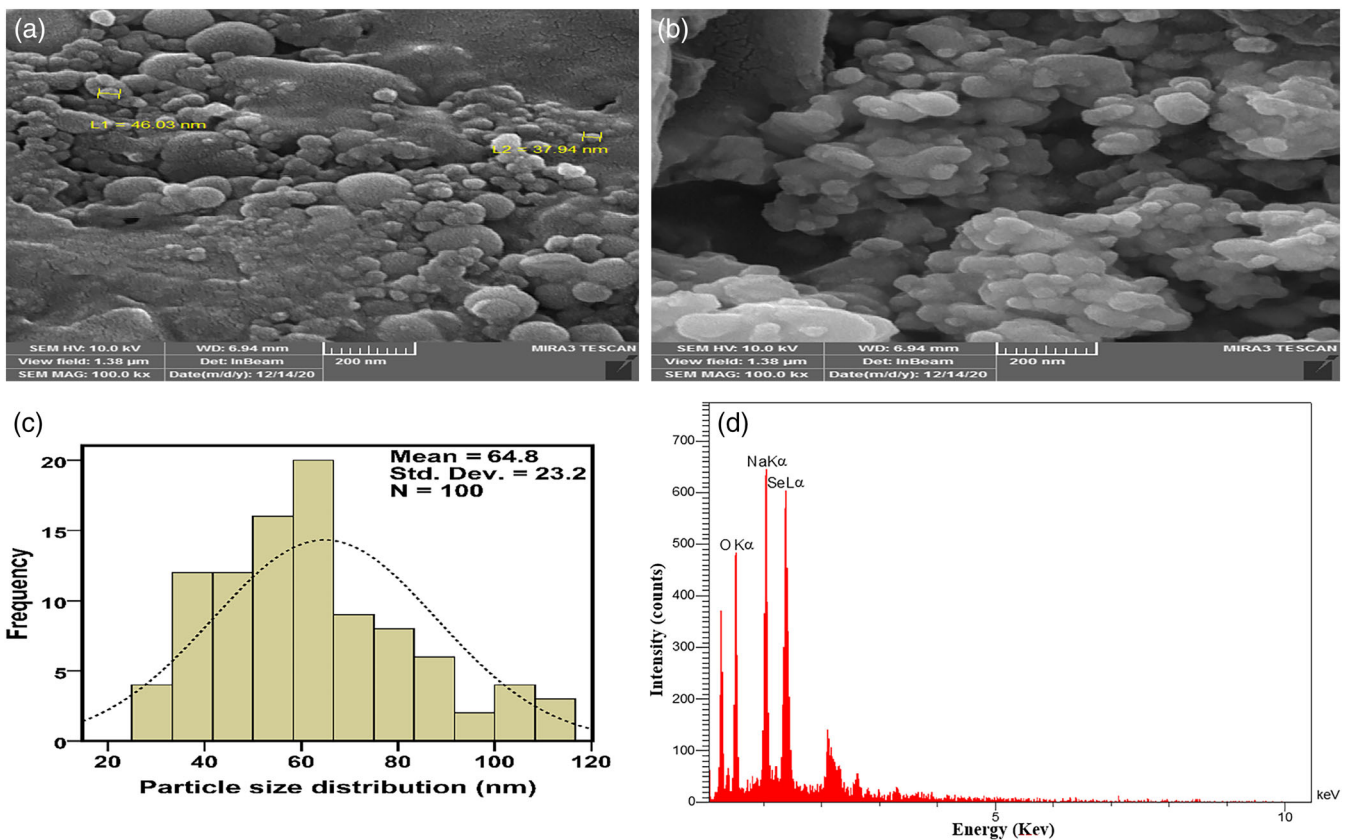


FIGURE 3 FESEM images (a, b), PSA (c), and EDX (d) of biosynthesized Se-NPs

3.2 | Results of MICs and RT-PCR

The MIC of three standard strains (antifungal-resistant) *A. fumigatus* TIMML-025, *A. flavus* TIMML-050, and *A. flavus* TIMML-379 was obtained for itraconazole (ITC) or amphotericin B (AMB) at the concentration 4 $\mu\text{g}/\text{ml}$, while the MIC of NPs was lower in all strains with 0.5 $\mu\text{g}/\text{ml}$. Simultaneously, biosynthesized Se-NPs showed a more acceptable MIC that is presented in Table 2. The inhibitory effect of Se-NPs on the expression of antifungal resistance genes, *CYP51* in

A. fumigatus and *HSP90* in *A. flavus*, was investigated using the real-time PCR method too. Based on the obtained results, the $\Delta\Delta\text{CTs}$ calculated for the three samples were -0.18 , -1.46 , and -1.14 . The $\Delta\Delta\text{CTs}$ for treated samples with biosynthesized Se-NPs were -0.25 , -1.76 , and -1.68 , which showed a low expression of *CYP51A* and *HSP90* genes after treatment with NPs (Table 3). It means that the expression of *CYP51A* and *HSP90* as target genes was down-regulated. Decreased relative expression of genes compared to different drug-treated, NPs -treated, and untreated

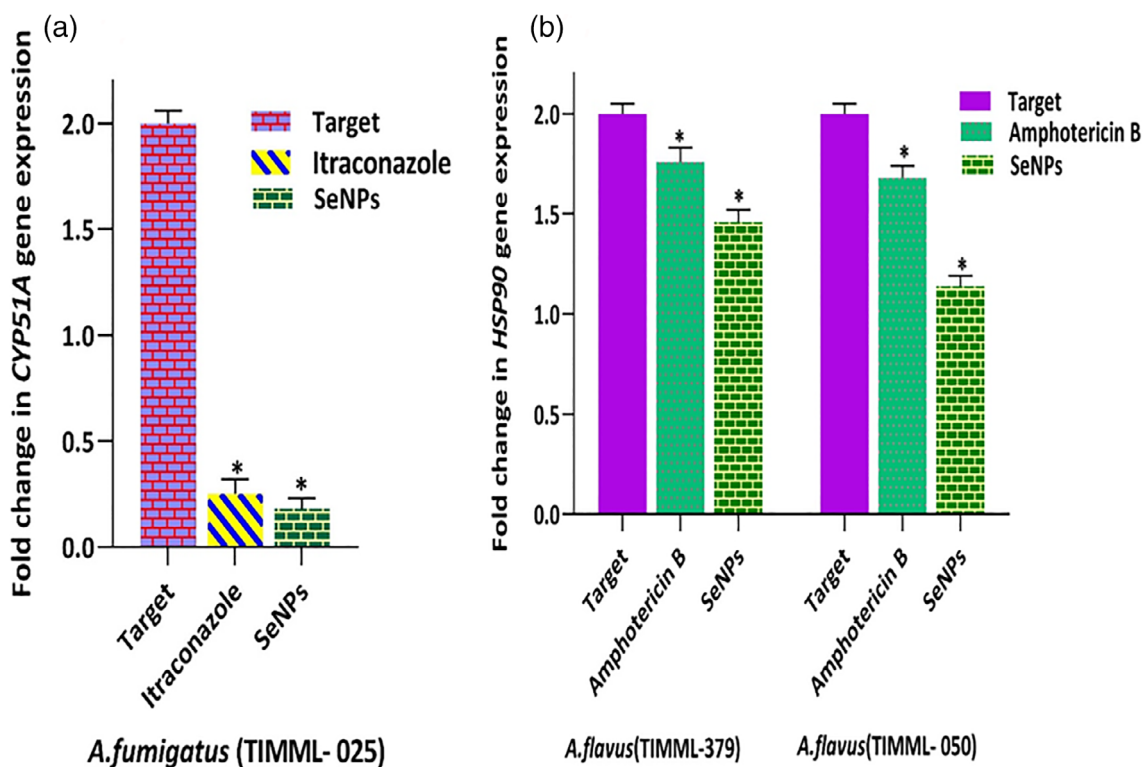
TABLE 2 The MIC results of ITC, AMB, and Se-NPs on the growth of *Aspergillus* strains

Name	1	2	3	4	5	6	7	8	9	10	11	12
<i>Aspergillus</i> strains/antifungal agent	64 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml	2 µg/ml	1 µg/ml	0.5 µg/ml	0.125 µg/ml	0.625 µg/ml	PC	NC
<i>A. fumigatus</i> : TIMML-025 Itraconazole	S	S	S	S	S	R	R	R	R	R	+	-
<i>A. fumigatus</i> : TIMML-025 Se-NPs	S	S	S	S	S	S	S	S	R	R	+	-
<i>A. flavus</i> : TIMML-379 Amphotericin B	S	S	S	S	S	R	R	R	R	R	+	-
<i>A. flavus</i> : TIMML-379 Se-NPs	S	S	S	S	S	S	S	R	R	R	+	-
<i>A. flavus</i> : TIMML-050 Amphotericin B	S	S	S	S	S	R	R	R	R	R	+	-
<i>A. flavus</i> : TIMML-050 Se-NPs	S	S	S	S	S	S	S	S	R	R	+	-

Abbreviations: NC, negative control; PC, positive control; R, resistance; S, sensitive.

TABLE 3 The results of expression of target genes (*CYP51A* and *HSP90*) and reference gene (β -tubulin) including CTs, Δ CTs, and $\Delta\Delta$ CTs

	MIC (mg/ml)	Gene (target)	CT (control)	CT (sample)	Δ CT (target)	Gene (reference)	CT (control)	CT (sample)	Δ CT (reference)	$\Delta\Delta$ CT (reference)
<i>Aspergillus fumigatus</i> TIMML-025	-	<i>CYP51A</i>	26.38	-	-	<i>TUB1</i>	21.86	-	-	-
<i>A. fumigatus</i> TIMML-025 Itraconazole	4 μ g/ml	<i>CYP51A</i>	-	28.41	-2.03	<i>TUB1</i>	-	23.71	-1.85	-0.18
<i>A. fumigatus</i> TIMML-025 Se-NPs	0.5 μ g/ml	<i>CYP51A</i>	-	31.85	-5.47	<i>TUB1</i>	-	27.08	-5.22	-0.25
<i>A. flavus</i> TIMML-379	-	<i>HSP90</i>	22.45	-	-	β -tubulin	"	-	-	-
<i>A. flavus</i> TIMML-379 Amphotericin B	4 μ g/ml	<i>HSP90</i>	-	26.27	-3.82	β -tubulin	-	25.34	-2.36	-1.46
<i>A. flavus</i> TIMML-379 Se-NPs	1 μ g/ml	<i>HSP90</i>	-	28.11	-5.66	β -tubulin	-	26.88	-3.90	-1.76
<i>A. flavus</i> TIMML-050	-	<i>HSP90</i>	22.93	-	-	β -tubulin	22.89	-	-	-
<i>A. flavus</i> TIMML-050 Amphotericin B	4 μ g/ml	<i>HSP90</i>	-	26.36	-3.43	β -tubulin	-	25.12	-2.29	-1.14
<i>A. flavus</i> TIMML-050 Se-NPs	0.5 μ g/ml	<i>HSP90</i>	-	28.52	-5.59	β -tubulin	-	26.80	-3.91	-1.68

**FIGURE 4** Decreased relative expression of *CYP51A* (a) and *HSP90* (b) antifungal resistance genes (* $p < 0.05$)

conditions are shown in Figure 4 using GraphPad Prism 8.0 software (* $p < 0.05$).

In Figure 5(a,b) the melt curve indicated that the efficiency and single discrete species of amplified double-stranded DNA products.

We also electrophoresed the PCR products on 2% agarose gel to confirm the presence of *CYP51A* and *HSP90* genes. The bands were observed in the range of 134 and 98 bp that was to be expected (Figure 6).

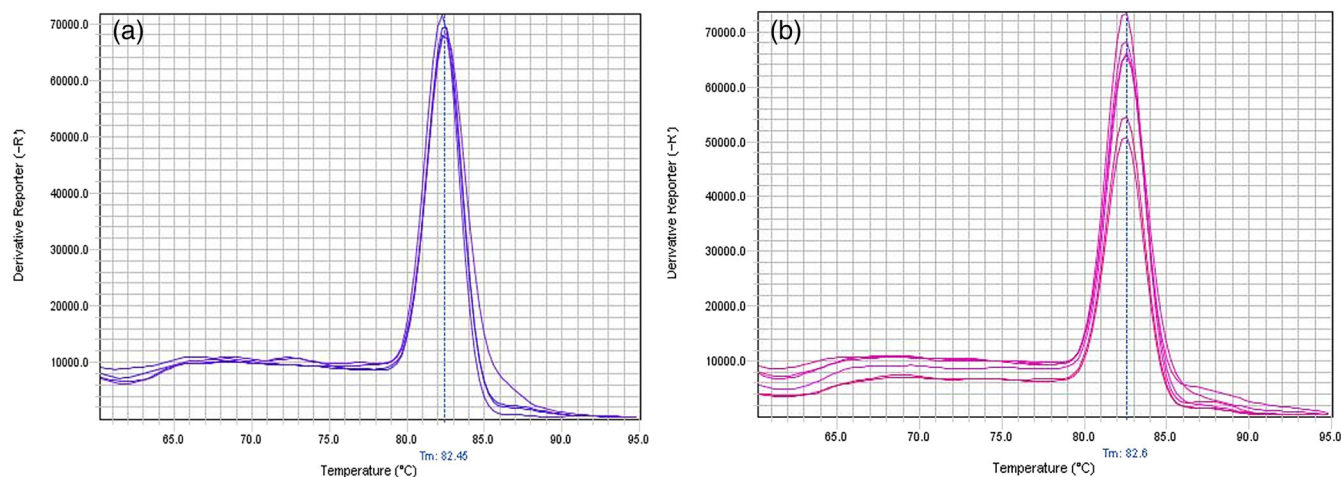


FIGURE 5 The melt curve of the *CYP51A* (a) and *HSP90* (b) genes

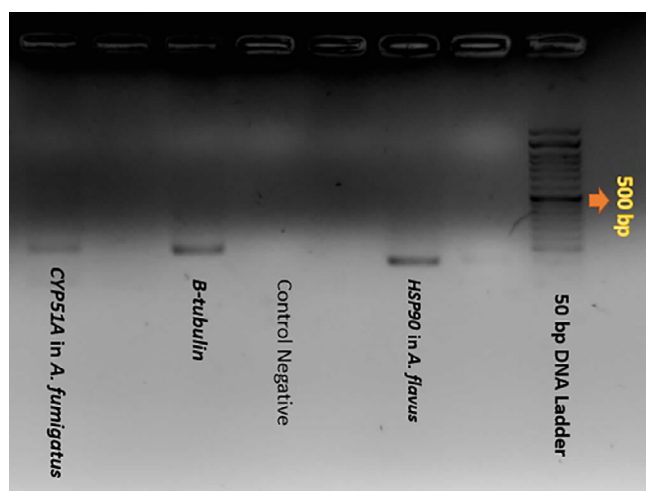


FIGURE 6 Agarose gel electrophoresis of PCR products to confirm the presence of *CYP51A* and *HSP90* genes

4 | DISCUSSION

Due to the increasing use of antifungal agents for the treatment of *Aspergillus* infections, antifungal resistance is rapidly growing in most *Aspergillus* species.^{32,33} Therefore, it is necessary to pay more attention to the factors other than antifungal drugs, which are discussed regarding the use of NPs and their applications as auxiliary agents to reduce or eliminate this problem.^{34,35} Meanwhile, the synthesis of biological NPs using plant extracts and microorganisms such as bacteria, parasites, and fungi is valuable, which is a safe and biocompatible method.³⁶ Studies by Feng et al. have also shown that the production of bioparticles and their application instead of chemical methods is a faster and cheaper method than chemical procedures, and has less risk to humans and the environment.³⁷ NPs can play a role as a growth inhibitor in pathogenic *Aspergillus*. This ability is achieved through the more effortless transfer of biosynthesized NPs from the cell membrane

pores and their entry into the intracellular space and the mechanism of inactivation of microorganisms' drug resistance genes and reducing the gene expression in the genome transcription stage.^{38,39} These NPs may even interfere with cell translation and protein synthesis, eliminating harmful, inefficient, and toxic cell products.⁴⁰ In the current study, the Se-NPs have been biosynthesized biologically using *Aspergillus* species to evaluate and compare their effect on growth and gene expression. Shoeibi et al., biosynthesized Se-NPs by green chemistry method using fungal agents.⁴¹ Also, Citrarasu et al., biosynthesized these NPs in the same conditions mentioned and observed discoloration of the colloidal solution, from yellow to orange, as the first sign, and by several methods approved the biosynthesis of NPs formation.⁴² Furthermore, Vahdati et al. revealed that The amount of MIC for NPs treated on *Staphylococcus aureus* and *Escherichia coli* has decreased compared to conventional antibiotics, which in this concept is consistent with our present study.⁴³ Mahmoudi et al. also showed in their recent study that Se-NPs inhibit the growth of fungi better than conventional antifungal agents even at a lower concentration.⁴⁴ In a similar study, Özmerdiven et al. determined azole resistance in *A. fumigatus* carrying the TR34/L98H Mutations. The susceptibility testing showed that the MIC values of these isolates were shifted upwards.⁴⁵

Our finding demonstrated that the Se-NPs could reduce the expression of *CYP51A* and *HSP90* genes associated with azole and polyene antifungal drugs. Mellado et al. investigated the expression of the *CYP51A* gene in 14 clinical isolates of *A. fumigatus* that exhibited a pattern of reduced susceptibility to azole antifungal drugs.⁴⁶ Their quantitative expression analysis using real-time PCR showed up an eightfold increase in the level of expression of the *CYP51A* gene compared to that by the susceptible strain. Chowdhary et al. explored the azole antifungal drug resistance in *A. fumigatus* with special reference to mutations in the *CYP51A* gene and its expression.⁴⁷ In another study by Shishodia et al., the expression of the *HSP90* gene in *A. flavus* was evaluated along with susceptibility testing in treated conditions with the relevant agent.⁴⁸ Also, Thakur et al. analyzed the differential expression pattern of heat shock protein genes in toxigenic and

atoxicogenic isolates of *A. flavus*.⁴⁹ Similarly, they measured the mRNA of heat shock proteins (*hsp60*, *hsp70*, and *hsp90*) using relative quantitative real-time PCR at different conditions. The importance of these studies will be more apparent when observing no evidence of side effects of biosynthesized NPs in humans. Ziaei et al., in their research on the influence of NPs on biological and ecological systems, showed that there is currently no evidence of the adverse effects of used NPs in the products on humans.⁵⁰ With the increase of drug-resistant fungal species to chemical antifungals, alternative ways are required to apply an effective treatment to avoid any economic and health costs too. Wang et al. expressed that due to the predicted drug resistance to antibiotics in the future, treatment of infections would be complicated and NPs could be a viable alternative to them.⁵¹

5 | CONCLUSION

The results of this study showed that the expression of *CYP51A* and *HSP90* genes could be significantly down-regulated through the use of Se-NPs against *A. fumigatus* and *A. flavus*.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS CONTRIBUTION

Conceptualization: Mahdi Hosseini Bafghi, Razieh Nazari, and Hossein Zarrinfar; Data curation: Mahdi Hosseini Bafghi, Razieh Nazari, Majid Darroudi, and Hossein Zarrinfar; Formal analysis: Mahdi Hosseini Bafghi, Razieh Nazari, Majid Darroudi, and Hossein Zarrinfar; Investigation: Mahdi Hosseini Bafghi, Razieh Nazari, and Hossein Zarrinfar; Methodology: Mahdi Hosseini Bafghi, Majid Darroudi, and Hossein Zarrinfar; Project administration: Mahdi Hosseini Bafghi and Hossein Zarrinfar; Resources: Mahdi Hosseini Bafghi, Majid Darroudi, and Hossein Zarrinfar; Software: Razieh Nazari, Mohsen Zargar, and Hossein Zarrinfar; Supervision: Razieh Nazari, Majid Darroudi, Mohsen Zargar, and Hossein Zarrinfar; Validation: Mahdi Hosseini Bafghi and Hossein Zarrinfar; Visualization: Mahdi Hosseini Bafghi, Majid Darroudi, and Hossein Zarrinfar; Writing – original draft: Mahdi Hosseini Bafghi; Writing – review and editing: Razieh Nazari, Majid Darroudi, Mohsen Zargar, and Hossein Zarrinfar. All authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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