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Green synthesis of silver nanoparticles using *Zingiber officinale* and *Thymus vulgaris* extracts: characterisation, cell cytotoxicity, and its antifungal activity against *Candida albicans* in comparison to fluconazole

Mohsen Mohammadi[1], Sabrieh Assadi Shahisaraee[2], Atiyeh Tavajjohi[3], Negin Pournoori[4], Samad Muhammadnejad[5], Shahla Roodbar Mohammadi[2], Reza Poursalehi[1], Hamid Delavari H[1] ✉

[1]*Department of Materials Engineering, Tarbiat Modares University, P.O. Box 14115-143, Tehran, Iran*
[2]*Department of Medical Mycology, Tarbiat Modares University, Tehran, Iran*
[3]*Department of Biology, Islamic Azad University of Shahr Rey, Tehran, Iran*
[4]*Department of Medical Physics and Biomedical Engineering, Tehran University of Medical Sciences, Tehran, Iran*
[5]*Cell-Based Therapies Research Center, Digestive Disease Research Institute, Tehran University of Medical Sciences, Tehran, Iran*
✉ *E-mail: hamid.delavari@modares.ac.ir*

Abstract: Fluconazole (FLZ) application as a highly successful commercial antifungal azole agent to treat the fungal infections is limited due to emergence of FLZ-resistant candida. In this study, the potential of green synthesised silver nanoparticles (NPs) as an antifungal agent against *Candida albicans* fungal pathogen is investigated. The extract of ginger (*Zingiber officinale*) and thyme (*Thymus vulgaris*) plays as reducing agent, capping agent and antifungal agent. The UV–visible spectroscopy shows the peak of surface plasmon resonance of synthesised Ag NPs after a period of time. The synthesised Ag NPs are spherical, with average sizes of 12 and 18 nm based on ginger and thyme extract, respectively. Fourier transform infrared spectroscopy confirms the adsorption of the plant extract on the surface of the as-prepared Ag NPs. Based on the minimum inhibitory concentration (MIC) method against Candida albicans, the antifungal activity of as-prepared green synthesised Ag NPs shows higher inhibitory in comparison to FLZ. Finally, the Ag NPs synthesised via thyme extract shows no cytotoxicity with concentration below 3.5 ppm, which can be considered as an appropriate candidate instead of FLZ to treat the superficial fungal infections.

1 Introduction

Superficial fungal infections (SFIs) are increasing worldwide due to direct contact by cross-border travel. SFIs are generally caused by various fungal organisms such as *Candida albicans*, which is the most common fungal pathogen for most of the fungal infections in humans [1–3]. Topical and oral (systemic) therapies are used for the treatment of SFIs [4, 5]. Topical treatment offers several advantages such as avoiding extensive drug absorption, decreasing prolonged systemic exposure, and reducing drug interactions [6]. Nevertheless, oral therapy achieves comparable higher cure rates than do topical treatment [7]. The most commonly used antifungal agents to treat SFIs are azole agents. There are several effective azole agents in different types of formulation [7]. One of the highly successful antifungal azole agents to treat the superficial and invasive fungal infections of *C. albicans* is fluconazole (FLZ). However, FLZ application is limited due to emergence of FLZ-resistant candida by the wide spread use of FLZ [8].

By developing nanotechnology, several nanostructures have been introduced as antifungal agents [9–11]. Among these nanomaterials, silver (Ag) nanoparticles (NPs) have been studied extensively more than others as antibacterial and antifungal agents [12–17]. Although there are a variety of chemical methods [18–21] in the preparation of Ag NPs, the 'green synthesis' has attracted a lot of interest due to avoiding hazardous waste, synthesising high purified product, economic benefits, and low-energy consumption [22].

The extract of different parts of various plants such as root, leaf, stem, and fruit can be used in the preparation of Ag NPs as green synthesis method [22–26]. It has been clarified that the biomolecules extracted from plants are responsible for metal ion reduction, forming the shape of synthesised NPs [27, 28], and colloidal stability by making capping [29–31] on the particles' surface during the growth processes. Therefore, the antifungal and antibacterial activities of green synthesised Ag NPs can be improved due to the intrinsic antifungal and antibacterial properties of plant extract. For this purpose, the green synthesis of Ag NPs by thyme (*Thymus vulgaris*) and ginger (*Zingiber officinale*) extracts and their antifungal properties against *C. albicans* versus FLZ antifungal agent have been investigated. It should be noted that thyme and ginger extracts demonstrated anti-inflammatory, antioxidant [32–35], and antimicrobial [36, 37] properties.

To the best of our knowledge, thyme and ginger extracts have not been used in the synthesis of Ag NPs. The main purpose of this work is the green synthesis of Ag NPs based on the thyme and ginger extracts and investigate its antifungal properties against Candida albicans in comparison to commercial antifungal agent (FLZ). Eventually, cytotoxicity of the as-synthesised Ag NPs via ginger and thyme on a human dermal fibroblast cell line (HDF-1) was assessed. Our results show that the as-prepared Ag NPs can be used instead of FLZ to treat SFIs with much lower concentrations.

2 Materials and methods

2.1 Extract preparation

To prepare the plant extract, in the initial stage, ginger rhizome and thyme's leaves were cleaned and washed with deionised (DI) water. After that, the plants were sliced into fine pieces and dried in the dark for 4 days. A total of 0.2 g of the prepared pieces of each plant were added to 100 mL of DI water and stirred at 80 °C for 40 min. For further purification, the extract solution was filtered through Whatmann No.1 filter paper and centrifuged at 4000 rpm for 5 min. It should be noted that the obtained extract was filtered by a 0.22

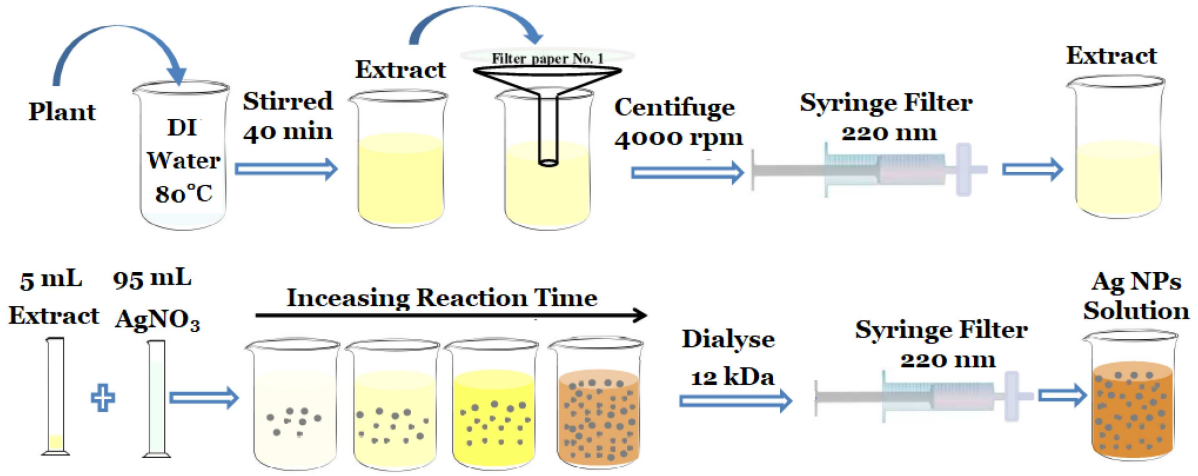

Fig. 1 *Schematic of the extract preparation and Ag NPs synthesis via plant extract (best viewed online in colour)*

μm syringe filter and kept at 4 °C in a dark place for the green synthesis of Ag NPs.

2.2 Green synthesis of Ag NPs

The 1 mL of the prepared extract was added to 20 mL of 2 mM aqueous solution of $AgNO_3$ (0.0068 g $AgNO_3$ into 20 mL of DI water). Silver nitrate was purchased from Merck Company by 99.9% purity). In the beginning, the colour of the solution is light yellow and changes to dark brown by progress of reaction which confirms the formation of Ag NPs. This colour alteration could be attributed to the formation of Ag NPs. To characterise the prepared particle and investigate the antifungal activity, the final solution was dialysed against water for 24 h by 12 kDa dialysis bag and finally filtered by a 0.22 μm syringe filter. The schematic of the extract preparation steps and Ag NPs synthesis are shown in Fig. 1.

2.3 Characterisation of Ag NPs

To detect and confirm the formation of Ag NPs during the synthesis of Ag NPs via ginger and thyme extracts, the UV–visible spectroscopy has been performed at room temperature using a SPUV-26 SC-Tech spectrophotometer. Fourier transform infrared spectroscopy (FTIR) was used to confirm the capping of the extract on the surface of Ag NPs by a Frontier PerkinElmer spectrometer. Transmission electron microscopy (TEM) was used to find the morphology of the as-synthesised Ag NPs via a Leo 912 AB microscope.

2.4 Antifungal study

The antifungal activity was examined by considering the minimum inhibitory concentration (MIC) according to broth micro-dilution method based on Clinical and Laboratory Standards Institute M27-A3 guidelines [38]. MIC is the lowest concentration of antifungal agent that prevents the visible growth of particular microorganisms.

A standard strain of *C. albicans* fungi (ATCC10231obtained from Pasteur Institute of Iran) was grown on Sabouraud Dextrose Agar growth medium containing chloramphenicol and kept in an incubator at 35°C. A total of 100 μL of Ag NPs solution (100 ppm) was added into a 100 μL sterile Roswell Park Memorial Institute (RPMI) medium and then subjected to twofold serial dilution with RPMI medium in a 96-well plate. Then, 100 μL of *C. albicans* culture in phosphate-buffered saline (1×10^6 cell/mL) was introduced into each well containing compound solution. Two wells were considered as positive and negative controls. The positive control well includes 100 μL of the growth medium and 100 μL of fungal suspension, and the negative control well contains 200 μL of the growth medium. After overnight incubation at 28°C, optical density measurements were conducted using a micro-plate spectrophotometer (Stat Fax 4300, Awareness Company, Elisa Plate Reader). It should be noted that all experiments were conducted in triplicates.

2.5 Cytotoxicity of the as-synthesised Ag NPs on a human dermal fibroblast cell line

To evaluate the cytotoxicity of Ag NPs, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay was carried out according to the previously described protocol [39]. A HDF-1 was purchased from Iranian Biological Resource Center (Tehran, Iran). In brief, HDF-1 cells were transferred to a 96-well microtitration plate in 200 μl of low glucose Dulbecco's modified Eagle's medium containing 10% foetal bovine serum. The seeding density was 2500 cells per well. After 3 days, the cells entered the logarithmic phase of growth and exposed to green synthesised Ag NPs with various concentrations (0.5, 1.25, 2.5, 5, and 10 ppm). This procedure was repeated for three times to achieve dependable results. Afterward, the Ag NPs were removed from the wells after 72 h. In order to display the regenerative capacity of the exposed cells that survived, 4 days were considered for the recovery period. During this step, fresh medium was harnessed to feed the plates daily. After that, at the end of the recovery period, 50 μl of MTT solution (5 mg/ml) was added to each well.

Then, the plates were further incubated for 4 h. Next, all remained supernatant were effaced. Thereupon, to dissolve the shaped unsolvable formazan crystals, 200 μl of dimethyl sulfoxide (DMSO) was added. To regulate the final pH, 25 μl of glycine buffer was poured to each well. Afterward, by a microtitration plate reader (BioTek®, USA) absorbance was instantly recorded at 570 nm. The utter values of the absorbance changed to surviving fraction data as the percentage of living cells of the control.

3 Results and discussion

3.1 Green synthesis of Ag NPs

The colour changes of synthesis solution are demonstrated in Fig. 2 in which the colour of mixture of $AgNO_3$ and extract solution is changed from colourless to reddish brown and there is no more change in the colour after the reaction goes to completion. To confirm the formation of Ag NPs, UV–visible spectra are recorded against water. The UV–visible spectra related to the synthesis of Ag NPs via ginger and thyme extracts are shown in Figs. 2*a* and *b*. The appeared peak in the range of 400–460 nm is related to the surface plasmon resonance (SPR) of the formed Ag NPs [40]. It can be observed that the SPR intensity increases by increasing the reaction time which confirms the synthesis of more Ag NPs. Indeed, Ag clusters are formed continuously by increasing the reaction. In addition, it can be observed that the rate of reaction for thyme extract is higher than that for ginger. The initial SPR band of Ag NPs appeared after 30 and 60 min in the case of using thyme and ginger as reducing agents of Ag NPs, respectively. Moreover, for the same reaction times, the intensity of SPR for thyme is

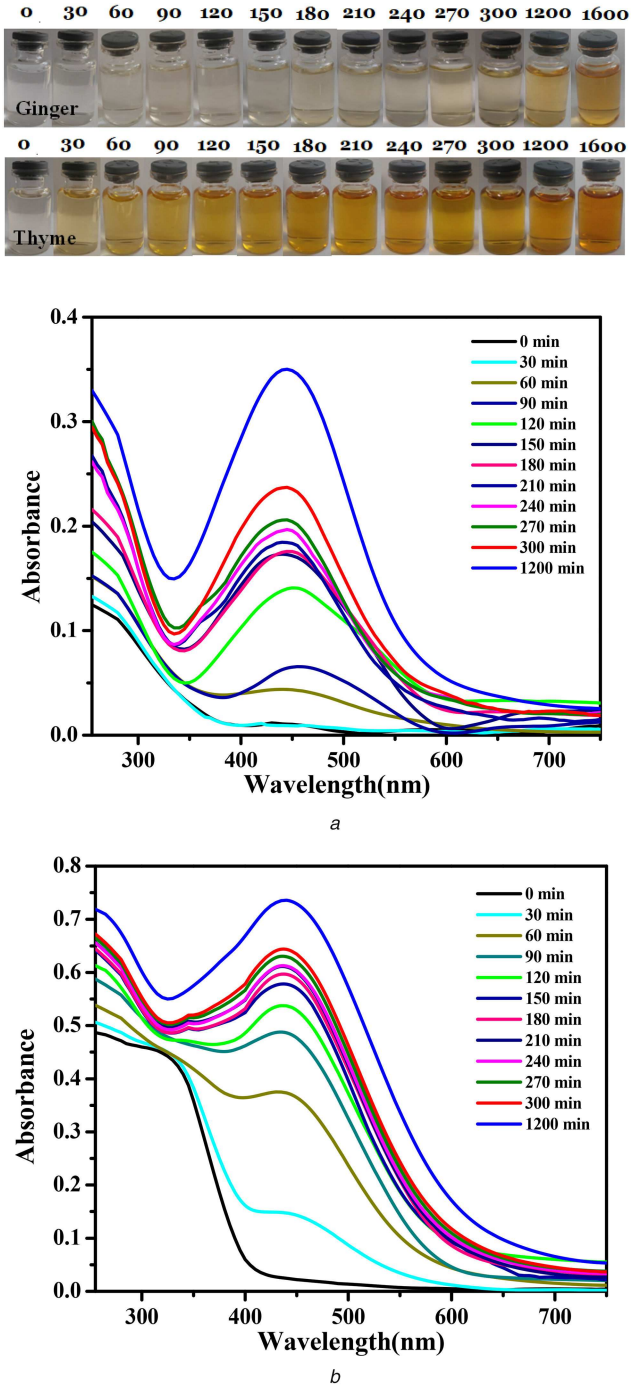

Fig. 2 *Colour changes of synthesis solution and their corresponding UV–visible spectra of Ag NPs synthesised in different times (in minutes) via* ***(a)*** Ginger extracts, ***(b)*** Thyme extracts

higher than that for ginger. Indeed, the thyme extract reduces Ag ions faster than ginger in the same concentration. Similar results can be observed for higher concentrations of the extract which is reported in supplementary information 1. It should be noted that at high concentration of the extract, the rate of reaction increased and more Ag NPs are formed due to more reducing agent.

Fig. 3 shows the typical TEM images of synthesised Ag NPs via ginger (a) and thyme (b) extracts. Ag NPs are spherical in both cases, with the mean sizes of 12 and 18 nm for ginger and thyme, respectively. Here, it should be noted that the ginger extract concentration (5 g of ginger in 100 mL of DI water) is 25 times more than that of the thyme extract. At this condition, Ag ions are reduced more by thyme extract and the final Ag NPs' size is increased more in comparison to ginger extract which again confirms higher reduction power of the thyme extract.

Various mechanisms [41–43] have been proposed to explain the formation of Ag NPs via plant extracts. Releasing or sharing of

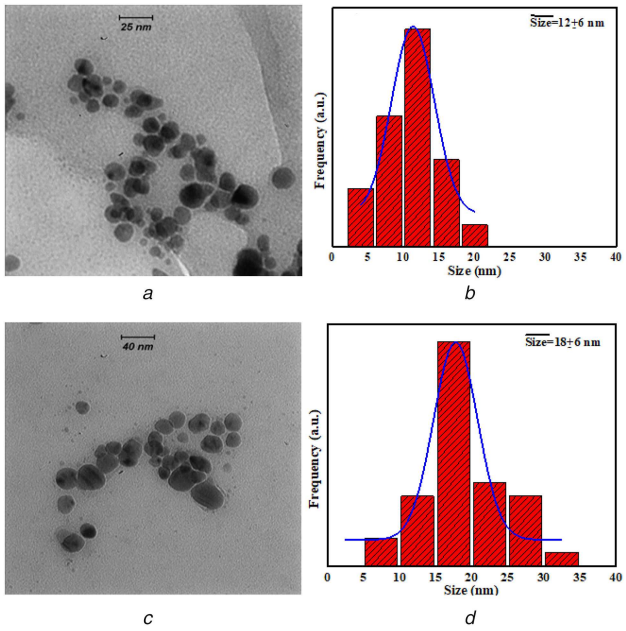

Fig. 3 *Typical TEM images of Ag NPs synthesised via* ***(a)*** Ginger extracts, ***(b)*** Their size distribution histograms, ***(c)*** Thyme extracts, ***(d)*** Their size distribution histograms

electrons from the plant extract is responsible for reduction of positive ions, and the phenolic compounds, OH, C=O, and CH group are main sources of electron supplier. Here, we also showed that the light or heating is necessary for reducing Ag ions via plant extract. Indeed, to supply electrons from the plant extract the reaction solution should be in elevated temperature or radiated with environmental light. Fig. 4 shows the effect of light and temperature on the synthesis of Ag NPs after 90 min of reaction. Sample-A was synthesised in a normal condition, i.e. at day light and room temperature. Both B and C samples were kept in dark, and sample-C was also heated around 60°C. There is no sign of the synthesis of Ag NPs after 90 min for sample B; however, Ag NPs have been synthesised for both samples A and C, which is an evidence for the catalytic role of light and temperature on the synthesis of Ag NPs via plant extracts. Broader peak in sample-B in comparison to sample-A may be due to broader size distribution and or more uneven NPs [44]. It is well established that the effect of temperature on size is dual. It means that as long as there are free electrons and positive ions, temperature promotes the reaction and more Ag NPs are synthesised. In parallel, temperature also increases the size of NPs after nucleation.

FTIR spectroscopy was carried out to recognise functional groups of extract and its effect as reducing and capping agents on the formation of Ag NPs. Fig. 5 shows FTIR spectra of extracts and synthesised Ag NPs. Fig. 5*a* shows the FTIR analysis of ginger extract and Ag NPs synthesised via ginger extract. The spectra at 3288 and 3275 cm^{-1} belong to OH stretch bonds. The weak peaks at 2936 and 2392 cm^{-1} are related to OH stretching of carboxylic acid group [45]. The bands at 1644 and 1764 cm^{-1} are due to C=O stretch of alkyne and the band at 1610 cm^{-1} are related to C=C stretch [46]. The bands created at 1388 cm^{-1} are CH_3 symmetric bonds of the alkene group. The band at 1077 cm^{-1} and the weak peaks at 1250 cm^{-1} could be due to C–O–C ether group. Other weak peaks at 1110 and 1044 cm^{-1} are related to C–OH stretching [47]. OH phenolic bonds are seen at 617 and 618 cm^{-1} [46]. The bands below 700 cm^{-1} can be related to Ag NPs and Ag-extract vibration.

Similarly, Fig. 5*b* shows the bands of thyme extract and synthesised Ag NPs via thyme extract. The bands at 3402 and 3225 cm^{-1} indicate the OH bond. The peaks at 2932, 2924, and 2848 cm^{-1} point to CH stretching vibration related to CH_2 and CH_3. The medium peaks at 1760, 1627, and 1598 cm^{-1} are due to C=O stretching. The bands at 1403 and 1384 cm^{-1} are related to

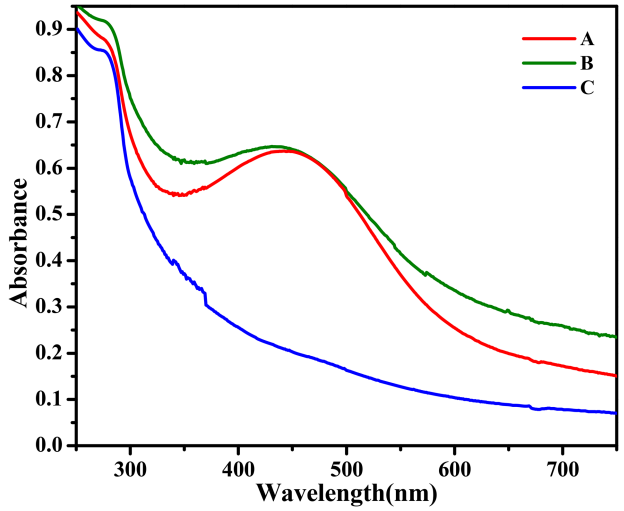

Fig. 4 *UV–visible spectra of Ag NPs synthesised via ginger extract synthesised*
(a) At room temperature in environmental light, ***(b)*** At temperature of 60°C in dark, ***(c)*** At room temperature in dark

CH symmetric stretching of the amide group. Therefore, substantial reduction in peak intensity could indicate the formation of Ag NPs that its peak has occurred at 825 cm^{-1}. The presence of bands with weak strength was also observed at 1037, 1127, 1116, and 1110 cm^{-1} which confirmed C–O bond of carbohydrates. Finally, the weak peaks at 680 and 617 cm^{-1} are interrelated to OH bond of the phenolic group.

According to the FTIR results of extracts and the Ag NPs synthesised via extracts, it is obvious that extracts are responsible for Ag NPs synthesis. The bond variation indicates that extracts provide required electrons for Ag NPs formation by the OH bond separation. Furthermore, this bond collapse leads to attach extracts to the Ag NPs. Therefore, FTIR confirms that extracts are adsorbed onto the surface of Ag NPs and play a capping role.

3.2 Antifungal activity

The antifungal activity of Ag NPs, pure extracts, silver nitrate, and FLZ against *C. albicans* was investigated by MIC method and the results are shown in Table 1. The extract of ginger and thyme demonstrated MIC at 1900 and 1100 ppm, respectively. Silver nitrate demonstrated MIC of 5.2 ppm. The results showed that the MIC of silver nitrate and Ag NPs synthesised by ginger and thyme were 5.2, 0.7, and 0.5 µg/ml, respectively. It can be inferred that the Ag NPs prepared via ginger and thyme extract demonstrated a higher inhibitory effect against *C. albicans* in comparison to silver nitrate and FLZ with MIC of 16 ppm. Indeed, Ag NPs synthesised with thyme and ginger extract demonstrated lower MIC which can be attributed to the synergetic effect of Ag NPs and plant extract. It has been shown that the antifungal activity of the extract of thyme and ginger may be involved due to their hydrophobic properties of these compounds which can attach to the fungal plasma membrane and interfere fungal proliferation by increasing the membrane permeability or inhibit spore germination and cell respiration [48]. On the other hand, Ag NPs demonstrated antifungal activity by attaching to cell membrane of fungi and interrupting the membrane integrity and finally destructing the membrane structure [49]. As mentioned previously, the FTIR analysis confirmed that the extract of ginger and thyme was coated on the surface of the as-synthesised Ag NPs. Therefore, prepared Ag NPs attach more to the cell membrane and destruct fungi plasma membrane, and finally prohibit the growth of *C. albicans*. It should be noticed that

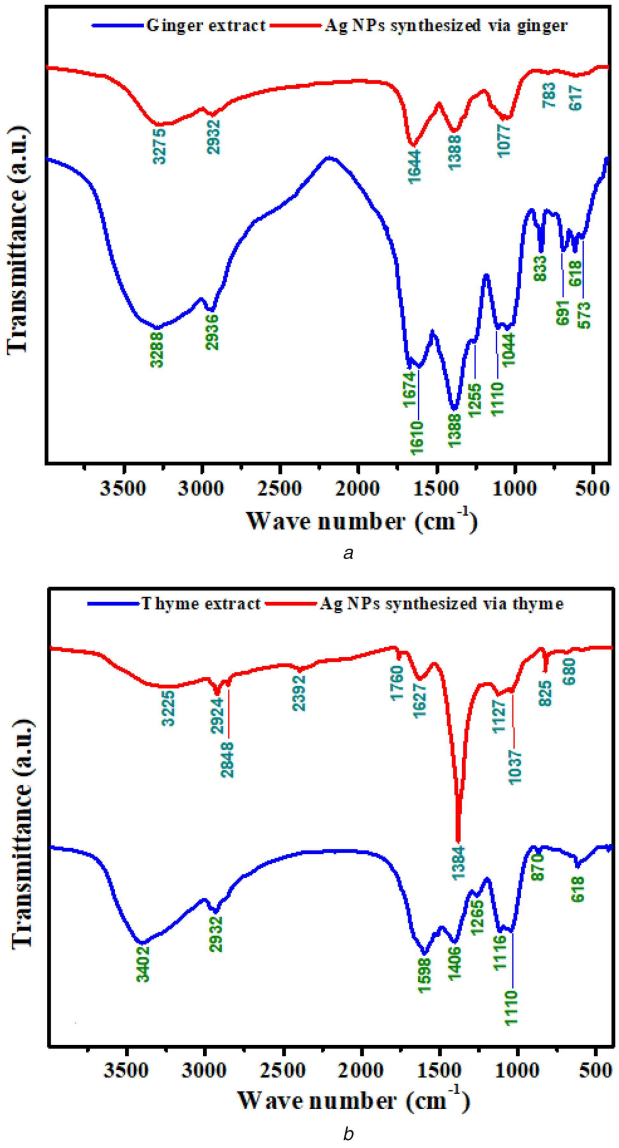

Fig. 5 *FTIR spectra of extracts and Ag NPs synthesised via*
(a) Ginger, ***(b)*** Thyme

the combination of natural extract with Ag NPs may open diverse antifungal mechanisms.

3.3 MTT-based cytotoxicity

Fig. 6 shows the cell cytotoxicity of green synthesised Ag NPs via plant extracts which evaluated on a human dermal fibroblast cell line based on MTT assay. The results show that Ag NPs synthesised by ginger extract is toxic at the concentrations above 1.25 ppm, meanwhile, Ag NPs synthesised by thyme extract demonstrated no cytotoxicity effect below the concentration of 3.5 ppm. Indeed, the results indicated that Ag NPs synthesised by thyme extract show lower cytotoxicity in comparison to Ag NPs synthesised by the ginger extract. By considering similar MIC for both Ag NPs synthesised via ginger and thyme extract, and lower toxicity for Ag NPs synthesised via thyme extract, it can be inferred that Ag NPs synthesised via thyme extract can be considered as an appropriate candidate instead of FLZ to treat the SEIs.

Table 1 MIC of Ag NPs, pure extracts, silver nitrate, and FLZ against *C. albicans*

Sample	Ag NPs via ginger	Ag NPs via thyme	$AgNO_3$	Ginger extract	Thyme extract	FLZ
concentration in µg/ml, ppm	0.7	0.5	5.2	1900	1100	16

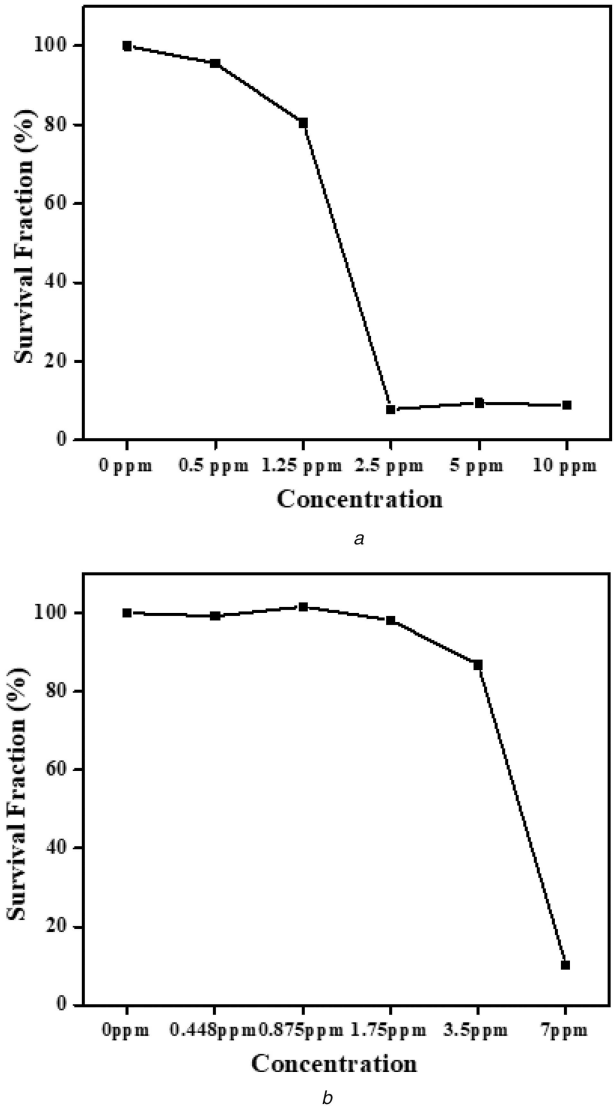

Fig. 6 *Cytotoxicity study of green synthesised Ag NPs against HDF-1 via* ***(a)*** Ginger extracts, ***(b)*** Thyme extracts

4 Conclusion

In this study, Ag NPs were prepared based on the green method by considering the intrinsic antifungal activity of natural extract (ginger and thyme). UV–visible spectra approved the formation of Ag NPs based the formation of SPR peak in the range of 440–450 nm. Moreover, the UV–visible results showed that thyme extract has higher reducing power in comparison to ginger extract. Spherical Ag NPs have been synthesised via ginger and thyme extract with average diameters of 12 and 18 nm, respectively. The Ag NPs prepared via ginger and thyme extract demonstrated higher inhibitory (MIC ∼ 0.5 ppm) in comparison to FLZ (MIC ∼ 16 ppm) against *C. albicans*. The cell cytotoxicity results demonstrated that Ag NPs are toxic at the concentration above 1.25 and 3.5 ppm based ginger and thyme extracts synthesis, respectively. Ag NPs synthesised via thyme extract can be considered as an appropriate candidate instead of FLZ to treat the SEIs due to its lower toxicity and MIC.

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