Original Article

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Antifungal activity of polymeric micelles of silver nanoparticles prepared from *Psidium guajava* aqueous extract

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Summary **In the present study, silver nanoparticles (AgNPs) were synthesized by green synthesis using *Psidium guajava* aqueous extract (PE) as a reducing agent and silver nitrate ($AgNO_3$) as a precursor. The obtained AgNPs showed maximum absorbance at 455 nm. The results from energy-dispersive X-ray spectroscopy demonstrate Ag signal at 88.33% weight. The particle image under scanning electron microscopy is spherical shape. The average size of the freshly prepared AgNPs is 96 ± 4 nm but is dramatically increases during storage due to particle aggregation. Coating AgNPs with polymeric micelles of poloxamer 407 (F127) at the suitable ratio can decrease the size of the freshly prepared AgNPs to 70.4 ± 0.8 nm and significantly prevent AgNPs from aggregation. The obtained coated AgNPs showed high effective on inhibition of *Candida albicans*. Isotonic solutions of 0.9% NaCl and phosphate buffer solution pH 7.4 can cause some extend of aggregation and increase the particle size of the coated AgNPs but the increased size is in the colloidal range that no precipitation occurs during 90 days at room temperature. From our results, it is suggested that the 1:1 ratio of AgNPs/F127 is the most suitable ratio to obtain the AgNPs loaded polymeric micelles with high stability, small particle size, and high inhibitory activity against *C. albicans*. These AgNPs are the promising antifungal nanomaterials for further study in animal model.**

Keywords: Silver nanoparticles, green synthesis, polymeric micelles, *Psidium guajava* antifungal activity

1. Introduction

Metal nanoparticles have been recently applied in various fields. Silver nanoparticle (AgNPs) are being successfully used in medical and pharmaceutical fields because of their potential on antimicrobial activity. Moreover, AgNPs also pronounce anticancer and antioxidant activities (*1*). Synthesis of AgNPs can be performed in small scale as in laboratory works or larger scale as in industries by redox reaction between silver salt such as silver nitrate ($AgNO_3$) as a precursor and reducing agent to reduced Ag^+ to Ag^o (*2*). However, some chemical reducing agents can cause harmful to human and environment. Green synthesis using reagents from eco-friendly resources such as bacteria (*3*), fungi (*4*), algae (*5*), and plants (*6*) are received increasing interest nowadays. The extracts from certain plants such as *Plectranthus amboinicus* (*7*), *Lycium barbarum* (*8*), *Alternanthera dentata* (*9*), *Coriandrum sativum* (*10*), *Emblica officinalis* (*11*), and *Sargassum incisifolium* (*12*) have been reported to act as good reducing agents in the green synthesis of AgNPs. The reducing activity is expected to obtain from some phytochemicals in plant extracts (*13*).

Psidium guajava is a plant in family Myrtaceae and is an original native plant of Mexico that extends throughout the South America, European, Africa and Asia (*14*). Many reports indicate that *P. guajava* leaves possess many biological effects that can support the good health of human being such as hepatoprotective

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effect (*15*), antioxidant activity (*16*), anti-inflammatory activity (*17*), antimicrobial activity (*18*), and anti-hyperglycemic as well as anti-hyperlipidemic effects (*19*). The main constituents of *P. guajava* are in the groups of vitamins, tannins, phenolic compounds, flavonoids, sesquiterpene alcohols and triterpenoid acids (*20*). The important phytochemicals of *P. guajava* leaves are gallic acid, catechin, epicatechin, rutin, naringenin, and kaempferol (*14,21,22*). *P. guajava* was previously used as a reducing agent in AgNPs synthesis (*23,24*), however the size of the obtained AgNPs were quite large according to the particle aggregation. Therefore, searching for suitable stabilizing agent is necessary in order to protect AgNPs from aggregation and to maintain the obtained particles with stable small size.

Polymeric micelles have spherical and nanosize (10-100 nm) supramolecular core/shell structures formed by self-assembly of amphiphilic copolymers in aqueous solution. Poloxamers are triblock copolymers composed of poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) (PEO-PPO-PEO). Polymeric micelles of poloxamers have been reported to stabilize AgNPs obtained from chemical and natural reducing agents (*25,26*), however, they have not yet been used with AgNPs obtained from *P. guajava* aqueous extract (PE).

In the present study, Poloxamer 407 (F127) micelles were investigated for possible potential to stabilize AgNPs prepared by using PE as a reducing agent. The effects of F127 micelles on physicochemical characteristics of the obtained AgNPs were evaluated using dynamic light scattering (DLS), scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX). Moreover, the physical stability and antifungal activity of the obtained AgNPs against *Candida albicans* were also studied.

2. Material and Methods

2.1. *Materials*

2,4,6-Tripyridyl-s-triazine (TPTZ) and 2-diphenyl-1-picrylhydrazyl (DPPH), 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were from Sigma-Aldrich, Inc (St. Louis, MO, USA). $AgNO_3$ and sodium hydroxide (NaOH) 97% were from RCI Lab-scan Co., Ltd. (Bangkok, Thailand). Hydrochloric acid (HCl) 37% was from Carlo Erba reagents (Rodano, Metropolitan City of Milan, Italy). Ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) was from RFCL limited (New Delhi, India). Iron (III) chloride hexahydrate ($FeCl_3$) was from Honeywell Riedel-de-Haën™ (Seelze GmbH Manufacturing Facility, Seelze, Hanover, Germany). Poloxamer 407 (F127) were from O-BASF Co. (Ludwigshafen, Germany). Sabouraud dextrose agar (SDA) and broth (SDB) were purchased from BBL™ (Baltimore, MD, USA). All other chemicals and solvents were of analytical reagent grade or the highest grade available. They were used without further purification.

2.2. *Preparation of PE*

Exact amount of 2 g of dried powder of *P. guajava* leaves was mixed with 100 mL deionized water and stirrer at room temperature for 24 h. The mixture was filtered through a Whatman No.1 filter paper and the filtrate was evaporated using rotary evaporator until the solvent was completely removed. PE obtained was kept in the refrigerator at 4ºC for further study.

2.3. *Synthesis of AgNPs*

A solution containing 0.1 mg/mL of PE in deionized water was firstly prepared and heated to 70ºC. Exact amount of 1 mL $AgNO_3$ 10 mM solution was added drop wise to the heated solution with continuous stirring at 100 rpm for 60 min. The obtained mixture was diluted with deionized water and subjected to centrifugation (Heraeus™ Megafuge™ 40 Centrifuge Series, Thermo Fisher Scientific, Waltham, MA, USA) at 8,000 rpm for 15 min to remove any trace un-utilized phytochemicals. This washing process was done in triplicate. The aqueous colloidal AgNPs were lyophilized using Freeze Dryer (Virtis®, Warminster, PA, USA) to obtained AgNPs powder.

2.4. *AgNPs loaded polymeric micelles*

Certain amount of AgNPs powder was dispersed in ultrapure water to obtain AgNPs at a concentration of 0.1 mg/mL. Aqueous solution containing 5% F127 was prepared and heated to 70°C with continuous stirring at 100 rpm. The AgNPs solution was dropped wise into F127 solution to obtain mixtures with different volume ratios of AgNPs solution to F127 solution (AgNPs/F127) at 1:1, 1:3 and 1:5 in order to load AgNPs in F127 micelles. The obtained AgNPs loaded micelles were called coated AgNPs. The mixtures were continuously stirred for 30 min and washed with ultrapure water at 10,000 rpm for 15 min 3 times by using centrifugation. The uncoated AgNPs were prepared in the same manner as the coated AgNPs but ultrapure water was used instead of F127 solution. The obtained AgNPs were kept at 4ºC for further use.

2.5. *Characterization of AgNPs*

The obtained AgNPs were characterized using UV-visible spectrophotometer (UV-vis). The size, size distribution, and zeta potential of AgNPs particles were investigated using DLS (Malvern, Nano series, UK). The surface morphology was investigated by using

SEM (IT-JSM-300) and the Ag signal was detected by using EDX, a high resolution SEM, in order to confirm Ag elemental from the AgNPs. For this experiment, the AgNPs sample was placed on a stub with graphite tape. The element standard used were C ($CaCO_3$), O (SiO_2), Cl (KCl), K (MAD 10 Feldspar), Cu (Cu) and Ag (Ag).

2.6. *Antifungal activity*

Antifungal activity of the obtained AgNPs was tested by using a method Kirby-Bauer (*27*) with some modification. Fungal strain of *C. albicans* ATCC 10231 was used as a test microorganism. The fungal strain was cultured in SDA at 37ºC for 36-48 h. The fungal suspension was adjusted to a final density of 0.5 McFarland constant by observing the optical density at 600 nm under UV-vis to obtain microbial concentration of 1-2 × 10^5 CFU/mL in SDB. Then the fungal suspension was swabbed on the surface of SDA. The wells were made on the agar plates using a sterile cork borer having a diameter of 6 mm. Each 40 µL sample was filled in the well whereas PE and F127 solutions were used as negative controls. The plates were incubated at 37 ± 2ºC for 48 h. The antimicrobial activity of AgNPs was evaluated by determining the diameter of clear zone of inhibition expressed in millimeter (mm). All samples were done in triplicate.

2.7. *Stability study*

The obtained AgNPs were kept at room temperature for 90 days to study their physical characteristics and antifungal activity. The change in physical characteristics such as particle size, size distribution, and zeta potential was investigated using DLS. Antifungal activity of the samples after keeping was compared to the freshly prepared using the antifungal test method mentioned above.

2.8. *Effect of diluting media*

The coated AgNPs containing 1:1 ratio of AgNPs/F127 was diluted with deionized water, 0.9% sodium chloride (NaCl) and phosphate buffer solution (PBS) pH 7.4 to investigate the effect of diluting media on particle size of AgNPs. The obtained suspensions were kept at room temperature for 90 days. The particles size and antifungal activity of the obtained AgNPs after keeping were investigated using the same methods as mentioned above. The results were compared with those of freshly prepared.

2.9. *Statistical analysis*

Data were reported as a mean ± standard deviation. Statistic analysis was done by means of a one-way analysis of variance (ANOVA) and Duncan's multiple range test ($p < 0.05$) using Statistic a software version 17.

3. Results

3.1. *Biosynthesis of AgNPs*

The original color of PE solution is green-brown whereas that of $AgNO_3$ solution is colorless. After mixing these two solutions, the color of the obtained mixture was light green-brown. During synthesis, the color of the mixture turned to brown color at 30 min. After completely reaction at 60 min, the mixture was dark brown color. The obtained AgNPs powder after dispersing in deionized water showed maximum absorbance at 455 nm as shown in Figure 1A. Morphology of AgNPs observed under SEM was spherical shape as shown in Figure 1B. Confirming Ag element using EDX, the spectrum indicated that the obtained AgNPs were composed of Ag 88.33% weight as seen in Figure 1C.

3.2. *AgNPs loaded polymeric micelles*

Synthesizing AgNPs using several ratios of AgNPs/F127 yielded AgNPs loaded polymeric micelles that so called coated AgNPs and those without F127 yielded

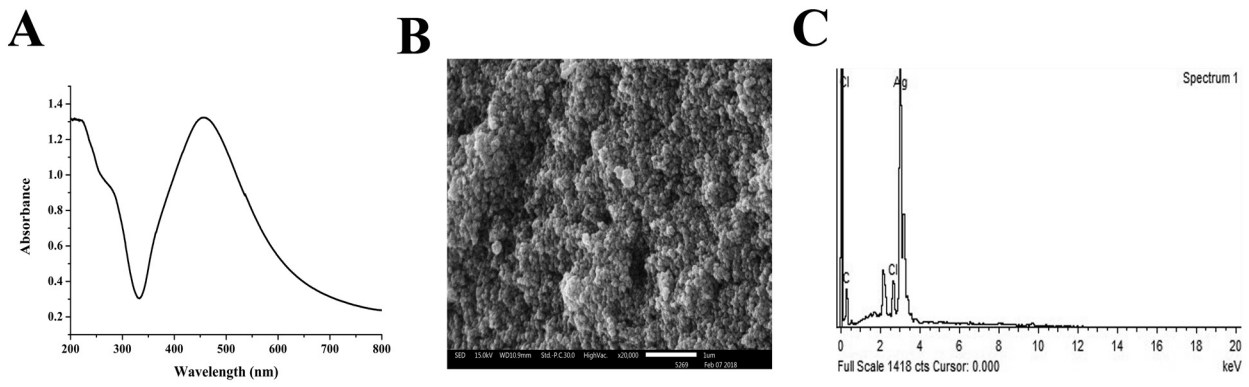

Figure 1. The UV-vis spectrum (A), surface morphology (B), EDX spectrum of Ag signal (C) of AgNPs.

Table 1. Particles size, size distribution, and zeta potential of uncoated AgNPs and AgNPs coated with polymeric micelles

Ratio of AgNPs/F127	Classification of AgNPs	Particles size (nm)	PdI	Zeta potential (mV)
1:0	uncoated	96.4 ± 3.8	0.24 ± 0.1	-18.4 ± 1.6
1:1	coated	70.4 ± 0.8	0.18 ± 0.1	-31.9 ± 0.5
1:3	coated	171.2 ± 2.4	0.22 ± 0.1	-25.5 ± 0.7
1:5	coated	258.6 ± 11.4	0.31 ± 0.1	-22.4 ± 1.2

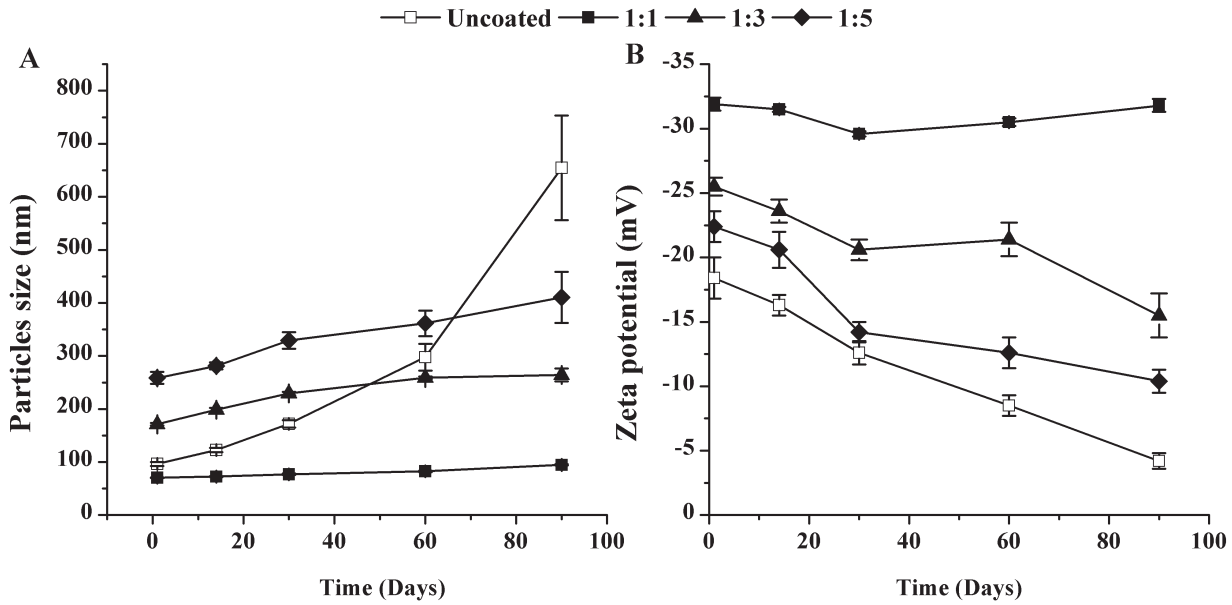

Figure 2. Changes of particles size (A) and zeta potential (B) of the uncoated AgNPs and AgNPs coated F127 with AgNPs/F127 ratios of 1:1, 1:3, and 1:5 after keeping for 90 days.

uncoated AgNPs. The color of the coated AgNPs colloid was lighter than the uncoated AgNPs. The particle size, size distribution, and zeta potential of the coated and uncoated AgNPs were shown in Table 1. It was found that the particle size of the coated AgNPs obtained from the 1:1 ratio of AgNPs/F127 was significantly smaller than the uncoated AgNPs. Increasing this ratio to 1:3 and 1:5 increased the size of the particles. Size distribution of the AgNPs obtained from all ratios was in the range of narrow and acceptable. The negative zeta potential value of the coated AgNPs at 1:1 ratio was higher than those from 1:3 and 1:5 ratios. It is noted that the negative zeta potential value of the coated AgNPs from all ratios was significantly higher than that of the uncoated AgNPs.

3.3. *Stability of AgNPs*

The particles size and zeta potential of the coated and uncoated AgNPs keeping at room temperature for 90 days are shown in Figure 2. It was found that the particle size of the uncoated AgNPs increased promisingly with storage time whereas the size of the coated AgNPs increased at very low rate, particularly those obtained from the ratio of 1:1. The zeta potential of the coated AgNPs obtained from the ratio of 1:1 was not significantly different between the freshly prepared and those kept for 90 days. However, the zeta potential of the uncoated AgNPs was significantly decreased dramatically along the storage time. After 90 days of keeping, the zeta potential of the uncoated AgNPs was nearly zero.

3.4. *Effect of diluting media*

In this study, the coated AgNPs obtained from 1:1 ratio of AgNPs/F127 was used and compared with the uncoated AgNPs. The particles size of AgNPs at day 1, 3, 7, 14, 30, 60, and 90 after contacting with three different diluting media was determined using DLS. The results are shown in Figure 3. It was found that the freshly prepared particles after diluting with 0.9% NaCl and PBS were bigger than those diluted with deionized water. Keeping at room temperature, the relatively high rate of size increase was seen within 15 days after contacting with 0.9% NaCl and PBS but after that the size was not much influenced. The size increase in the uncoated AgNPs occurred during 90 days could be seen more obviously than the coated AgNPs.

3.5. *Antifungal activity of AgNPs*

Antifungal activity of the obtained AgNPs against *C. albicans* was investigated using the diffusion method.

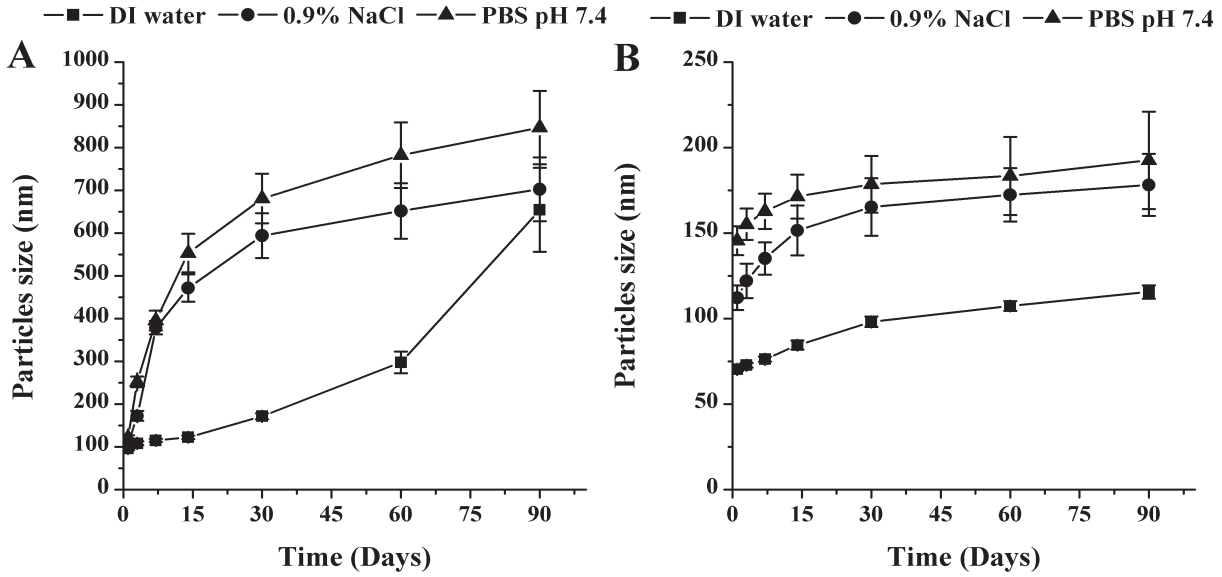

Figure 3. Changes of particle size of the uncoated AgNPs (A) and coated AgNPs (B) after contacting with different media.

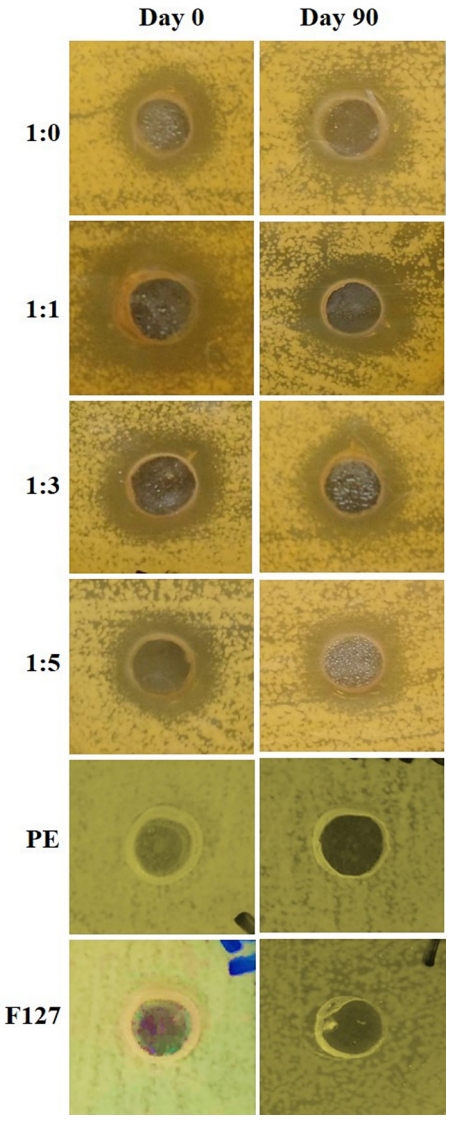

Figure 4. Inhibitory activity of the freshly prepared AgNPs (A) and the AgNPs after keeping for 90 days in comparison with the negative controls.

Table 2. Inhibition zones of the freshly prepared AgNPs *C. albicans* in comparison with AgNPs kept at room temperature for 90 days

Samples	Classification of sample	Day 0	Day 90
PE	negative control	NZ	NZ
F127	negative control	NZ	NZ
1:0 AgNPs/F127	uncoated AgNPs	13.8 ± 0.3	7.6 ± 0.7
1:1 AgNPs/F127	coated AgNPs	14.2 ± 0.7	13.8 ± 0.7
1:3 AgNPs/F127	coated AgNPs	11.4 ± 0.9	9.7 ± 1.2
1:5 AgNPs/F127	coated AgNPs	9.6 ± 0.7	8.4 ± 0.7

NZ: no inhibition zone.

The antifungal activity of the freshly prepared AgNPs was higher than that kept for 90 days as seen in Figure 4. The diameters of the inhibition zones are shown in Table 2. It is noted that the significant decrease in diameter of inhibition zone after keeping for 90 days could be found in both uncoated and coated AgNPs synthesized with 1:3 and 1:5 ratios. The coated AgNPs synthesized with 1:1 ratio showed significantly stably high antifungal activity. PE and F127 as negative controls showed no inhibition zone.

4. Discussions

P. guajava has been reported to have high reducing and antioxidant activities (*21,28,29*). Many active compounds for these activities such as myricetin, apigenin, cathechic, gallic acid, ferutic acid, saponins, oleanolic acid were found in PE (*14,16,20*). Previously, our group reported that quercetin, morin, and quercetin-3-*O*-glucopyranoside isolated from PE possess high reducing power (*21*). Thus, in the present study, PE was used as a reducing agent to synthesized AgNPs from $AgNO_3$. Adding PE solution to an aqueous $AgNO_3$ solution, resulting in color change to dark brown color

within 60 min due to excitation of surface plasmon vibration in metal nanoparticles (*30*). UV-vis absorption is widely used for characterization of AgNPs. Previous report revealed that the UV-vis spectra of AgNPs generally appear in the range of approximately 400-450 nm, depending on the type of plant extract and duration time of synthesis. For example, AgNPs obtained from the extract of *Hibiscus sabdariffa* showed the maximum absorbance at 400 and 410 nm at the reaction time of 10 and 90 min, respectively (*31*). In the current study, the spectra of the mixtures obtained after PE was completely reacted with $AgNO_3$ showed a strong broad peak at 455 nm indicating the presence of AgNPs (*32*). The obtained AgNPs showed spherical shape in the nanosize range corresponding to the size measured by DLS. Moreover, the results presented strong signal from the silver atoms confirming the development of silver nanostructures and the signals of C and O indicating the presence of the metabolites in PE. From these results, it is obviously shown that AgNPs can be obtained from the reduction of $AgNO_3$ by PE.

Stability study indicates that the uncoated AgNPs are not stable. Keeping at room temperature for 90 days, their particle size increases rapidly indicating that particle aggregation occurs. Entrapment of AgNPs inside the polymeric micelles of F127 yields a so-called coated AgNPs with different characteristics and particle stability depending on the ratios of AgNPs/F127 used. The particles size of the uncoated freshly prepared AgNPs was as small as the coated AgNPs obtained from the system of 1:1 ratio and significantly smaller than those from 1:3 and 1:5 ratios. The size and zeta potential of the coated AgNPs particularly of 1:1 ratio is not significantly changed, whereas the size of the uncoated AgNPs significantly increased and their zeta potential value dramatically decreased until almost zero at the end of the studied period. It is considered that negatively charged surfaces of AgNPs can help in preventing the aggregation. F127 as a poloxamer block copolymer has an ability to form micelles in solution and/or on the surface of particles (*33*). It is, therefore, considered that the negative charges in the relative hydrophilic part (PEO) of F127 surrounded at AgNPs surfaces influence the high negative value of the particles and prevent the particles from aggregation. It is also considered that besides enhancing the stability of AgNPs, F127 micelles also play an important role on controlling shape and size of the particles. From our results, it is also suggested that the ratio of AgNPs/F127 is important. Only the 1:1 ratio is found to be suitable for synthesizing AgNPs that the highest stable AgNPs can be achieved.

Green synthesis of AgNPs has been reported using many plant extracts (*7-12*). However, from those reported, it has been shown that AgNPs obtained from different reducing plant extracts possess different characteristics and activity. AgNPs have been reported on activity against *C. albicans* that only the small size of AgNPs showed the effective antifungal activity (*34,35*). The mechanism of action of AgNPs on *C. albicans* has been explored that AgNPs can disrupt fungal cell membrane structure and inhibit normal budding process due to the destruction of the membrane integrity (*36*). AgNPs obtained from the reduction of $AgNO_3$ by PE were previously reported by other groups but the particle size obtained was quite large due to particle aggregation (*23,24*). In the present study, our results show that the aggregation of AgNPs can be prevented by coating AgNPs with polymeric micelles of F127 at suitable ratio of AgNPs/F127. The antifungal activity of the coated AgNPs is higher than that of the uncoated. The activity of the uncoated and the coated AgNPs at ratios of 1:3 and 1:5 decreases dramatically after storage for 90 days. The antifungal activity of the 1:1 ratio AgNPs kept at room temperature is as high as that of freshly prepared, indicating that only the suitable ratio of 1:1 is the best formulation for producing AgNPs with the smallest size, the most stable, and the most effective antifungal activity.

As mentioned above, our results suggest that AgNPs with only small particle size is essential for antifungal activity, if AgNPs are aggregated and the particle size becomes large in the diluting vehicle, it is not possible for AgNPs to have this activity potential. Investigation of effects of diluting media on the particle size is therefore necessary. As the isotonic solutions of 0.9% NaCl and PBS pH 7.4 are often used as diluting vehicle for intravenous injection. The effects of these two solutions are therefore investigated. The results demonstrate that these two solutions cause the increase in AgNPs size compared with those diluted with deionized water. It is considered that the electrolytes in both solutions play an important role on this effect. Our results are in line with the other groups who found that the freshly prepared AgNPs were aggregated and precipitated in a few minutes after contacting with 0.9% NaCl and PBS pH 7.4 (*37*). However, our AgNPs showed no precipitation. The increased size of the AgNPs in the current study are still in the colloidal range that all particles can be suspended in the systems. This is considered to be due to F127 micelles that play an important role on prevention of precipitation. Keeping at room temperature for 90 days, the size of AgNPs in both isotonic and deionized water systems gradually increased but not dramatically. Deionized water is not a good vehicle for intravenous injection as it is not isotonic solution. Comparing between 0.9% NaCl and PBS pH 7.4, the size of AgNPs in 0.9% NaCl is smaller than that in PBS pH 7.4. From these results, it is considered that both isotonic diluting solutions influence the size of AgNPs coated with F127 micelles rapidly at the fresh synthesis but not much effect along the period of storage. It is concluded that entrapment of AgNPs prepared from PE with F127 micelles yield

the promising antifungal AgNPs suitable for further investigation in animal model.

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