TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2015) 39: 556-561
© TÜBİTAK
doi:10.3906/biy-1406-81

Biological synthesis of silver nanoparticles and evaluation of antibacterial and antifungal properties of silver and copper nanoparticles

Azam JAFARI[1,*], Latifeh POURAKBAR[1], Khalil FARHADI[2], Lida MOHAMADGOLIZAD[1], Yobert GOOSTA[3]

[1]Department of Biology, Faculty of Science, Urmia University, Urmia, Iran
[2]Department of Chemistry, Faculty of Science, Urmia University, Urmia, Iran
[3]Department of Plant Protection, Faculty of Agriculture, Urmia University, Urmia, Iran

Received: 29.06.2014 • **Accepted/Published Online:** 29.01.2015 • **Printed:** 30.07.2015

Abstract: There is increasing attention being paid to metallic nanoparticles because of their intensive applications in different areas of science such as medicine, chemistry, agriculture, and biotechnology. In addition, there has been growing interest in using environmentally friendly methods of synthesizing nanoparticles without making or using substances risky to the environment and human health. Biological methods for the synthesis of nanoparticles have been considered as possible ecofriendly alternatives to chemical synthesis. In the present study, biosynthesis and characterization of silver nanoparticles (SNPs) using marshmallow flower (*Althaea officinalis* L.), thyme (*Thymus vulgaris* L.), and pennyroyal (*Mentha pulegium* L.) leaf extracts is reported for the first time. Copper nanoparticles (CuNPs) were formed by reduction of $CuCl_2$ with L-ascorbic acid. The antibacterial and antifungal effects of SNPs and CuNPs in comparison with silver nitrate ($AgNO_3$) and copper chloride ($CuCl_2$) (respective nanoparticle constrictive salts) and synthetic antibiotics and fungicides were studied. Fungi (*A. flavus* and *P. chrysogenum*) and bacteria (*E. coli* and *S. aureus*) showed clear hypersensitivity to silver and copper nanoparticles, and the effects of SNPs were more notable than those of CuNPs. Data analysis showed that copper chloride and silver nitrate had a lower inhibitory effect in their nanoparticles, especially against the tested fungi.

Key words: Metallic nanoparticles, antifungal, antibacterial, *Althaea officinalis*, *Thymus vulgaris*, *Mentha pulegium*, plant extract

1. Introduction

Increasing strains with resistance to antibiotics and the resulting failure to treat infectious diseases is a major challenge in the medical and health fields. Drug resistance, especially in recent decades, had led to a search for different approaches and methods for finding new compounds against bacteria and fungi. Scientists have accepted the idea that nanotechnology is a novel area of science that combines biology, chemistry, and physics (Rezaei-Zarchi et al., 2012; Demir et al., 2014). Nanoparticles have dimensions of 100 nm or less. They have gained remarkable attention because of their unusual properties and the various applications they are suited for, when compared to their bulkier counterparts (Kato, 2011; Metzler et al., 2012). These features have helped spread the use of nanomaterials at a faster rate day by day. They can be used in the fight against germs, diagnosis and cure of diseases, water and air purification, food production, cosmetics, and clothing (Aitken et al., 2006). Silver is the most commonly used engineering nanomaterial in all consumer products (Akinoglu et al., 2014).

Silver and copper have long been known to display a strong toxicity against a wide range of microorganisms. Thus, silver- and copper-based compounds have been used intensively in medicine to treat burns and infections. These antimicrobial effects have been studied by many researchers (Mirzajani et al., 2011; Yasa et al., 2012). The antimicrobial potential of these metals led to their use in health-related products (Selvaraj et al., 2011). Research showed the strong antibacterial effect of silver nanoparticles on both gram-positive and gram-negative bacteria, mainly multidrug-resistant strains (Ashrafi et al., 2013; Sangiliyandi et al., 2014). It can be used as an antifungal agent, and this effect has been intensively studied (Jo et al., 2009). Antibacterial properties of copper nanoparticles (CuNPs) have been compared with triclosan, and a strong antibacterial effect has been reported for both (Cubillo et al., 2006). The mechanisms causing nanoparticles to act on bacteria have not yet been fully clarified. However, the four most common theories proposed are: (1) the uptake of free silver ions leads to a disturbance in ATP production and DNA replication. (2) Silver nanoparticles interact

* Correspondence: st_az.jafari@urmia.ac.ir

with membrane proteins and accumulate in the cell membrane, which affects their correct role and membrane permeability. (3) Silver nanoparticles and silver ions produce ROS. (4) Silver nanoparticles directly damage the cell membranes (Mirzajani et al., 2011).

With the quick growth of nanotechnology, applications have expanded further, and now silver engineered nanoparticles are most commonly used in consumer products (Forough and Farhadi, 2010). Thus, many chemical, physical, biological, and hybrid methods have been designed to synthesize various types of nanoparticles (Grass et al., 2010; Liu et al., 2011). Although chemical and physical methods are more widespread for nanoparticle synthesis, the use of toxic compounds limits their application. Developments in biological production are now of more interest due to simplicity of the procedures and their ability to take on a biological role, as in medicine (Mohanpuria et al., 2008; Popescu et al., 2010).

Many articles have been published about the effects of nanoparticles on plants, bacteria, and fungi. In our study, the effects of selected doses of nanoparticles and their constituent salts are considered. The effects of these nanoparticles has never been evaluated against the tested organisms. The aims of this study are: 1) biosynthesis of silver nanoparticles using different plant extracts. 2) Evaluation of the toxicity of nanoparticles and their salts against two fungi, *Aspergillus flavus* and *Penicillium chrysogenum*, and two bacteria, *Escherichia coli* and *Staphylococcus aureus*. 3) To compare the toxicity effects of silver and copper nanoparticles.

2. Materials and methods

2.1. Synthesis of silver nanoparticles

Marshmallow flowers (*Althaea officinalis* L.), thyme (*Thymus vulgaris* L.), and pennyroyal (*Mentha pulegium* L.) leaves were collected from the plains around Urmia in northwestern Iran. The collected samples were washed thoroughly under deionized water and dried. Marshmallow flowers (not pennyroyal and thyme leaves) were crushed with a grinder to a fine powder and extracted by bain marie. For preparation of colloidal silver nanoparticles, 1 mM silver nitrate (Sigma company) solution was added to 2 mL of each plant extract, to make a final volume up to 100 mL. This was heated in a bain marie for 15 min at 62 °C. The liquid changed from colorless to brown at the previously mentioned temperature and room temperature (Figure 1). Deionized water was used in all stages. The formation of silver nanoparticles was studied by TEM microscopy (Figure 2). TEM analysis was performed in Kefa Nano Laboratory (Zeiss, EM 900).

2.2. Synthesis of copper nanoparticles

Copper nanoparticles were prepared by chemical decrease of $CuCl_2$ in deionized water. L-ascorbic acid was used as a reducer and stabilizer (Jing et al., 2011). Briefly, 3.40 g of $CuCl_2.2H_2O$ and 7.044 g of L-ascorbic acid (Merck, Germany) were dissolved separately in 100 mL of deionized water. $CuCl_2.2H_2O$ aqueous solution was heated to 80 °C with magnetic stirring in an oil bath. CuNPs was produced by the addition of drops of L-ascorbic acid solution into the $CuCl_2$ solution while stirring. The mixture was kept at 80 °C until a dark brown solution formed (10–12 h). This dispersion was centrifuged at 7500 rpm for 15 min.

2.3. Study of antimicroorganism activity

The tested fungi, *A. flavus* (IR 111) and *P. chrysogenum* (ATCC 10003), and bacteria, *E. coli* (ATCC 25922) and *S. aureus* (ATCC 12228), were bought from the regional center collection of the Iran Industrial Molds and Bacteria in lyophilized form. Antifungal properties of these nanoparticles and their salts were studied by disk diffusion method using potato dextrose agar (PDA; Merck, Germany). The fungi were grown on PDA culture medium and allowed to sporulate. Then a suspension of 10^5 conidia/mL was prepared and spread over petri plates containing PDA medium. Fifty microliters (of 1 mM) of the prepared silver and copper nanoparticles, silver nitrate, copper chloride, and Benomyl (synthetic fungicide) were poured onto blank disks (6.6 mm diameter). The disks were placed on the surface of inoculated plates. In controls, deionized water was used. The plates were incubated at 26–27 °C in darkness for 60 h. The inhibitory effects were examined by zones of inhibition, which appeared as a clear area around the disks (Cheesbrough, 2000). The diameter of the zones of inhibition measured and the mean value for each organism were calculated in millimeters.

To find the antibacterial properties, bacteria were grown in Mueller Hinton medium (Merck, Germany), and then suspensions with a concentration of 10^6 CFU/mL were prepared and spread over the petri plates. Fifty microliters (of 1 mM) of the synthesized silver and copper nanoparticles, silver nitrate, and copper chloride were poured onto blank disks (6.6 mm diameter). Four antibiotics: penicillin (10 μg), gentamicin (10 μg), tetracycline (30 μg), and cephalexin (30 μg) in ready disks (Antimicrobial Susceptibility Test Disk, Merck, Germany) were placed on the culture medium. For the controls, deionized water was used. All plates were incubated at 37 °C for 24 h, and the inhibition zones were measured. All experiments were repeated 3 times for both fungi and bacteria.

3. Results

Synthesis of silver nanoparticles using watery extracts of the aforementioned medicinal plants is reported for the first time. The reduction of silver ions to silver nanoparticles during exposure to plant extracts was accompanied by a color change (Figure 1). TEM images showed the forming

Figure 1. Color change during synthesis of silver nanoparticles. Silver nitrate (colorless liquid); SNPs synthesized from a) thyme, b) pennyroyal, and c) marshmallow.

nanoparticles, their spherical shape, and a size of 50 nm (Figure 2).

The inhibitory effect of SNPs against mycelia growth of the tested fungi, *Aspergillus flavus* and *Penicillium chrysogenum*, was almost identical (Table 1). Analysis of *Aspergillus flavus* data showed there was a significant difference in mycelia growth in treatments with SNPs in comparison with CuNPs and $CuCl_2$. The effects of Benomyl and $AgNO_3$ on *A. flavus* were not significantly different than SNPs (Figure 3). In *P. chrysogenum*, SNPs were more effective, with a significant difference toward $AgNO_3$, CuNP, and $CuCl_2$. Benomyl showed the maximum inhibitory effect, and $CuCl_2$ had the least inhibitory effect on the growth of the tested fungi (Figures 3 and 4). *P. chrysogenum* had a higher inhibition zone than *A. flavus*.

The inhibitory effects of silver and copper nanoparticles against the tested bacteria were almost identical; however, SNPs and CuNPs had a greater inhibitory effect against *E. coli* than *S. aureus* (Table 2). Of the four tested antibiotics, penicillin and cephalexin had no inhibitory effect on *E. coli*. In addition, there was not a significant inhibitory effect of silver and copper nanoparticles with gentamycin and tetracycline against *E. coli* (Figure 5). Data analyses of *S. aureus* showed a significant difference in SNPs to $CuCl_2$ and antibiotics. Copper chloride and silver nitrate had fewer inhibitory effects than their nanoparticles (Figure 6).

4. Discussion

Toxicity refers to any harmful impacts on an organism during exposure to nanoparticles and their salts. If the aim is to sterilize or disinfect a specific organism, toxicity may be interpreted as a positive result (antibacterial, antiviral). However, if the same materials affect other organisms in an unplanned or undesired manner, such toxicity is a potential

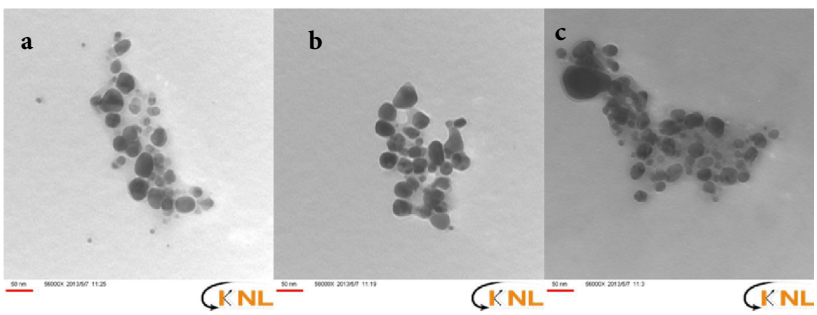

Figure 2. TEM image of the synthesized silver nanoparticles from a) thyme, b) pennyroyal, and c) marshmallow.

Table 1. Inhibition zones of different treatments against fungi. Synthesized silver nanoparticles from 1) marshmallow, 2) pennyroyal, 3) thyme, and 4) CuNP; 5) $CuCl_2$; 6) $AgNO_3$; 7) Benomyl.

	Zone of inhibition (mm)						
Microorganisms	1	2	3	4	5	6	7
Aspergillus	36	35	35	24	16	35	39
Penicillium	37	36	37	27	12	28	47

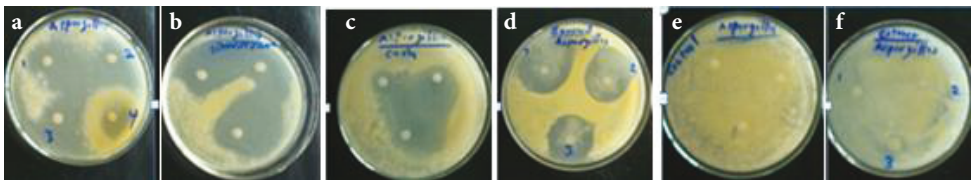

Figure 3. Inhibitory effect of different treatments on *A. flavus*. a) Synthesized SNPs from 1) marshmallow, 2) pennyroyal, 3) thyme, and 4) CuNP; b) $AgNO_3$; c) $CuCl_2$; d) Benomyl; e) control; f) plant extract of 1) marshmallow, 2) pennyroyal, and 3) thyme.

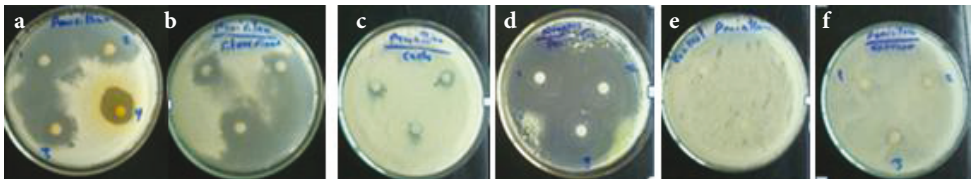

Figure 4. Inhibitory effect of different treatments on *P. chrysogenum*. a) Synthesized SNPs from 1) marshmallow, 2) pennyroyal, 3) thyme, and 4) CuNP; b) $AgNO_3$; c) $CuCl_2$; d) Benomyl; e) control; f) plant extract of 1) marshmallow, 2) pennyroyal, and 3) thyme.

Table 2. Inhibition zones of different treatments against bacteria. Synthesized silver nanoparticles from 1) marshmallow, 2) pennyroyal, 3) thyme, and 4) CuNPs; 5) gentamycin; 6) penicillin; 7) tetracycline; 8) cephalexin; 9) $CuCl_2$; 10) $AgNO_3$.

	Zone of inhibition (mm)									
Microorganism	1	2	3	4	5	6	7	8	9	10
E. coli	18	18	18	17	19	0	19	0	15	16
S. aureus	15	16	16	16	13	17	19	12	14	15

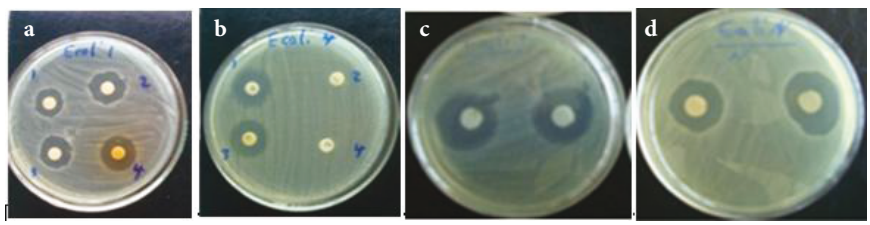

Figure 5. Inhibitory effects of different treatments on *E. coli*. a) Synthesized SNPs from 1) marshmallow, 2) pennyroyal, 3) thyme, and 4) CuNP; b) antibiotics: 1) gentamycin, 2) penicillin, 3) tetracycline, 4) and cephalexin; c) $CuCl_2$; d) $AgNO_3$.

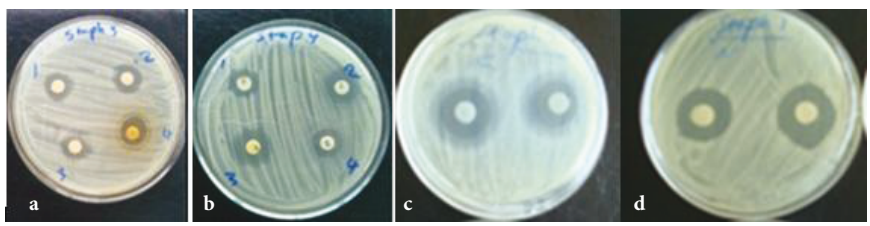

Figure 6. Inhibitory effects of different treatments on *S. aureus*. a) Synthesized SNPs from 1) marshmallow, 2) pennyroyal, 3) thyme, and 4) CuNP; b) antibiotics: 1) gentamycin, 2) penicillin, 3) tetracycline, 4) and cephalexin; c) $CuCl_2$; d) $AgNO_3$.

hazard (Marambio-Jones and Hoek, 2010). The current fundamental need in nanotechnology is the development of ecofriendly and reliable methods for synthesis of metallic nanoparticles. We have affirmed the use of biological reducing agents that are natural, low-cost, and ecofriendly materials for producing silver nanoparticles, to avoid the presence of risky and toxic solvents. David et al. (2010) synthesized silver nanoparticles by *Euphorbia hirta* L. extract and showed its efficient antifungal affect against *Candida albicans*, *C. kefyr*, and *A. niger*. In addition, *Ocimum sanctum* L. and *Vitex negundo* L. extracts were used with silver nanoparticles. These silver nanoparticles were tested against *Staphylococcus aureus*, *Vibrio cholerae*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* (Prabhu et al., 2010). Results showed the inhibitory effect achieved at 150 µL of plant leaf nanoparticles. According to Agnihotri et al. (2014), sub-10 nm nanoparticles increased antibacterial influences. Due to their size the smallest particles easily penetrate microorganisms and have greater toxicity effects; however, the larger ones cause less toxicity (Veerasamy et al., 2011; Azam et al., 2012). Other researchers tested antimicrobial features of nanoparticles against selected bacteria and achieved promising results (Hajipour et al., 2012). Commercially combined silver nano powders had the ability to decrease *E. coli* and *S. aureus* colony-forming units of 2×10^4 CFU/mL to 0 and <20, respectively (Smetana et al., 2008). More work may be needed to compare the antimicrobial effects of commercially and biologically combined SNPs and find the most effective ones as well as effective doses and sizes. The antibacterial effects of copper nanoparticles are less than those of silver nanoparticles, and Ag-Cu bimetallic nanoparticles showed higher antimicrobial activity (Nazeruddin et al., 2014).

In conclusion, the susceptibility of a microorganism to silver and copper nanoparticles has been clearly pointed out. Our results showed these microorganisms had sensitivity against the tested nanoparticles; however, *E. coli* showed higher sensitivity than *S. aureus* to both nanoparticles. Among the tested fungi, *P. chrysogenum* had higher inhibition zones. In addition, copper chloride had less toxicity in both nanoparticles, especially against the tested fungi.

Acknowledgments
The authors wish to thank Dr Mehrdad Forough and Dr Rahim Molaei from the chemistry department of Urmia University for their help with the synthesis of nanoparticles.

References

Agnihotri S, Mukherji S, Mukherji S (2014). Size-controlled silver nanoparticles synthesized over the range 5–100 nm using the same protocol and their antibacterial efficacy. RSC 4: 3974–3983.

Aitken RJ, Chaudhry MQ, Boxall ABA, Hull M (2006). Manufacture and use of nanomaterials: current status in the UK and global trends. Occup Med 56: 300–306.

Akinoglu EM, Morfa AJ, Giersig M (2014). Nanosphere lithography—exploiting self-assembly on the nanoscale for sophisticated nanostructure fabrication. Turk J Phys 38: 563–572.

Ashrafi M, Sarajuoghi M, Mohammadi K, Zarei S (2013). Effect of nanosilver application on agronomic traits of soybean in relation to different fertilizers and weed density in field conditions. EEB 11: 53–58.

Azam A, Ahmed AS, Oves M, Khan MS, Habib SS, Memic A (2012). Antimicrobial activity of metal oxide nanoparticles against Gram-positive and Gram-negative bacteria: a comparative study. Int J Nanomedicine 7: 6003–6009.

Cheesbrough M (2000). District Laboratory Practice in Tropical Countries, Part 2. 2nd edition. Fakenham, Norfolk, UK: Cambridge University Press.

Cubillo EA, Pecharromán C, Aguilar E, Santarén J, Moya JS (2006). Antibacterial activity of copper monodispersed nanoparticles into sepiolite. J Mater Sci 41: 5208–5212.

David E, Elumalai EK, Prasad TNVKV, Venkata K, Nagajyothi PC (2010). Green synthesis of silver nanoparticle using *Euphorbia hirta* L. and their antifungal activities. J Appl Sci Res 2: 76–81.

Demir E, Kaya N, Kaya B (2014). Genotoxic effects of zinc oxide and titanium dioxide nanoparticles on root meristem cells of *Allium cepa* by comet assay. Turk J Biol 38: 31–39.

Forough M, Farhadi K (2010). Biological and green synthesis of silver nanoparticles. Turkish J Eng Env Sci 34: 281–287.

Grass LRN, Athanassiou EK, Stark WJ (2010). Bottom-up fabrication of metal/metal nanocomposites from nanoparticles of immiscible metals. Chem Mater 22: 155–160.

Hajipour MJ, Fromm KM, Ashkarran AA, de Aberasturi DJ, de Larramendi IR, Rojo T, Serpooshan V, Parak WJ, Mahmoudi M (2012). Antibacterial properties of nanoparticles. Trends Biotechnol 30: 499–511.

Jing X, Ye W, Qunji X, Xuedong W (2011). Synthesis of highly stable dispersions of nanosized copper particles using L-ascorbic acid. Green Chem 13: 900–904.

Jo YK, Kim BH, Jung G (2009). Antifungal activity of silver ions and nanoparticles on phytopathogenic fungi. Plant Dis. 93: 1037–1043.

Kato H (2011). In vitro assays: tracking nanoparticles inside cells. Nat Nanotechnol 6: 139–140.

Liu J, Qiao SZ, Hu QH, Lu GQ (2011). Magnetic nanocomposites with mesoporous structures: synthesis and applications. Small 7: 425–443.

Marambio-Jones C, Hoek EMV (2010). A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. J Nanopart Res 12: 1531–1551.

Metzler DM, Erdem A, Tseng YH, Huang CP (2012). Responses of algal cells to engineered nanoparticles measured as algal cell population, chlorophyll a, and lipid peroxidation: effect of particle size and type. J Nanotechnol 2012: 1–12.

Mirzajani F, Ghassempour A, Aliahmadi A, Esmaeili MA (2011). Antibacterial effect of silver nanoparticles on *Staphylococcus aureus*. Res. Microbiol 162: 542–549.

Mohanpuria P, Rana NK, Yadav SK (2008). Biosynthesis of nanoparticles: technological concepts and future applications. J Nanopart Res 10: 507–517.

Nazeruddin GM, Prasad RN, Shaikh YI, Shaikh AA (2014). Synergetic effect of Ag-Cu bimetallic nanoparticles on antimicrobial activity. Der Pharmacia Lettre 3: 129–136.

Popescu M, Velea A, Lorinczi A (2010). Biogenic production of nanoparticles. Dig J Nanometers Bios 5: 1035–1040.

Prabhu N, Divya TR, Yamuna GK, Siddiqua AS, Puspha JD (2010). Silver phytho nanoparticles and their antibacterial efficacy. Dig J Nanomater Bios 5: 185–189.

Rezaei-Zarchi S, Imani S, Mohammad Zand A, Saadati M, Zaghari Z (2012). Study of bactericidal properties of carbohydrate-stabilized platinum oxide nanoparticles. Int Nano Lett 2: 40–45.

Sangiliyandi G, Jae WH, Deug-Nam K, Jin-HK (2014). Enhanced antibacterial and anti-biofilm activities of silver nanoparticles against Gram-negative and Gram-positive bacteria. Nanoscale Res Lett 9: 1–17.

Selvaraj KRN, Krishnamoorthi E, Singh BK (2011). Effect of biologically synthesized nanoparticles with plant products and chemotherapeutics against biofilm of clinical isolates of *Staphylococcus aureus* and *Candida tropicalis*. Int J Pharma Inf J Biotechnol Biother 1: 1–10.

Smetana A, Klabunde K, Marchin G, Sorensen C (2008). Biocidal activity of nanocrystalline silver powders and particles. Langmuir 24: 7457–7464.

Veerasamy R, Xin TZ, Gunasagaran S, Xiang TFW, Yang EFC, Jeyakumar N, Dhanaraj SA (2011). Biosynthesis of silver nanoparticles using mangosteen leaf extract and evaluation of their antimicrobial activities. J Saudi Chem Soc 15: 113–120.

Yasa I, Lkhagvajav N, Koizhaiganova M, Celik E, Sari O (2012). Assessment of antibacterial activity of nanosized Ag doped TiO_2 colloids. World J Microbiol Biotechnol 28: 2531–2539.