Digest Journal of Nanomaterials and Biostructures Vol. 8, No. 1, January - March 2013, p. 273 - 280

BIOSYNTHESIS, ANTIMICROBIAL AND CYTOTOXIC EFFECTS OF SILVER NANOPARTICLES USING ROSMARINUS OFFICINALIS EXTRACT

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The biosynthesis of nanoparticles has received increasing attention due to the growing need to develop safe, cost-effective and environmentally friendly technologies for nano-materials synthesis. In the present investigation, we report, biomedical potential of silver nanopaticles synthesized from extract of *Rosmarinus officinalis* on different pathogenic bacteria, yeast and toxicity against human acute promyelocytic leukemia (HL-60) cell line. 10 mL of extract was mixed with 90 mL of 2 mM or 1mM aqueous $AgNO_3$ and heated at 70 °C for 3min. A change from yellowish to dark brown colour was observed. Characterization using UV-VIS spectrophotometery and X- ray diffraction analysis were performed. Antimicrobial activity against six microorganisms was tested using disc diffusion method and cytoxicity test using MTT was obtained on the human leukemia cell line (HL-60). The synthesized silver nanoparticles efficiently inhibited various pathogenic organisms and reduce viability of the HL-60 cells in a dose-dependent manner. It has been demonstrated that the extract of *Rosmarinus officinalis* are capable of producing silver nanoparticles and these particles are quite stable in solution and further studies are needed to fully characterize the antiproliferative potential to examine whether this would prove to be a novel approach for accelerating anticancer potentials for other cases as well.

(Received November 26, 2012; Accepted January 25, 2013)

Keywords: Rosmarinus officinalis; Silver Nanoparticles; Antimicrobial activity; Cytotoxic effect

1. Introduction

The field of nanotechnology is one of active areas of research in modern materials science. Nanoparticles exhibit enhanced properties on specific characteristics such as size, distribution and morphology. Nanoparticles and nanomaterial's applications are increasing rapidly [1]. Silver nanoparticles have found tremendous applications in the areas of catalysis, optoelectronics, detection and diagnostic, antimicrobials and therapeutics [2-5]. Many attempts have been made to use silver nanoparticles as an anti-cancer agent and they have all turned up positive [6]. However, the role of silver nanoparticles as an anti-cancer agent should open new doors in the field of medicine.

There is still need for economic, commercially doable in addition environment friendly synthesis route to synthesize silver nanoparticles. Several approaches are out there for the synthesis of silver nanoparticles for example, chemical reduction [7], photochemical [8], reverse micelles [9], thermal decomposition [10], radiation assisted [11], electrochemical [12], and recently via green chemistry method [5].

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Biological method of nanoparticles synthesis using microorganisms [13], enzyme [14], and plant or plant extract offers numerous benefits over chemical and physical methods [5, 16]. Among the various known synthesis methods, plant-mediated nanoparticles synthesis is preferred as it is cost-effective, environmentally friendly, and safe for human therapeutic use [15, 17]. Many reports are available on the biogenesis of silver nanoparticles using several plant extracts, particularly *Lantana camara* [5], *Moringa oleifera* [18], *Catharanthus roseus* [19], *Eucalyptus hybrid* [20], *Cassia auriculata* [21]. However, potential of the plants as biological materials for the synthesis of nanoparticles is still under utilization.

Rosmarinus officinalis L. (Family: Lamiaceae), commonly referred to as rosemary, belongs to mint family. It is a popular herb in many western countries, with global cultivation and an exceptionally wide usage in the Mediterranean countries from where it originated. Rosemary has a long list of claims pertaining to its medicinal usage including antibacterial [22] and antioxidant properties [23]. It is known to be an effective chemo preventive agent, an antimutagenic [24]. The extract of plant has also been reported for its anti-carcinogenic, cognition-improving and certain glucose level lowering properties which makes it useful as a natural feed additive [25]. Here in, we report for the first time synthesis of Ag nanoparticles, reducing the silver ions present in the solution of silver nitrate by the aqueous extract of *R. officinalis*. Further these biologically synthesized nanoparticles were found highly toxic against different pathogenic microorganisms tested. Toxicity against human acute promyelocytic leukemia (HL-60) cell line was also evaluated.

2. Materials and Methods

2.1. Materials

The chemical silver nitrate ($AgNO_3$), Mueller-Hinton agar (MHA) and Sabouraud Dextrose agar (SDA, Oxoid) were purchased from Merck, Germany. Penicillin and streptomycin were purchased from Bio Source International, Belgium. Tissue culture plastic wares were obtained from BD Bioscience (USA). All organic solvents used were of HPLC grade. RPMI 1640, fetal bovine serum (FBS) and MTT (3-(4,5-Dimethyl-thiazol-2-yl)-2, S-diphenyltetrazolium bromide) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of the extract

Leaves plant extract was prepared by Soxhlet extraction method. About 100 g of powder material was uniformly packed into a thimble and run in Soxhlet extractor. It was exhaustible extracted with methanol for the period of about 48 hour or 22 cycles or till the solvent in the siphon tube of an extractor become colourless. After that extracts were filtered with the help of filter paper and solvent was evaporated from extract in rotary evaporator to get the syrupy consistency. Then extract was kept in refrigerator at 4 °C for future experiments.

2.3. Synthesis of silver nanoparticles

An aqueous solution of silver nitrate was prepared by adding 2mM or 1 mM of $AgNO_3$ to 90 ml of distilled water at room temperature. The aqueous solution was mixed with 10 ml of leaf extracts at a temperature of 70 °C while stirring magnetically at 1000 rpm for 3min. The bio-reduced aqueous component was used for the UV–Vis spectroscopy characterization.

2.4. Characterization of silver nanoparticles

UV-Vis spectral analysis was done by using UV-Vis spectrophotometer (PG- $T80^+$ UV/Vis spectrophotometer, England) from 350-700 nm at a resolution of 1 nm. XRD measurements of the silver nanoparticles solution drop-coated on glass were done on Shimadzu XRD-6000 model with 40 kv, 30 mA with Cu k α radiation at 2 θ angel. X-ray powder diffraction is a rapid analytical technique primarily used for phase identification of a crystalline material and can provide information on unit cell dimensions. The crystallite domain size was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherrer formula.

$$D = 0.94 \lambda / \beta \cos \theta$$

2.5. Evaluation of antibacterial activity

The silver nanoparticles synthesized using *R. officinalis* extract was tested for antimicrobial activity by agar well diffusion method against different pathogenic microorganisms *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus volgaris*, *Klebsiella pneumoniae* (Gram Negative), *Staphylococcus aureus* (Gram Positive) and *Candida albicans* (Yeast). The pure cultures of bacteria were subcultured on MHA and SDA for yeast. Each strain was swabbed uniformly onto the individual plates using sterile cotton swabs. Wells of 8 mm diameter were made on nutrient agar plates using gel puncture. Using a micropipette, 50 µL of nanoparticle solution was poured onto each well on all plates. After incubation at 37◦C for 24 hours, the diameter of zone inhibition was measured in millimeter, and was recorded as mean ± SD of the triplicate experiment.

2.6. Viability of HL-60 cells

Cell viability was evaluated by the MTT colourimetric technique. Briefly, 100 µl of the yellow tetrazolium MTT (3- (4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) without phenol red, are yellowish in colour solution (5 mg mL^{-1} in PBS) was added to each well. The plates were incubated for 3-4 h at 37 °C, for reduction of MTT by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan solubilized the MTT crystals by adding and quantified by spectrophotometric mean and then the supernatants were removed. For solubilization of the MTT crystals, 100 µL of isopropanol or DMSO was added to the wells. The plates were placed on a shaker for 15 min for complete solubilization of crystals and then the optical density of each well was determined. The quantity of formazan product as measured by the amount of 545 nm absorbance is directly proportional to the number of living cells in culture. Each experiment was done in triplicate. The relative cell viability (%) related to control wells containing cell culture medium without nanoparticles as a vehicle was calculated as follows:

$$\text{Percentage of cell viability } (\%) = \left(\frac{\text{Sample Absorbance}}{\text{Control Absorbance}} \right) \times 100$$

2.7. Statistical analysis

The grouped data were statistically evaluated using ANOVA with SPSS/14 software. Values are presented as the mean ± S.D. of the three replicates of each experiment.

3. Results

Green synthesis of silver nanoparticles using 2mM $AgNO_3$ is shown in Figure 1. The fresh suspension of *R. officinalis* was yellowish in colour. However, after addition of $AgNO_3$ and heated at 70 °C for 3 min, the suspension turned dark brown. Formation of silver nanoparticles was confirmed using UV-Vis spectral analysis and showed silver surface plasmon resonance band at 460 nm (Figure 2).

The X-ray diffraction pattern of the biosynthesized silver nanostructure produced by the leaf extract was further demonstrated and confirmed by the characteristic peaks observed in the XRD image (Figure 3). The Braggs reflections were observed in the XRD pattern at 2 θ were 32.37°, 38.40° and 44.85° in the whole spectrum of value ranging from 20 to 60 and indicated that the structure of silver nanoparticles is face-centred cubic (fcc). These are corresponding to [111] and [200] planes for silver, respectively. The lattice constant calculated from this pattern was a = 4.086 A° and the data obtained was matched with the database of Joint Committee on Powder Diffraction Standards (JCPDS) file No. 04-0783. The average grain size of the silver nanoparticles formed in the bioreduction process was determined using Scherr's formula and was estimated as 60 nm.

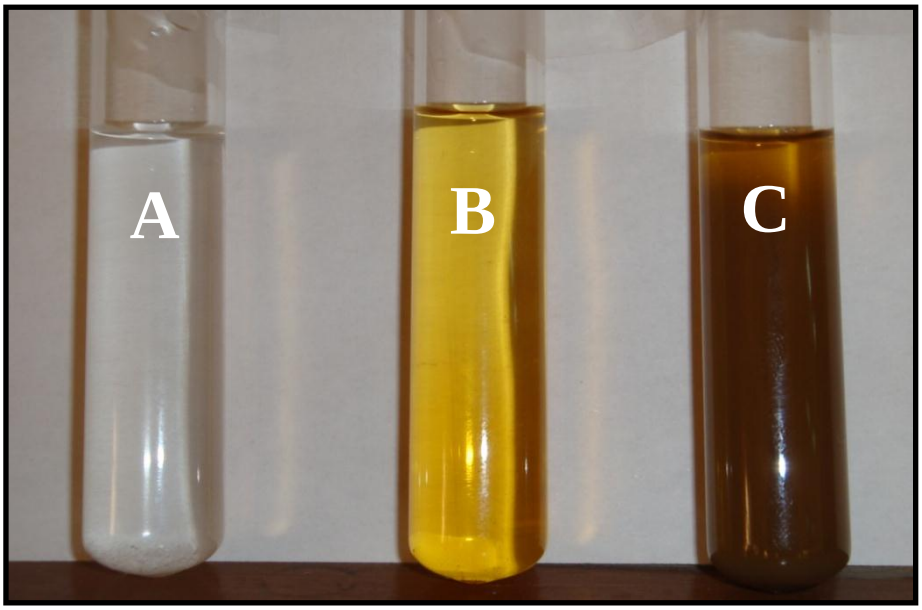

Fig. 1. Photograph showing colour changing. (a) 2mM AgNO3 without R. officinalis extract (b) Aqueous leaf extract of R. officinalis (c) Changing colour from yellowish to dark brown after adding 2mM AgNO3 and heated at 70 °C for 30 min.

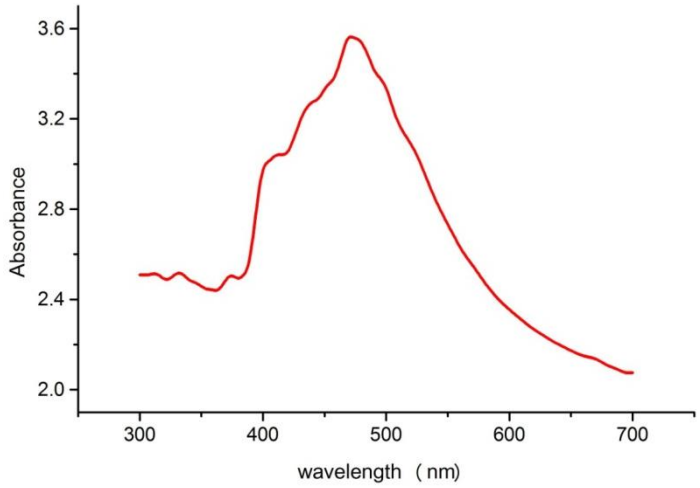

Fig. 2. UV/Vis absorption spectra of reduction of silver ions to silver nanoparticles after heating at 70 °C for 30 min.

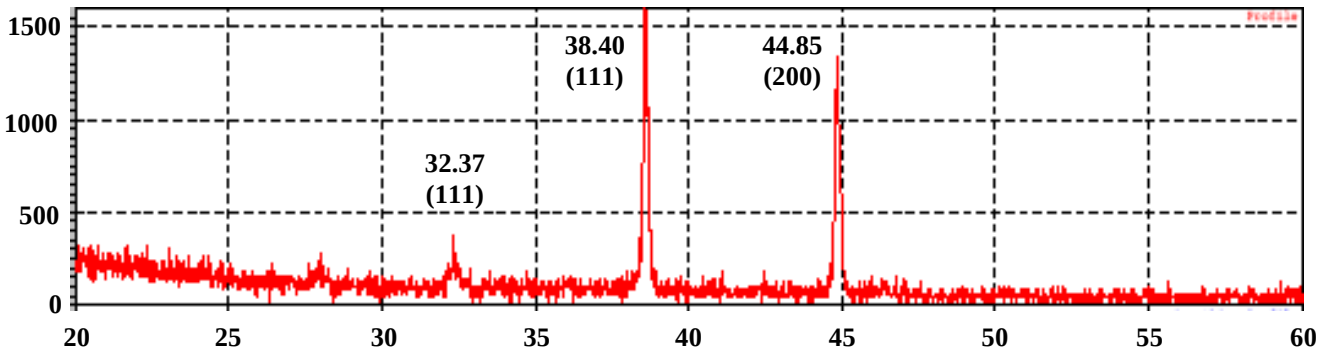

Fig. 3. XRD patterns recorded from drop-coated films on glass substrate of silver nanoparticles synthesized by treating R. officinalis extract with $AgNO_3$ aqueous solutions.

The antimicrobial activity of silver nanoparticles against various pathogenic organisms including bacteria and yeast was investigated. Compared with the control, the diameters of inhibition zones increased for all the test pathogens (Figure 4). It has been reported that antimicrobial effect was dose dependant. At 2mM silver nanoparticles, the 25 mm clear inhibitory zone appeared around 100 µl against *S. aureus* and *S. pneumoniae* after incubation for 24 h followed by *C. albicans* (24 mm), then 22 mm for Gram-negative bacteria *E. coli*, *K. pneumonia*, *P. aeruginosa* and *Proteus volgaris* suggesting that synthesized nanoparticles have good antibacterial action against Gram-positive organism than Gram-negative organisms.

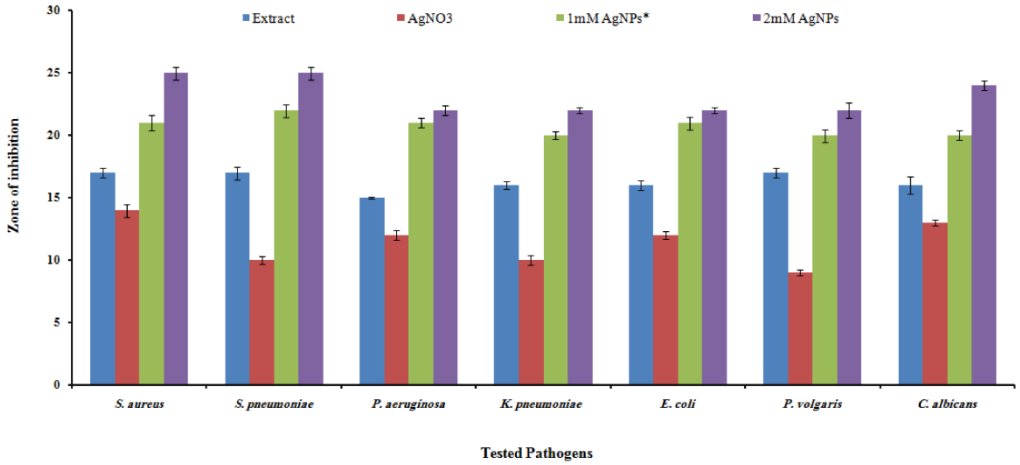

*Fig. 4. Size of the inhibition zone for silver nanoparticles synthesized by treating R. officinalis extract against the tested microorganisms. * AgNPs = Silver nanoparticles*

The in vitro cytotoxic effects of silver nanoparticles were screened against HL-60 cell line and viability of tumour cells was confirmed using MTT assay. The silver nanoparticles were able to reduce viability of the HL-60 cells in a dose-dependent manner as shown in (Figure 5). After six hours of treatment, the silver nanoparticles at concentration of 2 mM decreased the viability of HL-60 cells to 44% of the initial level. Longer exposures resulted in additional toxicity to the cells and reached to 80% dead cells after 24 h of incubation. However, the toxicity of AgNPs appeared much higher than that of $AgNO_3$ (22%) or *R. officinalis* extract only (33%) at the same period of incubation (Figure 5).

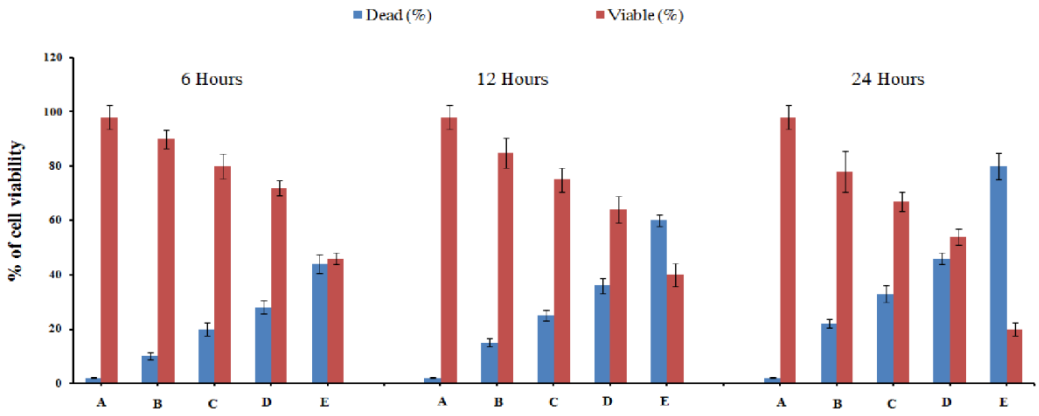

Fig. 5. Percent viability measured on HL-60 cells (A) Control (HL-60+RPMI) (B) AgNO (C) R. officinalis extract (D) 1 mM AgNPs (E) 2 mM AgNPs for 6, 12 and 24 h, by MTT assay. The data are expressed as Mean ± Standard deviation (SD) of three independent experiments.

4. Discussion

Reduction of silver ion into silver nanoparticles during exposure to the plant extracts could be followed by colour change. In the present study, silver nanoparticles exhibited dark brown colour in aqueous solution. These characteristic colour variations is due to the excitation of the of the surface plasmon resonance in the metal nanoparticles [26].

The frequency and width of the surface plasmon absorption depends on the size and shape of the metal nanoparticles as well as on the dielectric constant of the metal itself and the surrounding medium [21, 27]. It is generally recognized that UV–VIS spectroscopy could be used to examine size- and shape-controlled nanoparticles in aqueous suspensions [18, 28].

XRD is commonly used for determining the chemical composition and crystal structure of a material; therefore, detecting the presence of silver nanoparticles in plants extracts can be achieved by using XRD to examine the diffraction peaks of the plant [19]. In present study the X-ray pattern of synthesized silver nanoparticles matches the FCC structure of the bulk silver. The X-ray diffraction results clearly show that the silver nanoparticles formed by the reduction of Ag^+ ions by the *R. officinalis* might are crystalline in nature.

In this study, the application of silver nanoparticles as an antimicrobial agent was investigated and exhibited better antimicrobial activity against all human pathogens. However, the antimicrobial effect was dose-dependent, and was more pronounced against Gram-positive bacteria than Gram-negative bacteria. Additionally, the silver nanoparticles showed good inhibition activity towards *C. albicans*.

The mechanism of inhibitory action of silver nanoparticles on microorganisms, though not very clearly understood, could be by their adhesion to the cell membrane and further penetration inside or by interaction with phosphorus containing compounds like DNA disturbing the replication process or preferably by their attack on the respiratory chain. It has also been suggested that a strong reaction takes place between the silver ions and thiol groups of vital enzymes thus inactivating them. Some studies reported that the attachment of the nanoparticles on to the surface of the cell membrane disturbs the permeability and respiration functions of the cell. Experimental evidence advocated the loss of replication ability by the DNA when treated with silver ions which results in loss of cell viability and eventually resulting in cell death [29-32].

In this study, we have employed a time- and dose dependent approach to evaluate the toxicity of the nanoparticles on human acute promyelocytic leukemia (HL-60). The viability of HL-60 cells considerably decreased with increasing doses and time of incubation. The mortality data obtained in these results allow us to predict their potential not only because of the cytotoxic effect, but also in terms of the potential for tumour reduction. The cytotoxic effects of silver are the result of active physicochemical interaction of silver atoms with the functional groups of intracellular proteins, as well as with the nitrogen bases and phosphate groups in DNA [33- 35].

5. Conclusion

In conclusion, the rapid biological synthesis of silver nanoparticles using *R. officinalis* extract provides a stable, environmental friendly, simple and efficient route for synthesis of nanoparticles. These obtained silver nanoparticles have potential applications in the biomedical field and this simple procedure has several advantages such as cost effectiveness, compatibility for medical and pharmaceutical applications, as well as large scale commercial production. The biosynthesized silver nanoparticles showed excellent antimicrobial activity and possessed considerable cytotoxic effect against HL-60. Future application of silver nanoparticles as an antiproliferative agent could be limited by the fact that it is equally toxic to normal cells. Hence it is imperative that the biological applications employing silver nanoparticles should be given special attention besides embracing the antimicrobial potential. Further studies must be conducted in antiproliferative potential to examine whether this would prove to be a novel approach for accelerating anticancer potentials for other cases as well.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgment

The authors are sincerely thankful to Miss Wasnna H. Mohammed for the microbiological aspect of this work and facilities to accomplish the present research project within time.

References

[1] C. Silvestre, D. Duraccio, S. Cimmino, Prog. Polym. Sci. **36**, 766 (2011).
[2] D. Jain, H.K. Daima, S. Kachhwaha, S.L. Kothari, Dig. J. Nanomater. Biostruct. **4**, 723 (2009).
[3] S. Gurunathan, K. Kalishwaralal, R. Vaidyanathan, V. Deepak, S.R.K. Pandian, J. Muniyandi, Colloids Surf. B **74**, 328 (2009).
[4] V. Parashar, R. Parashar, B. Sharma, A.C. Pandey, Dig. J. Nanomater. Biostruct. **4**, 45 (2009).
[5] P. Sivakumar, C. Nethra Devi, S. Renganathan, Asian J. Pharm. Clin. Res. **5**, 97 (2012).
[6] R. Vaidyanathan, K. Kalishwaralal, S. Gopalram, S. Gurunathan. Biotechnol. Adv. **27**, 924 (2009).
[7] I.P. Santos, L.M.L. Marzan. Langmuir **15**, 948 (1999).
[8] R.K. Bera, A.K. Das, C.R. Raj. Chem. Mater. **22**, 4505 (2010).
[9] K.T. Lim, H.S. Hwang, W. Ryoo, K.P. Johnston. Langmuir **20**, 2466 (2004).
[10] I.J.L. Plante, T.W. Zeid, P. Yangab, T. Mokari. J. Mater. Chem. **20**, 6612 (2010).
[11] Y. Cheng, L. Yin, S. Lin, M. Wiesner, E. Bernhardt, J. Liu. J. Phys. Chem. C **115**, 4425 (2011).
[12] T. Hirsch, M. Zharnikov, A. Shaporenko, J. Stahl, D. Weiss, O. S. Wolfbeis, V. M. Angew. Chem. Int. Ed. **44**, 6775 (2005).
[13] S. Sunkar, C. V. Nachiyar. Global J. Med. Res. **12**, 43 (2012).
[14] H. Schneidewind, T. Schuler, K. K. Strelau, K. Weber, D. Cialla, M. Diegel, R. Mattheis, A. Berger, R. Moller, J. Popp. Beilstein J. Nanotechnol. **3**, 404 (2012).
[15] P. Kouvaris, A. Delimitis, V. Zaspalis, D. Papadopoulos, S. A. Tsipas, N. Michailidis. Materials Lett. **76**, 18 (2012).
[16] Y.S. Jae, S.K. Beom. Bioprocess Biosyst. Eng. **32**, 79 (2009).
[17] V. Kumar, S. K. Yadav. J. Chem. Technol. Biotechnol. **84**, 151 (2009).
[18] T.N.V.K.V. Prasad, E.K. Elumalai. Asian Pac. J. Trop. Biomed. **1**, 439 (2011).
[19] C. Panneerselvam, S. Ponarulselvam, K. Murugan, K. Kalimuthu, S. Thangamani. Asian Pac. J. Trop. Biomed. 574 (2012).
[20] M. Dubay, S. Bhadauria, B.S. Kushwah. Dig. J. Nanomater. Biostruct. **4**, 537 (2009).
[21] C. Udayasoorian, R.V. Kumar, M. Jayabalakrishnan. Dig. J. Nanomater. Biostruct. **6**, 537 (2011).
[22] K. Karamanoli, D. Vokou, U. Menkissoglu, I. H. Constantinidou. J. Chem. Ecol. **26**, 2035 (2000).
[23] K. Ozcan. J. Med. Food **6**, 267(2003).
[24] M. Minnunni, U. Wolleb, O. Mueller, A. Pfeifer, H.U. Aeschbacher. Mutat. Res. **269**, 193 (1992).
[25] Z.R. Faixov, S. Faix. Foliav Eterinaria. **3-4**, 135 (2008).
[26] P. Kouvaris, A. Delimitis, V. Zaspalis, D. Papadopoulos, S.A. Tsipas, N. Michailidis. Materials Lett. **76**, 18 (2012).
[27] A.R. Bijanzadeh, M. R. Vakili, R. Khordad. Internat. J. Phys. Sci. **7**, 1943 (2012).
[28] B.J. Wiley, S.H. Im, Z.Y. Li, J. McLellan, A. Siekkinen, Y. Xia. J. Phys. Chem. B **110**, 15666 (2006).
[29] Y. Matsumura, K. Yoshikata, S. Kunisaki, T. Tsuchido. Appl. Environ. Microbiol. **69**, 4278 (2003).

[30] S.K. Gogoi, P. Gopinath, A. Paul, A. Ramesh, S.S. Ghosh, A. Chattopadhyay Langmuir **22**, 9322 (2006).
[31] S.H. Kim, H.S. Lee, D.S. Ryu, S.J. Choi, D.S. Lee. Korean J. Microbiol. Biotechnol. **39**, 77 (2011).
[32] M. Rai, A.Yadav, A. Gade Biotechnol. Adv. **27**, 76 (2009).
[33] S. Moaddab, H. Ahari, D. Shahbazzadeh, A.A. Motallebi, A.A. Anvar, J. Rahman-Nya, M. R. Shokrgozar. Int. Nano. Lett. 1, 11 (2011).
[34] K. Satyavani, S. Gurudeeban, T. Ramanathan, T. Balasubramanian. J. Nanobiotechnol. **9**, 2 (2011).
[35] P.V. AshaRani, G. Low Kah Mun, M.P. Hande, S. Valiyaveettil. ACS Nano. **3**, 279 (2009).