PLANT-DERIVED NANOPARTICLES ENHANCE ANTIVIRAL ACTIVITY AGAINST COXSAKIEVIRUS B3 BY ACTING ON VIRUS PARTICLES AND VERO CELLS

ABID NABIL BEN SALEM[a,*], ROUIS ZYED[a], MOHAMED ALI LASSOUED[b], SOUALEH NIDHAL[c], SOUAD SFAR[b], AOUNI MAHJOUB[a]

[a]*Laboratory of Transmissible Diseases and Biological Actives Substances LR99-ES27, Faculty of Pharmacy, Avenue Avicenne, 5000, Monastir, University of Monastir, Tunisia*
[b]*Laboratory of Galenic Pharmacy, Faculty of Pharmacy, Avenue Avicenne, 5000, Monastir, University of Monastir, Tunisia*
[c]*Laboratory for Research on Biologically Compatible Compounds, Faculty of Dentistry, Avenue Avicenne, 5019 Monastir, University of Monastir, Tunisia*

Ricinus communis L. (Euphorbiaceae) is widely cultivated in the tropics and warm regions for castor oil. It possesses various biological activities such as hepatopro-tective, insecticidal, contraceptive, and antifertility activity. The antimicrobial potential of this plant need to be evaluated in order to more characterize the content of its bioactive compounds. The aims of the present study were the biosynthesis of nanoparticles derived from plant and the evaluation their antiviral activity as well as their mode of action by incubating the test samples with the virus prior to infection or with cell culture before inoculation with the virus suspension. Nanoparticles showed greater antiviral activity than the aqueous extracts and they act on the virus and the cell culture. The concentration of these NPs at which infectivity was inhibited by 50% (IC$_{50}$) ranged from 344 to 375 µg/mL. We have reported for the first time the synthesis of nanoparticles derived from *Ricinus communis* aqueous extracts. The results in the present study showed promising findings that need to be more evaluated.

1. Introduction

The field of nanotechnology is one of the most active areas of research in modern materials science. Nanoparticles exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology. New applications of nanoparticles and nanomaterials are emerging rapidly [1, 2, 3]. Nanocrystalline silver particles have found tremendous applications in the field of high sensitivity biomolecular detection and diagnostics, antimicrobials and therapeutics, Catalysis and micro-electronics [6-8]. However, there is still need for economic, commercially viable as well environmentally clean synthesis route to synthesize silver nanoparticles. A number of approaches are available for the synthesis of silver nanoparticles for example, reduction in solutions [9], chemical and photochemical reactions in reverse micelles [10], thermal decomposition of silver compounds [11], radiation assisted [12], electrochemical

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*Corresponding author: nabilabid@udbukltd.com

[13], sonochemical [14], microwave assisted process [15] and recently via green chemistry route [16-18].

The use of environmentally benign materials like plant leaf extract [19], bacteria [20], fungi [21] and enzymes [22] for the synthesis of silver nanoparticles offers numerous benefits of eco-friendliness and compatibility for pharmaceutical and other biomedical applications as they do not use toxic chemicals for the synthesis protocol. Chemical synthesis methods lead to presence of some toxic chemical absorbed on the surface that may have adverse effect in the medical applications. Green synthesis provides advancement over chemical and physical method as it is cost effective, environment friendly, easily scaled up for large scale synthesis and in this method there is no need to use high pressure, energy, temperature and toxic chemicals. Silver has long been recognized as having inhibitory effect on microbes present in medical and industrial process [23, 24]. The most important application of silver and silver nanoparticles is in medical industry such as topical ointments to prevent infection against burn and open wounds [25].

Metal nanoparticles have been studied for their antimicrobial potential and have proven to be antibacterial agents against both Gram-negative and Gram-positive bacteria [4, 5, 26, 27, 36]. Theoretically, any metal could be analysed for antiviral activity, however, little effort has been done to determine the interactions of metal nanoparticles with viruses, and only recently some studies have emerged showing that metal nanoparticles can be effective antiviral agents against HIV-1 [37–40], hepatitis B virus [41], respiratory syncytial virus [42], herpes simplex virus type 1 [43, 44], monkeypox virus [45], influenza virus [46] and Tacaribe virus [47].

Ricinus communis L. (Euphorbiaceae) is widely cultivated in the tropics and warm regions for castor oil [49]. It possesses various biological activities such as hepatopro-tective [50, 51], insecticidal [52], contraceptive [53] and antifertility activity [54]. The antimicrobial potential of this plant need to be evaluated in order to more characterize the content of its bioactive compounds.

The aim of the present study was the evaluation of the antiviral activity of *Ricinus communis* aqueous extracts of different plant aerial parts and their corresponding biologically synthesized NPs.

2. Experimental

2.1. Plant material and preparation of the Extract
Ricinus communis fruit and leaf extracts were used to make the aqueous extract. Leaf and fruit weighing 20g each were thoroughly washed in distilled water, dried, cut into fine pieces and were crushed into 100 ml sterile distilled water and filtered through Whatman No.1 filter paper (pore size 25 μm).

2.2. Biosynthesis and characterization of AgNPs
UV-Vis Spectra analysis. Aqueous solution of Silver nitrate ($AgNO_3$) was prepared and used for the synthesis of silver nanoparticles at a concentration of 1mM. 10 ml of plant extract was added into 90 ml of aqueous solution of 1mM Silver nitrate for reduction into Ag+ ions and kept at room temperature in the dark for 5 hours. The reduction of pure Ag+ ions was monitored by measuring the UV-Vis spectrum of the reaction medium at 5 hours after diluting a small aliquot of the sample into distilled water. UV-Vis spectral analysis was carried out using UV-Vis spectrophotometer Evolution EV 60 (Thermo Fisher Scientific).

Granulometry. Particles size measurements of the powder samples were carried out with a Beckman-Coulter LS 230 laser granulometer in the 0.1 – 10000 nm range. The nanosize range was determined for the colloidal suspensions using a Zetasizer Nano system (Zetasizer Ver. 6.20) from Malvern Instruments.

The biosynthesized NPs were prepared in RPMI 1640 cell culture media. Following serial dilutions of the stock were made in culture media.

2.3. Cytotoxicity assay and antiviral activity
Cell culture and virus. The Vero cell line was maintained in RPMI 1640 supplemented with fetal bovine serum (10% v/v), L-Glutamin (2mM), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. Coxakievirus B3

Nancy strain (kindly provided by Pr. Bruno Pozzetto, Laboratory of Bacteriology-Virology, Saint-Etienne, France) was propagated in Vero cells.

Preparation of Virus Stock. Coxakievirus B3 Nancy strain suspension (0.1ml) was used to infect a confluent monolayer of Vero cells in 75 cm^2 culture flask and adsorbed for 1hour to allow the viruses to adhere onto the cells. Non-adherent particles were washed off using 2% RPMI 1640 medium and the infected cells overlaid with 20 ml of 2% RPMI 1640 (maintenance medium) and incubated until full cytopathic effect was observed in 5 to 6 days. This was further repassed twice and the harvested virus stored at –20°C until used.

Cytotoxicity assay. In this assay, aqueous extract and NPs are tested individually to see if they either cure an infected cell, or protect it from infection, pathogenic effects. The process is simple, and relies on a cell culture system able to support virus growth.

The evaluation is based on the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), by themitochondrial dehydrogenase of viable cells, to give a blue formazan product which can be measured spectrophotometrically. The MTT colorimetric assay was performed in 96-well plates. Cells were seeded in 96-well plates at a concentration of 5 x 10^4 cells/well and incubated for 24h at 37°C in a 5% CO_2 humidified atmosphere. Microscopic examination insured that stable normal cell layers were maintained in each well throughout every experiment. After treatment with various concentration of the test compound (78, 156, 312.5, 625, 1250, 2500, 5000, and 10000 μg/mL), the cells were incubated for an additional 48 h at 37°C. The cells were examined daily under a phase-contrast microscope to determine the minimum concentration of compound that induced alterations in cell morphology. After that, the medium was removed and cells in each well were incubated with 100μl of MTT solution (5 mg/ml) for 4 h at 37°C. MTT solution was then discarded and 50μl dimethyl sulfoxide (DMSO) was added to dissolve insoluble formazan crystal and the plates were incubated at 37°C for 30 min. Optical density (OD) was measured at 540 nm using a Perkin-Elmer ELISA reader (HTS 7000 plus). Data were obtained from triplicate wells. Cell viability was expressed with respect to the absorbance of the control wells (untreated cells), which were considered as 100% of absorbance. The percentage of cytotoxicity is calculated as [(A-B)/A]x100, where A and B are the OD$_{540}$ of untreated and of treated cells, respectively. The 50% cytotoxic concentration (CC$_{50}$) was defined as the compound's concentration (μg/ml) required for the reduction of cell viability by 50%, which were calculated by regression analysis.

Virus inhibition assay. Confluent Vero cell cultures were treated with nanoparticles during and after virus infection in three sets of experiments as follows: (1) 5 x 10^4 TCID$_{50}$ of the virus was exposed with three effective minimal cytotoxic concentrations of plant extracts (1250μg/mL, 2500μg/mL, 5000μg/mL) and silver NPs (125μg/mL, 250μg/mL, 500μg/mL) for one hr at 37°C. Then 100 μl of the mixture was added to the cells cultured fluently in 96-well flat-bottom microtiter plate (100 μl); (2) Cells were treated with three effective minimal cytotoxic concentrations of plant extracts and silver NPs (100 μl) for one hr at 37°C. After one hr incubation at 37°C, 5 x 10^4 TCID$_{50}$ of the virus (100 μl) were added.

All plates were incubated at CO_2-incubator for 48 hrs. The viability of the infected and non-infected cells was evaluated using absorbance values of formazan. The percent of protection was calculated as follows:

Percent protection = [(ODT) V-(ODC) V] / [(ODC) M-(ODC) V] ×100

Where (ODT) V, (ODC) V and (ODC) M indicate absorbance of the sample, the virus-infected control (no compound) and mock-infected control (no virus and no compound), respectively (10).

3. Results
3.1. UV-vis spectra and granulometric analysis
The absorption peak is obtained at 240 nm and 330 nm for *Ricinus communis* leaf and fruit extracts (Figure 1). The detection of two picks at different wavelengths indicated the presence of more than one population of NPs with different sizes and shapes. Size distribution by intensity

showed monomodal distribution with narrow band with picks at about 744 nm and 801 nm for *Ricinus communis* leaf and fruit extracts, respectively. The size of particles has increased for nanoparticles with picks at about 981 nm and 1047 nm for *Ricinus communis* leaf and fruit NPs, respectively (Figure 2). The difference in size of nanoparticles is governed by the concentration of phenolic compounds as discussed previously [48].
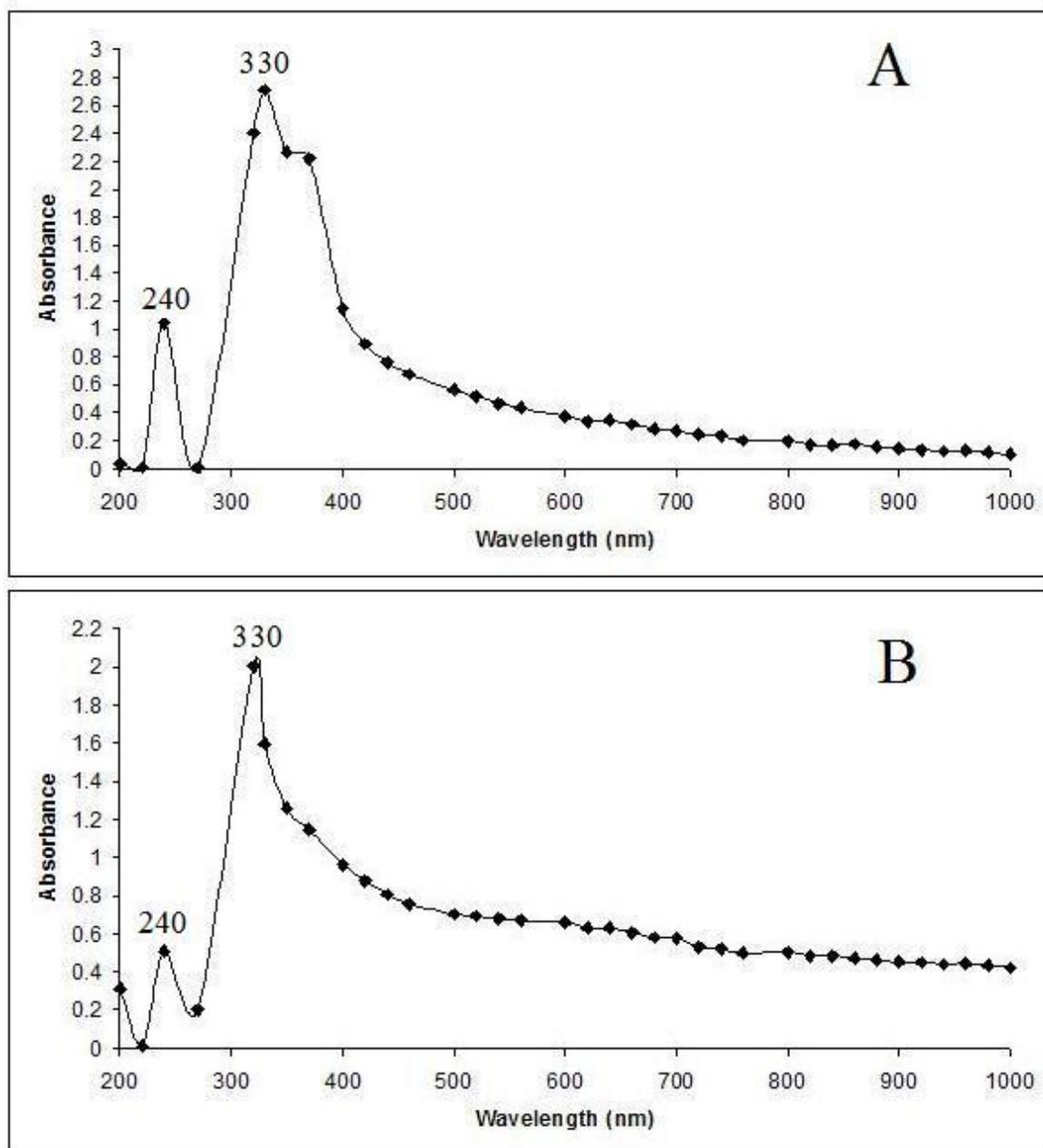
Fig. 1. UV-Vis absorption spectrum of silver nanoparticles synthesized by treating 1 mM aqueous AgNO3 solution with 10% Ricinus communis leaf (A) and fruit (B) extracts after 5 hrs.

3.2. Cytotoxic effect and antiviral activity

Vero cells were used as models to assess silver nanoparticles' cytotoxicity. By means of MTT-based assay, the 50% cytotoxic concentrations (CC_{50}) of plant extracts were defined as 16.5 ± 3.5 mg/mL and 14.0 ± 2.7 mg/mL against Vero cells for *Ricinus communis* leaf and fruit extracts, respectively, whereas CC_{50} was defined as 10.0 ± 1.9 mg/mL and 15.0 ± 2.4 mg/mL against Vero cells for *Ricinus communis* leaf and fruit NPs, respectively.

In order to elucidate the mode of antiviral action and to identify the target site, cells were pretreated with extracts before viral infection (pretreatment of cells) and virus was incubated with extracts before cell infection (pre-treatment of virus). All extracts tested were used at their maximum non-cytotoxic concentrations (125, 250, and 500 µg/mL). To evaluate the activity of antiviral agents in vitro, the selectivity index (SI = CC_{50}/IC_{50}) was determined. The selectivity index describes the ratio between the cytotoxic and the antiviral activity of a substance.

The *Ricinus communis* leaf and fruit extract showed no significant inhibition of viral infection, whereas the most significant antiviral activity was shown for *Ricinus communis* fruit NPs (1047 nm). The concentration of these NPs at which infectivity was inhibited by 50% (IC_{50}) ranged from 344 to 375 µg/mL (Table 1). The SI were 40 and 44 µg/mL for virus pretreatment and cell pretreatment with *Ricinus communis* fruit NPs, respectively.
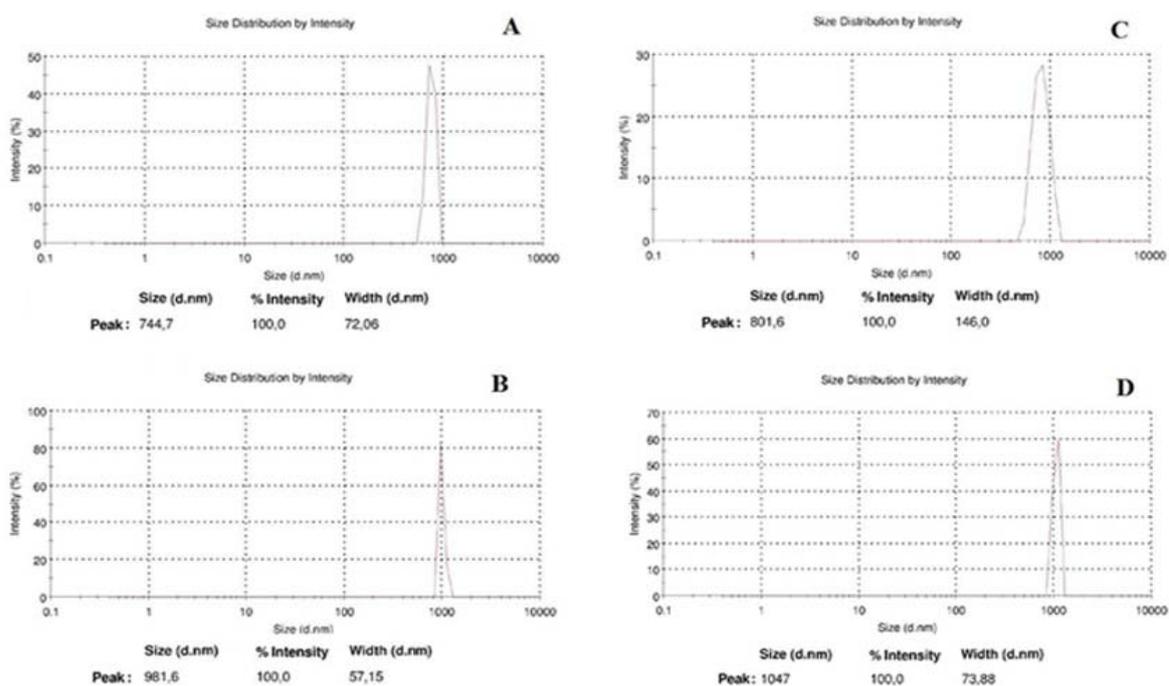
A: *Ricinus communis* leaf extract; B: *Ricinus communis* leaf extract NPs; C: *Ricinus communis* fruit extract; D: *Ricinus communis* fruit extract NPs.

Table 1. Determination of the inhibitory concentrations (IC$_{50}$) and selectivity index (SI = CC$_{50}$/IC$_{50}$) against coxakievirus B3

		% viability at different extract concentrations (µg/mL)									
		125	250	500			1250	2500	5000		
Cell pre-treatment	CC$_{50}$ (mg/mL)				IC$_{50}$ (µg/mL)	SI				IC$_{50}$ (µg/mL)	SI
Ricinus communis (A)	16.5	ND	ND	ND	ND	ND	0	0	0	ND	ND
Ricinus communis (B)	14.0	ND	ND	ND	ND	ND	0	0	0	ND	ND
Ricinus communis (C)	10.0	5.4	0	0	ND	ND	ND	ND	ND	ND	ND
Ricinus communis (D)	15.0	0	16	66	340	44	ND	ND	ND	ND	ND
Virus pre-treatment	CC$_{50}$ (mg/mL)				IC$_{50}$ (µg/mL)	SI				IC$_{50}$ (µg/mL)	SI
Ricinus communis (A)	16.5	ND	ND	ND	ND	ND	0	0	0	ND	ND
Ricinus communis (B)	14.0	ND	ND	ND	ND	ND	0	0.4	4	ND	ND
Ricinus communis (C)	10.0	0	0	6	ND	ND	ND	ND	ND	ND	ND
Ricinus communis (D)	15.0	0	26	70	375	40	ND	ND	ND	ND	ND

(A): *Ricinus communis* leaf extract; (B): *Ricinus communis* fruit extract; (C): *Ricinus communis* leaf extrat NPs; (D): *Ricinus communis* fruit extract NPs.
ND: Not determined.

4. Discussion

The course of viral infections is governed by complex interactions between the virus and the host cellular system. All viruses replicate via a broadly similar sequence of events. The virus must first bind to the cell, and then the virus or its genome enters in the cytoplasm. The genome is liberated from the protective capsid and, either in the nucleus or in the cytoplasm, it is transcribed and viral mRNA directs protein synthesis, in a generally well regulated fashion. Finally, the virus undergoes genome replication and together with viral structural proteins assembles new virions which are then released from the cell. Each of the single described phases represents a possible target for inhibition.

The aqueous extracts of *Ricinus communis* leaf and fruit extracts and their corresponding NPs did not show cytotoxic effect on Vero cell culture. However, the antiviral activity of these extracts showed different pattern. The nanoparticles synthesized from plant fruit extract showed the greatest antiviral activity when both incubated with cell culture and virus suspension.

The biosynthesized nanoparticles in the present study have a size greater than the virus and lower than the cell size. Thus the antiviral activity of *Ricinus communis* fruit NPs showed in the present study may be due to fusion inhibition between virus and cells.

5. Conclusions

Here in, we report for the first time synthesis of silver nanoparticles from *Ricinus communis* fruit and leaf extracts. Further these biologically synthesized nanoparticles were found non toxic against Vero cell culture. However, the synthesized NPs from fruit extract showed anti-enteroviral activity and further analysis need to be done to more characterize the mode of action of these NPs. Together with the risk of emerging or re-emerging viral agents, the field of antiviral compound discovery is very promising. 'The first and the second authors are equally contributed in this work'.

References

[1] W. Jahn, J. Struct. Biol. **127**, 106 (1999).
[2] H.S. Naiwa, Ed. HandBook of Nanostructural Materials and Nanotechnology Academic Press New York., 1-5 (2000).
[3] C. J. Murphy, J. Mater Chem. **18**, 2173 (2008).
[4] J.R. Morones, J.L. Elechiguerra, A. Camacho, K. Holt, J.B. Kouri, J.T. Ramírez, M.J. Yacaman, Nanotechnol. **16**, 2346 (2005).
[5] J.S. Kim, E. Kuk, K.N. Yu, J.H. Kim, S.J. Park, H.J. Lee, S.H. Kim, Y.K. Park, Y.H. Park, C.Y. Hwang, Y.K. Kim, Y.S. Lee, D.H. Jeong, M.H. Cho, Nanomed. **3**, 95 (2007).
[6] S. Schultz, D.R. Smith, J.J. Mock, D.A. Schultz, PNAS **97**, 996 (2000).
[7] M. Rai, A. Yadav, A. Gade, Biotechnol. Advances. **27**, 76 (2009).
[8] J.L. Elechiguerra, J.L. Burt, J.R. Morones, A. Camacho-Bragado, X. Gao, H.H. Lara, M.J. Yacaman, J. Nanobiotechnol. **3**, 6 (2005).
[9] D.V. Goia, E. Matijevic, J. Chem. **22**, 1203 (1998).
[10] C. Taleb, M. Petit, P. Pileni, Chem. Mater. **9**, 950 (1997).
[11] K. Esumi, T. Tano, K. Torigoe, K. Meguro, Chem. Mater. **2**, 564 (1990).
[12] A. Henglein, Langmuir. **17**, 2329 (2000).
[13] L. Rodriguez-Sanchez, M.C. Blanco, M.A. Lopez-Quintela, J. Phys. Chem. B. **104**, 9683 (2000).
[14] J.J. Zhu, S.W. Liu, O. Palchik, Y. Koltypin, A. Gedanken, Langmuir. **16**, 6396 (2000).
[15] Pastoriza-Santos, L.M. Liz-Marzan, Langmuir. **18**, 2888 (2002).
[16] N.A. Begum, S. Mondal, S. Basu, R.A. Laskar, D. Mandal, Colloids and Surfaces B: Biointerfaces. **71**(1), 113 (2009).

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[17] H. Bar, D.K. Bhui, G.P. Sahoo, P. Sarkar, S.P. De, A. Misra., Colloids and Surfaces A: Physicochem. Engineer. Aspects. **339**, 134 (2009).

[18] J.Y. Song, B.S. Kim,Bioprocess Biosyst. Eng. **32**, 79 (2009).

[19] V. Parashar, R. Parashar, B. Sharma, A.C. Pandey, Digest J. Nanomater. Biostr. **4**(1), 45 (2009).

[20] N. Saifuddin, C.W. Wong, A.A.N. Yasumira, E-Journal of Chemistry. 6(1), 61 (2009).

[21] K.C. Bhainsa, S.F. D'Souza,.Colloids and Surfaces B: Biointerfaces. **47**, 160 (2006).

[22] B. Willner, B. Basnar, B. Willner, FEBS J. **274**, 302 (2007).

[23] R.M. Jose, L.E. Jose, C. Alejandra, Nanotechnol. **16**, 2346 (2005).

[24] C. Lok, C. Ho, R. Chen, Q. He, W. Yu, H. Sun, P.K. Tam, J. Chiu, C. Che, J. Biol. Inorg. Chem. **12**, 527 (2007).

[25] M. Ip, S.L. Lui, V.K. M. Poon, I. Lung, A. Burd, J. Medical Microbiol. **55**, 59 (2006).

[26] A.R. Shahverdi, A. Fakhimi, H.R. Shahverdi, S. Minaian, Nanomed. **3**, 168 (2007).

[27] I. Sondi & B. Salopek-Sondi, J. Colloid Interface Sci. **275**, 177 (2004).

[36] M. Rai, A. Yadav, A. Gade, Biotechnol. Adv. **27**, 76 (2009).

[37] J.L. Elechiguerra, J.L. Burt, J.R. Morones, A. Camacho-Bragado, X. Gao, H.H. Lara, M.J. Yacaman, J. Nanobiotechnol. **29**, 3 (2005).

[38] R.W. Sun, R. Chen, N.P. Chung, C.M. Ho, C.L. Lin, C.M. Che, Chem. Commun. (Camb) **40**, 5059 (2005).

[39] H.H. Lara, N.V. Ayala-Nuñez, L. Ixtepan-Turrent, C. Rodriguez-Padilla, J. Nanobiotechnol. **8**, 1 (2010).

[40] H.H. Lara, L. Ixtepan-Turrent, E.N. Garza-Treviño, C. Rodriguez-Padilla, J. Nanobiotechnol. **8**, 15 (2010).

[41] L. Lu, R.W. Sun, R. Chen, C.K. Hui, C.M. Ho, J.M. Luk, G.K. Lau, C.M. Che, Antivir. Ther. **13**, 253 (2008).

[42] L. Sun, A.K. Singh, K. Vig, S. Pillai, R. Shreekumar, S.R. Singh, J. Biomed. Biotechnol. **4**, 149 (2008).

[43] D. Baram-Pinto, S. Shukla, N. Perkas, A. Gedanken, R. Sarid, Bioconjug. Chem. **20**, 1497 (2009).

[44] D. Baram-Pinto, S. Shukla, A. Gedanken, R. Sarid, Small **6**, 1044 (2010).

[45] J.V. Rogers, C.V. Parkinson, Y.W. Choi, J.L. Speshock, S.M. Hussain, Nanoscale Res. Lett. **3**, 129 (2008).

[46] I. Papp, C. Sieben, K. Ludwig, M. Roskamp, C. Böttcher, S. Schlecht, A. Herrmann, R. Haag, Small **6**, 2900 (2010).

[47] J.L. Speshock, R.C. Murdock, L.K. Braydich-Stolle, A.M. Schrand, S.M. Hussain, J. Nanobiotechnol. **8**, 19 (2010).

[48] M.N. Nadagouda, G. Hoag, J. Collins, R.S. Varma, Growth Des. **9**, 4979 (2009).

[49] A. Scarpa, A. Guerci, J. Ethnopharmacol. **5**, 117 (1982).

[50] B. Shukla, P.K.S. Visen, G.K. Patnaik, N.K. Kapoor, B.N. Dhawan, Drug Develop. Res. **26**, 183 (1992).

[51] P.K.S. Visen, B. Shukla, G.K. Patnaik, S.C. Tripathi, D.K. Kulshreshtha, R.C. Srimal, International J. Pharmacognosy **30**, 241 (1992).

[52] S.M. Upasani, H.M. Kotkar, P.S. Mendki, & V.L. Maheshwari, Pest Management Sci. **59**, 1349 (2003).

[53] F.K. Okwuasaba, S.C. Das, C.O. Isichei, M.M. Ekwenchi, O. Onoruvwe, A.O. Olayinka, Phytother. Res. **10**, 97 (1997).

[54] K. Sandhyakumary, R.G. Bobby, & M. Indira, Phytotherapy Res. **17**, 508 (2003).