IJBPAS, August, 2015, 4(8): 5539-5550

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

ANTIBACTERIAL ACTIVITY OF DOXYCYCLINE CONJUGATED WITH SILVER NANOPARTICLES AGAINST *BRUCELLA BACTERIA IN VITRO* AND *IN VIVO*

MOHSEN AJALLI[1], MOJTABA SALOUTI*[2], HAMED ALIZADEH[1], ALI REZA KAZEMIZADEH[3], ZAHRA HEIDARI[2]

[1]Young Researchers and Elite Club, Zanjan Branch, Islamic Azad University, Zanjan, Iran

[2] Biology Research Center, Zanjan Branch, Islamic Azad University, Zanjan, Iran

[3]Department of Chemistry, Zanjan Branch, Islamic Azad University, Zanjan, Iran

***Corresponding Author: E Mail: saloutim@yahoo.com**

ABSTRACT

Brucellosis caused by Brucella bacteria is an important public health problem throughout the world. The aim of this study was the conjugation of doxycycline with silver nanoparticles to evaluate its efficiency to kill Brucella bacteria. At the first, doxycycline molecules were conjugated with silver nanoparticles and the interaction was approved by FT-IR analysis. The antimicrobial activity of the doxycycline-silver nanoparticles conjugate was determined against B. abortus 544 by agar well diffusion method. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the conjugate were determined by macrodilution method. Then, the infected animal model was used to study the antimicrobial effect of the doxycycline-silver nanoparticles conjugate in vivo. The results showed the enhanced antimicrobial activity of doxycycline-silver nanoparticles conjugate in comparison with doxycycline or silver nanoparticles alone in vitro and in vivo.

Keywords: *Brucellaabortus 544*, Antibacterial effect, Silver Nanoparticles, Doxycycline

INTRODUCTION

Brucellosis is an important public health problem throughout the world, especially in the Mediterranean region, Arabian Peninsula, Indian Subcontinent, Mexico, and some parts of Central and South America (1, 2). In 1986, the World Health Organization (WHO)

recommended the use of a 6 week course of doxycycline plus rifampicin to provide a totally oral regimen in the treatment of human brucellosis (3). Roushan *et al* reportedthe combination use of doxycycline for 6 weeks plus streptomycin for 14-21 days the highest rate of cure and the lowest rate of relapse in 2006 (4). Numerous other antibiotic classes have been clinically tested in the treatment of brucellosis (5). However, the researchers believe that antibiotics not suitable for brucellosis treatment. Therefore, it is necessary to find a new treatment for brucellosis (5). Doxycycline is a tetracycline antibiotic with a potent antibacterial activity against a wide variety of bacteria. However, poor cellular penetration limits its use for the treatment of infectious diseases caused by intracellular pathogens. One potential strategy to overcome this problem may be the use of nanoparticles as a carrier that can help to target the intracellular sites of infections (6).Silver ions and silver salts have been used for decades as antimicrobial agents in various fields because of their growth-inhibitory capacity against microorganisms. It is well known that silver ions and silver compounds have strong antimicrobial effects and many investigators are interested in using inorganic nanoparticles as antibacterial agents. The antimicrobial properties of silver was previously reported by Russell and Hugo in 1982 and these studies have established the bactericidal effect of silver nanoparticles on *Brucella melitensis* (8, 9).In this study, we used glutathione for conjugation of silver nanoparticles to doxycycline. Glutathione (GSH) is an important antioxidant in plants, animals, fungi and some bacteria and archaea, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides. It is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side-chain and the amine group of cysteine which is attached by normal peptide linkage to a glycine (7). The structure of glutathione was shown in figure 1.

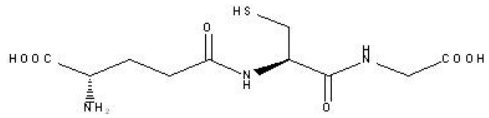


Figure1: The structure of glutathione

Greenhalgh et al. in 2009 prepared penicillin-conjugated polyacrylate nanoparticles as a new opportunity for development of a new anti-MRSA agent (10). They reported high antimicrobial activity of the prepared complex against methicillin-resistant *Staphylococcus aureus* in comparison with the non-conjugated form of the antibiotic. In another study Haider Naqvi et al. investigated antimicrobial activity of the combined form

of biologically synthesized silver nanoparticles and different antibiotics such as ciprofloxacin, imipenem, gentamycin, vancomycin, and trimethoprim against multidrug-resistant bacteria and showed the synergistic effect of the antibiotics and nanoparticles combination(11). They concluded that silver nanoparticles can be effectively used in combination with antibiotics in order to improve their efficacy against various pathogenic microbes (11).So far,the antibacterial activity of doxycycline conjugated with silver nanoparticles has not been investigated.

MATERIALS AND METHODS

Materials and media

Doxycycline was purchased from Sigma Aldrich, USA. *Brucella abortus 544* was obtained from microbial bank of the University of Tehran, Iran. Silver nanoparticles was purchased from NanoNasb Parscompany, Tehran, Iran in the size range of 3-18 nm and the concentration of 4000 ppm. The female BALB/c mice (6-8 weeks old and 25-35 g weight) were purchased from institute Pasture, Tehran, Iran. All the other chemical materials and microbial media were purchased from Merck, Germany.

Conjugation of doxycycline with silver nanoparticles

The conjugation of silver nanoparticles with doxycycline was performed in four steps as the followings:

1. **Conversion of Lipoic Acidto 5-(1,2-dithiolan-3-yl) pentanoyl chloride**

Lipoicacid**(1)** was converted to lipoyl chloride**(2)**to find capability for binding to amino groups of doxycycline in the following procedure:Lipoic acid (0.688 g, 3.33 mmol) was dissolved in 15 ml of dry dichloromethane (DCM) in 0^{o}C. Then,4-5 drops of *N*,*N*-dimethyl formamide (DMF) and oxalyl chloride (2.38 ml, 27.3 mmol) was added to the solution. When the gas bubbling was stopped, the solution was allowed to be stirred for an additional 20-30 min under the argon stream. Then,10 ml of DMF was added to the solution and the mixture was concentrated under the reduced pressure. Next, the residue was further dried under high vacuum at ambient temperature (12). The crude product was used in the next step without further purification (**Figure2**).

2. **Binding of lipoyl chloride to doxycycline**

Trifluoroacetic acid (TFA) (0.58 ml, 4.16 mmol) was added dropwise to freshly dissolved doxycycline **(3)** (2.77 mmol) in 30 ml of dichloromethane, and cooled to 0^{o}C under N_2gasstream. Lipoyl chloride **(2)**(3.30 mmol) was re-dissolved in 5 ml of

dichloromethane and added dropwise to the solution. The solution was allowed to be warmed to the ambient temperature overnight. Then, the reaction mixture was diluted with20 ml ethylacetate (EtOAc) and the pH was adjusted to 2 using the aqueous 1N HCl. Next, the precipitate was filtered and rinsed with ethylacetate. Then, it was washedusing50 ml of 1N HCl and brine, dried with Na_2SO_4, filtered and concentrated under the reduced pressure to obtain **(4)** (0.247 g, 27%) in powder form (12) (**Figure 3**).

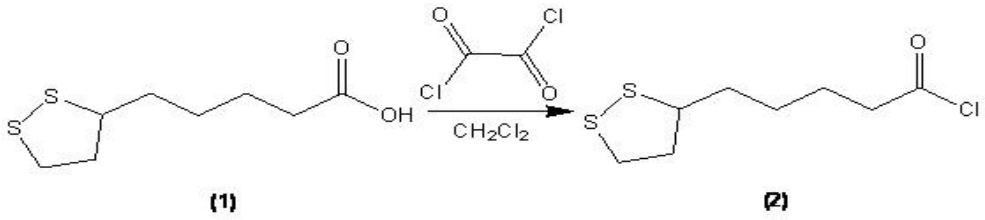


Figure2:Conversion of lipoic acid to lipoyl chloride

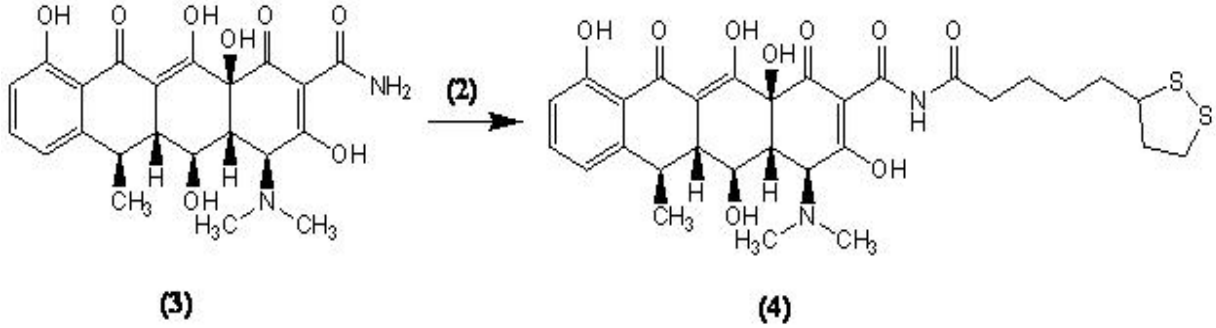


Figure 3: Binding of lipoyl chloride(2) to doxycycline(3)

3. Coating of silver nanoparticles with Glutathione

350µl of colloidal silver nanoparticles (SNPs) was mixed with 350µl of glutathione (GSH) and 100 mM of Tris-HCl, and was allowed to react at ambient temperature on a shaker for 3 days. The product was precipitated and washed with methanol (3X) and collected by centrifugating at 6000 g to obtain GSH-SNP. The GSH-SNP was then resuspended in 50% aqueous glycerol (12).

4. Conjugation ofSNP-GSH with lipoyl chloride-doxycycline

GSH-SN Pandlipoyl chloride-doxycycline were mixed in methanol/H_2O/glycerol (75:12.5:12.5, respectively) for 10 days. Then, the solution was centrifuged at 4°C for 5 min at 13000 rpm. The supernatant was removed and the product was washed with methanol (3X)and centrifuged at

mentioned conditions one more time. The supernatant was removed and the precipitate was kept for the next step. In order to remove the non-conjugated silver nanoparticles and free doxycycline molecules, the solution washed with methanol (3X) and centrifuged at 4°C for 5 min at 13000 rpm. Then, the supernatant was discarded and the precipitate was dissolved in the deionized water(12).

FT-IR analysis

The FT-IR spectra of GSH, GSH-SNP, doxycycline-Lipoylcholoride and doxycycline-Lipoylcholoride-GSH-SNP conjugate were recorded by Perkin-Elmer Fourier transform infrared spectroscopy (model FT/IR-Jasco 6300)to confirm the formation of the complex (13).

Antimicrobial activity assessment

Well diffusion Agar assay

Muller-Hinton agar was supplemented with 1% of sheep blood. 5 mm diameter wells were prepared and 1.5×10^8CFU/ml of *B. abortus 544* suspension was cultivated in plates with the sterile swab. Then, the wells were loaded with dilutions of SNP-doxycycline conjugate (1:2 to 1:256), doxycycline, silver nanoparticles and normal saline (control). The plates were incubated at 37°C under 7-10% CO_2gas for 72 h. Then, the zone of growth inhibition was measured by a ruler (9, 14).This process was repeated three times.

MIC and MBC determination assay

Serial dilutions of SNP-doxycycline conjugate (1:2 to 1:256) were prepared in Muller-Hinton broth supplemented with 1% of sheep blood in tubes. Then, 5×10^5CFU/ml of *B. abortus 544* suspension was added to each tube and incubated at 37°C under 7-10% CO_2 for 72 h. Next, the tubes were examined for turbidity. The lowest concentration of SNP-doxycycline conjugate that inhibited growth of the bacteria was designated as minimum inhibitory concentration (MIC). For calculation of minimum bactericidal concentration (MBC) of the conjugate, 0.1 ml of inoculums from each tube was sub-cultured on Muller-Hinton agar plates supplemented with 1% of sheep blood (9, 15). The number of colonies on agar was counted after 72 h of incubation at the same conditions and compared with the number of bacteria in the original inoculums. The lowest concentration of SNP-doxycycline conjugate that could kill 99.9 % of the bacteria was determined as MBC. The above steps were repeated for determination of MIC and MBC of silver nanoparticles alone and doxycycline alone for comparison.

Mouse Model

The animal study was conducted in 4 arms including: (a) SNP-doxycycline conjugate, (b) silver nanoparticles alone, (c) doxycycline alone and (d) normal saline (control). The twenty BALB/c mice were injected intraperitoneally with suspension of 5×10^5CFU/ml *B.abortus 544* at the first day. The mice were divided into 4 groups of 5mouse and 0.5 ml of MBC concentration of the above treatments was injected intraperitoneally to each group separately on the second day. After 7 days, the mice were killed with an anesthetic. Then, the mice spleens were removed aseptically and each was homogenized in 10 ml of sterile saline buffer. Next, the suspensions of spleens were cultured in plates containing Mueller Hinton agar media. The plates were incubated at 37°C in the presence of 7-5% CO_2gas for 72 h. Then, the bacteria colonies were counted by streaking (14).

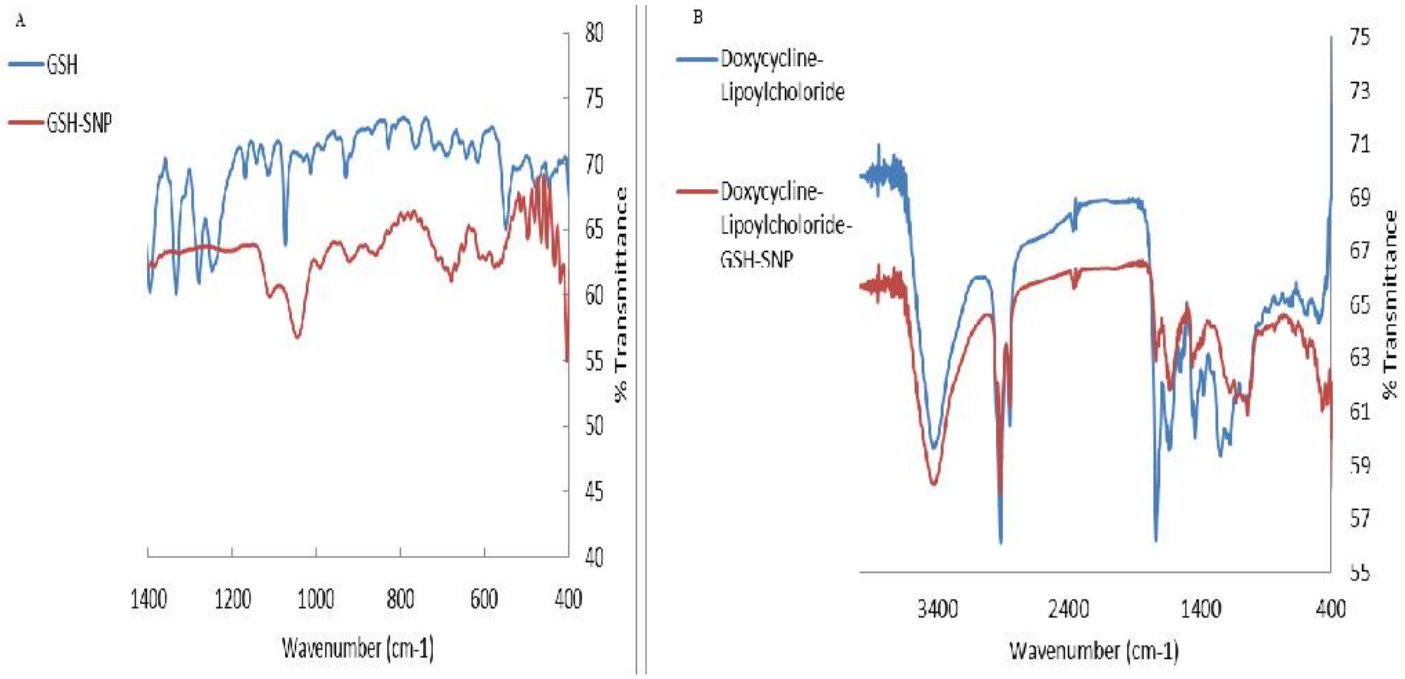
Statistical analysis

The results were analyzed by one way ANOVA test by SPSS 18 software. P-value in this test was less than 0.05.

RESULTS

FT-IR

The FT-IR spectrum GSH,GSH-SNP, conjugated SNP-GSH with lipoyl chloride andlipoyl chloride-doxycycline were shown in figure 4.TheS-H vibration (2524 cm-1 band), which appears in GSH spectrum was missed in GSH-SNP spectrum, because of the new S-Ag^0 bond. In GSH, the band at 1713 cm^{-1} was attributed to the -COOH group of the glycine residue, which is absent in GSH-SNP. This indicates interaction of -COOH with metal ions. In lipoyl chloride-doxycycline the S-S vibration frequencies were appeared in 498 cm^{-1}. This band is absent in conjugated SNP-GSH with lipoyl chloride-doxycycline because of S-S bond cleavage (figures 1-2).



(A) **(B)**

Figure 4: (A) FT-IR comparison of Glutathione (GSH) with Glutathione-Silver Nanoparticles (GSH-SNP) and (B) doxycycline-lipoylcholoride with doxycycline-lipoylcholoride-GSH-SNP

Well diffusion agar assay

The agar-well diffusion assessment showed that SNP-doxycycline conjugated has more antimicrobial effect against *B. abortus 544* in comparison with silver nanoparticle or doxycycline antibiotic alone. It can be seen that the concentration of silver nanoparticles and doxycycline in the conjugated formare a half of SNP and doxycycline alone **(Table 1)**.

MIC and MBC

The MIC and MBC of SNP-doxycycline conjugate against *B.abortus 544*were 1:16 and 1:8, respectively. The results showed that the SNP-doxycycline conjugate has more antibacterial activity in comparison with silver nanoparticles or doxycycline alone. So that it can be seen that the concentration of silver nanoparticles and doxycycline in the conjugate reduced by half for the same antimicrobial effect. The MIC and MBC ofthe conjugate, silver nanoparticles and doxycycline have been shown in table 2.

Mouse Model

The statistical analysis of *in vivo* results showed that the SNP-doxycycline conjugate sharply reduces the colonization of *B.abortus 544*.The results of colony counting in the spleen culture were listed in table 3.

Table 1: Antibacterial effect of silver nanoparticle (SNP)and doxycycline in different concentrations on *Brucellaabortus 544*.

SNPs (ppm)	Doxycycline (µg/ml)	Diameter of inhibition zone (mm)
4	1	5±1
8	2	10±1
12	3	15±2
16	4	20±2
32	8	25±2
64	16	30±5
128	32	35±5
256	64	40±7

Table 2: MIC and MBC of silver nanoparticles, doxycycline and the conjugate

Treatments		MIC	MBC
Silver nanoparticles (ppm)		8	10
Doxycycline (µg/ml)		8	16
SNPs-doxycycline Conjugated	SNP (ppm)	4	6
	Doxycycline (µg/ml)	4	8

Table 3: Mean ± Standard deviation of grown bacteria in spleen suspension culture

Groups	*Brucella abortus 544* CFU/Spleen
Silver Nanoparticles-Doxycycline Conjugate	45±8*
Silver Nanoparticles	521±156*
Doxycycline	$18\pm2 \times 10^5$ *
Normal Saline (control)	$3\times10^{10}\pm1.2\times 10^3$

*** Indicates a significant difference compared with control group**.

DISCUSSION

Due to the outbreak of infectious diseases caused by different pathogenic bacteria and the development of antibiotic resistance, the researchers are searching for new antibacterial agents (15, 16). Antibiotic resistance is a type of drug resistance where a microorganism has developed the ability to

survive when exposes to an antibiotic. The volume of antibiotic prescribed is the major factor in increasing rates of bacterial resistance rather than compliance with antibiotics (16). In recent years increasing of drug resistance of *Brucella* bacteria has created many problems. So trying to find the new treatments for brucellosis isone of the top priorities of the World Health Organization (17).Silver nanoparticles have emerged up as the novel antimicrobial agents owing to their high surface area to volume ratio and its unique chemical and physical properties (16). Previously, the highest synergistic antibacterial activity was observed with silver nanoparticles combined with some antibiotics such as penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin (18).Silver nanoparticles have high affinity for binding to amino groups after the sulfur groups(19). In this study, doxycycline was attached to silver nanoparticles to have a stable binding via the lipoyl chloride. FT-IR results showed that the conjugation of silver nanoparticles to doxycycline has been carried out correctly.

Agar well diffusion method was performed to evaluate the antimicrobial activity of the new agent as well as its dependent agent *B. abortus 544*(20). In the present study, it was observed that with increasing of the concentration of SNP- doxycycline, conjugated the bacterial growth was decreased. These results are quite consistent with findings of Ayala et al. in Mexico in 2009 (21).

Determination of MIC and MBC showed that MIC fell by half in the conjugate. In2005, Ping Li et al. attached the silver nanoparticles to the *β*-lactam compounds. They found that these complexes have more antibacterial effect in comparison with *β*-lactam compounds and concluded that the *β*-lactam and silver nanoparticles when complexed together show synergistic effect (18). Also, Shahverdi et al. in 2007showed that the antibacterial activities of penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin were increased in the presence of silver nanoparticles against *Staphylococcus aureus* and *E. coli*. In their study the highest enhancing effects were observed for vancomycin, amoxicillin, and penicillin G against *S. aureus* (23).Study of antimicrobial effects of silver nanoparticles-doxycycline conjugate in animal model revealed that there were significant differences between experimental group and the control group. The results showed that the numbers of bacteria grown out in the group treated with doxycycline-silver nanoparticles

conjugate was significantly less than other groups.

CONCLUSION

Overall, the results of this study showed that the antimicrobial effect of silver nanoparticles-doxycycline conjugate was greater than the silver nanoparticles ordoxycycline alone against *B. abortus 544in vitro* and *in vivo*.

ACKNOWLEDGMENT

The authors grateful to all of staff of biology research center of Islamic Azad University of Zanjan.

REFERENCES

[1] Sofian M, Aghakhani A, Velayati AA, Banifazl M, Eslamifar A, Ramezani A. Risk factors for human brucellosis in Iran: a case–control study. International Journal of Infectious Diseases 2008; 12(2): 157-161.

[2] Avdikou I, Maipa V, Alamanos Y. Epidemiology of human brucellosis in a defined area of Northwestern Greece. Epidemiology and infection2005; 133(5): 905-910.

[3] Joint Food and Agriculture Organization (FAO), World Health Organization (WHO). FAO-WHO expert committee on brucellosis. 6th report. WHO technical report series, no. 740. Geneva: World Health Organization; 1986. p. 56-7.

[4] Roushan, MRH, Mohraz M, Hajiahmadi M, Ramzani A, Valayati AA. Efficacy of gentamicin plus doxycycline versus streptomycin plus doxycycline in the treatment of brucellosis in humans. Clinical infectious diseases 2006; 42(8): 1075-1080.

[5] Joint Food and Agriculture Organization (FAO), World Health Organization (WHO). FAO-WHO expert committee on brucellosis. 6th report. WHO technical report series, no. 740. Geneva: World Health Organization; 1986. p. 56-7.

[6] Ariza J, Mile B, Antonio C, Juan DC, Michael JC, Matthew EF, et al. Perspectives for the treatment of brucellosis in the 21st century: the Ioannina recommendations. PLOS medicine 2007; 4(12): 1872-1878.

[7] Zakeri B, Gerard DW. Chemical biology of tetracycline antibiotics. This paper is one of a selection of papers published in this Special Issue, entitled CSBMCB-Systems and Chemical Biology, and has undergone the Journal's usual peer review process. Biochemistry and Cell Biology 2008; 86(2): 124-136.

[8] Pompella A, Visvikis A, Paolicchi A, Tata V, Casini AF. The changing faces of glutathione, a cellular protagonist. Biochemical Pharmacology 2003; 66 (8): 1499–503.

[9] Hugo WB, Russell AD. Principles and practice of disinfection, preservation and sterilization, Oxford, 1982; 0632041943, London.

[10] Alizadeh H, M Salouti, Shapouri R. Intramacrophage antimicrobial effect of silver nanoparticles against *Brucella melitensis 16M*. ScientiaIranica 2013; 20(3): 1035-1038.

[11] Greenhalgh K, Edward T. In vivo studies of polyacrylate nanoparticle emulsions for topical and systemic applications. Nanomedicine: Nanotechnology, biology and medicine 2009; 5(1): 46-54.

[12] Naqvi SZH, Urooj K, Muhammad IA, Asif J, Abdul H, Safia A, Naeem A. Combined efficacy of biologically synthesized silver nanoparticles and different antibiotics against multidrug-resistant bacteria. International journal of nanomedicine2013; 8: 3187.

[13] Rachel AK. Gold Nanocrystal Therapeutics: Treatment of Multidrug Resistant Pathogens and Disrupting Protein/Protein Interactions. A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy. 2010.

[14] Chang MC, Junzo T. FT-IR study for hydroxyapatite/collagen nanocomposite cross-linked by glutaraldehyde. Biomaterials 2002; 23(24): 4811-4818.

[15] Gottenbos B, Henny C, Flip K, Paul N, Henk JB. In vitro and in vivo antimicrobial activity of covalently coupled quaternary ammonium silane coatings on silicone rubber. Biomaterials 2002: 23(6):1417-1423.

[16] Rai M, Alka Y, Aniket G. Silver nanoparticles as a new generation of antimicrobials. Biotechnology advances 2009; 27(1): 76-83.

[17] Andersson DI, Diarmaid H. Antibiotic resistance and its cost: is it possible to reverse resistance?. Nature Reviews Microbiology2010; 8(4): 260-271.

[18] Pappas G, Javier S, Nikolaos A, Epameinondas T. New approaches to the antibiotic treatment of brucellosis. International journal of antimicrobial agents 2005; 26(2): 101-105.

[19] Li PJL, Changzhu W, Qingsheng W, Jian L. Synergistic antibacterial effects of β-lactam antibiotic combined with silver

nanoparticles. Nanotechnology 2005; 16(9): 1912.

[20] Gu H, Keming X, Chenjie X, Bing X. Biofunctional magnetic nanoparticles for protein separation and pathogen detection. Chemical Communications2006; 9: 941-949.

[21] Ncube NS, Afolayan AJ, Okoh AI. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. African Journal of Biotechnology2008; 7(12): 1797-1806.

[22] Ayala N, Nilda V, Humberto HLV, Lilianadel CIT, Cristina RP. Silver nanoparticles toxicity and bactericidal effect against methicillin-resistant *Staphylococcus aureus*: nanoscale does matter. Nanobiotechnology2009; 5(1-4): 2-9.

[23] Cho KH, Jong EP, Tetsuya O, Soo-Gil P. The study of antimicrobial activity and preservative effects of nanosilveringredient.ElectrochimicaActa 2005; 51(5): 956-960.

[24] Shahverdi AR, Fakhimi A, Shahverdi HR, Minaian S. Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against *Staphylococcus aureus* and *Escherichia coli*. Nanomedicine: Nanotechnology, Biology and Medicine 2007; 3(2): 168-171.