Improved delivery of voriconazole to Aspergillus fumigatus through solid lipid nanoparticles as an effective carrier

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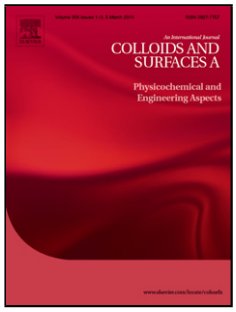

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Improved delivery of voriconazole to *Aspergillus fumigatus* through Solid lipid nanoparticles as an effective carrier

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Short title: Voriconazole loaded Solid lipid nanoparticles against *Aspergillus fumigatus*

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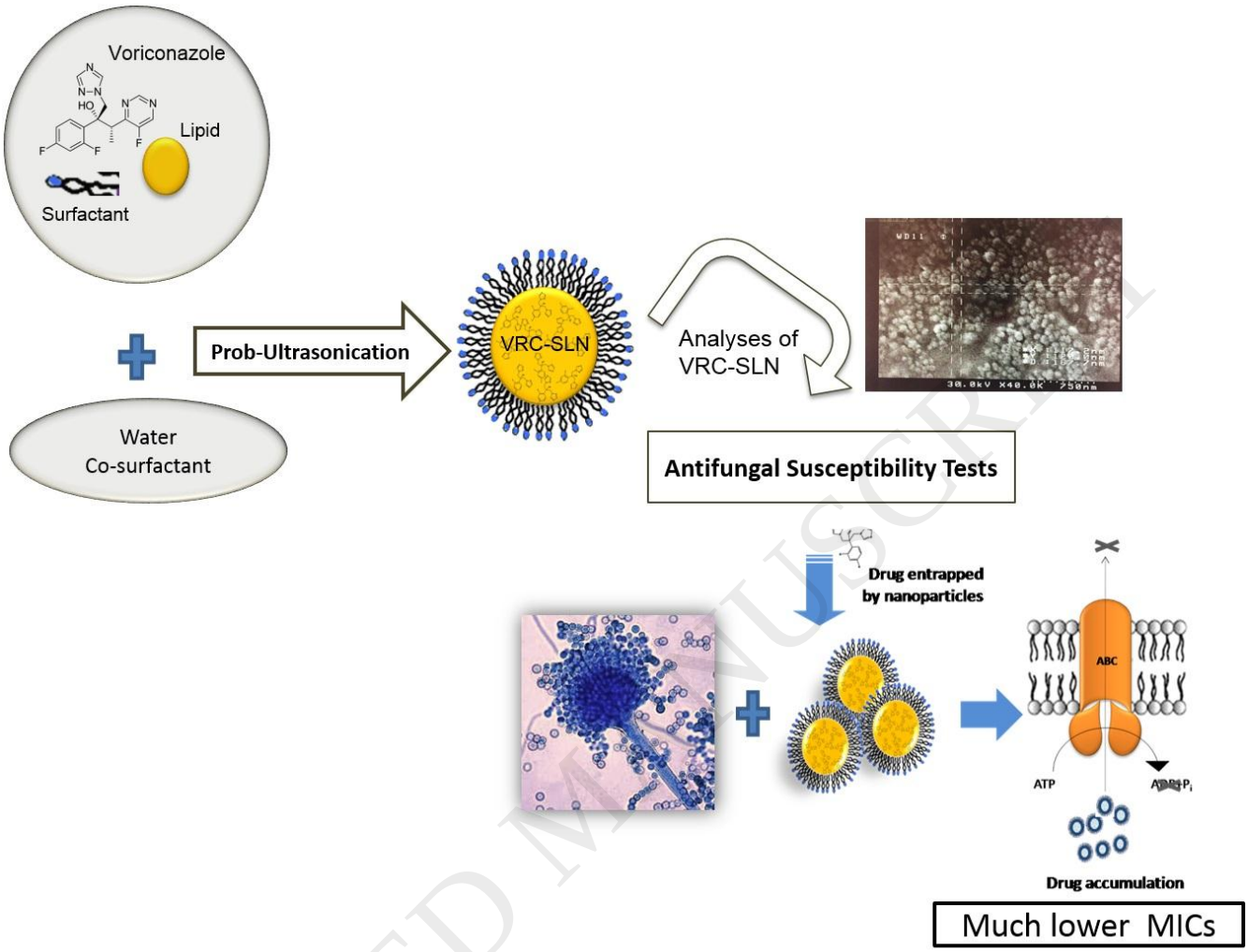
Graphical abstract



Abstract:

Novel voriconazole-loaded solid lipid nanoparticles (VRC-SLNs) were prepared via probe-ultrasonication method, and the resultant nanoparticles were tested on *A. fumigatus*. Voriconazole-loaded solid lipid nanoparticles were prepared using the probe ultrasonication technique. Photon correlation spectroscopy (PCS) was used to determine the average particle size and zeta potential of SLN formulations. Transmission electron microscopy was also used to determine the morphology of solid lipid nanoparticles. To determine MIC for all SLN

formulations against strains of *Aspergillus* the Clinical and Laboratory Standards Institute guidelines was followed. The results showed that SLNs containing voriconazole exhibited almost spherical shape with a diameter and zeta potential of 286.6± 4.7nm and -15 ± 4.1 mV respectively. This novel formulation of VRC led to a significant reduction in MICs for all *Aspergillus* either VRC-susceptible or VRC-resistant isolates ($P < 0.05$). The MIC_{50} drug concentration was obtained as 0.015 μg/ml for both VRC-susceptible strains of *A. fumigates* while it was 0.25 μg/ml against VRC ($p<0.05$). VRC-resistant strains showed a MIC_{50} of 0.015 μg/ml as well. These novel drug formulations may increase the bioavailability through an increase in the dissolution rate of voriconazole. This study showed, for the first time, VRC-SLNs can be employed as an effective delivery systems for VRC on *A. fumigatus* isolates.

Keywords: Aspergillus, Solid lipid nanoparticles, Voriconazole, MIC_{50}, Drug Release

Introduction

Azoles are the only class of oral antifungal available for the treatment of *Aspergillus* diseases. Aspergillus is a saprophytic conidial mold isolated abundantly from soil, construction dust and hospitals (1, 2) which causes a broad spectrum of pulmonary aspergillosis (allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA) and invasive pulmonary aspergillosis (IPA)) (3).

Voriconazole (VRC) is used as the first-line treatment for invasive aspergillosis [(4)] and is available in oral and intravenous formulations. Voriconazole is a second-generation broad-spectrum triazole that inhibits the cytochrome P450-dependent enzyme lanosterol 14 alpha-demethylase, and subsequently, interrupts the essential step for ergosterol biosynthesis in the fungal cell [(5)]. Apart from the clinical implications of resistance, exposure of the fungus to azoles in the environment is the second route for induction of resistance [(6)]. In a recent international surveillance study, the prevalence rate of azole-resistant *A. fumigatus* isolates was determined to be 3.2% [(7)]. Moreover, it has been reported that the prevalence of azole-resistant *A. fumigatus* in Iran has gone up remarkably from 3.3% to 6.6% [(8)]. Therefore, designing new antifungal agents/formulations or novel drug delivery systems is highly required to introduce a new phase in the management of invasive aspergillosis.

Generally, solid lipid nanoparticles (SLNs) contains biodegradable lipid which could be in a solid form at both room and body temperatures and their particle size varies between 50 and 1000 nm [(9, 10)]. They have numerous advantages such as drug protection against harsh environmental situations, ease of large scale production using high pressure homogenization technique, biocompatibility, and biodegradability (11). This carrier can be administered through general routes, which can transport the drug to the target site. Biodegradable lipid matrix, high

drug loading, increased drug stability, controlled drug release, and enhanced penetration of drugs into the skin or any other target are some of the other advantages of SLNs [(12, 13)].

Therefore, the aim of the present study was to develop a new drug delivery system using solid lipid nanoparticles loaded with VRC (VRC-SLNs) prepared by high-shear homogenization and ultrasonication methods. Large-scale productions of lipid nanoparticles are mainly obtained by high pressure homogenization technique (14). The influence of some formulation variables on the characteristics of the VRC-SLNs was also investigated. Drug susceptibility testing was performed according to the Clinical and Laboratory Standards Institute (CLSI) M38-A2 protocol for strains both resistant and susceptible to VRC.

2. Material and method

Voricnazole (Fanavaran Daroui Hakim, Tehran-Iran), Compritol®888 ATO (CO), Lipocire, Precirol®ATO5 and stearic acid (all were supplied by Gattefossé;Saint-Priest, Cedex, France), spans 80, 60 , Tween 80, sodium acetate, acetic acid, methanol, Sabouraud dextrose agar (SDA), RPMI medium (Merck, Germany) and morpholinepropanesulfonic acid (MOPS) from Sigma Chemical Co., St. Louis, MO (USA) were used. All other reagents and solvents used in the current study were either of analytical or HPLC grades.

2.1 Screening of lipids

The screening of lipids was performed by evaluating the solubility of voriconazole, VRC with varying concentrations ranging from 10 to 25% w/w with regard to lipid mass in different lipids (Compritol®888 ATO (CO), Lipocire, Precirol®ATO 5 and stearic acid). The physical mixtures of lipid and VRC were heated to 85ºC followed by the examination of the formulations to see

which lipid or lipid combinations could dissolve the drug completely. Those lipids were able to dissolve the drug completely were selected to make SLNs.

2.2 Preparation of VRC-SLNs

The solid lipid nanoparticles loaded with VRC (VRC-SLNs) was prepared by high-shear homogenization followed by probe ultrasonication technique which has been published previously [(15)]. Large-scale productions of lipid nanoparticles are mainly obtained by high pressure homogenization technique. Briefly, the mixtures of solid lipid (2 g), VRC (0.5 g) and span 80/60 (0.25 g) were thoroughly mixed and melted at 85 ºC. Then the molten mixture was transferred to the 1/3 aqueous solution containing 0.5 g of hydrophilic surfactant Tween 80 (Tn80). The mixture then was heated at the same temperature (85 ºC) followed by sonication of the mixture (Bandelinsonopuls, Berlin,Germany) for 5 min to form a coarse pre-emulsion. After sonication, the obtained mixture was thoroughly dispersed in the rest of the aqueous solution containing surfactants (Tn80) cooled in an ice bath. The final mixture was maintained in the ice bath for 10 min while sonicating. This cooling step caused the formation of the lipid nanoparticles.

2.3 Characterization of VRC-SLNs

2.3.1 Determination of particle size and zeta potentials

The Zeta Sizer Nano ZS (Malvern Instruments, UK) was employed to determined the average particle size and zeta potential of SLNs at 25 ºC at a fixed angle of 90º. The results are the means and standard deviations of at least three determinations.

2.3.2 Morphology measurement

In order to evaluate the morphology of drug particles field emission scanning electron microscope (FESEM, HITACHI S-4160, and U.S.A) was used. A drop of suspension was located on double-sided carbon tape and dried at 25◦C for 24 h followed by sputter coated with gold for 40 s before examination.

2.4 Antifungal susceptibility testing for VRC-SLN

2.4.1 Isolates

A total of 62 clinical and environmental isolates were employed. Clinical isolates (n=38, 61.3%) had been obtained from the lower respiratory tract, sinus, cerumen, nails. Biopsy samples via routine diagnostic procedures at hospital laboratories and environmental isolates (n=24, 38.7%) had been collected from the soil of gardens surrounding the hospitals and indoor air of hospital wards. Cultures were prepared on a Sabouraud dextrose agar plate (SDA; Difco), and incubated at 30 ºC for 72 h.

2.4.2 Antifungal agents

In order to determine antifungal susceptibility of nanoparticle formulations and pure VRC, the test (AFST) was carried. To this end, the drug solution in DMSO was prepared and then a two-fold dilution was obtained in Roswell Park Memorial Institute (RPMI) 1640 medium (with L-

glutamine, without bicarbonate) (Merck, Germany) and buffered to pH 7.0 using a 0.165 M solution of 3-N-morpholinepropanesulfonic acid. Two-fold dilutions of VRC-SLN were prepared with concentrations of 16 µg/ml to 0.1 µg/ml. All voriconazole-SLNs formulations for AFST test were freshly prepared and used within a week.

2.4.3 Antifungal susceptibility testing

MICs for both VRC and VRC-SLN formulations were obtained according to recommendations published in the CLSI M38-A2 documents [(16)]. Inoculums suspensions were prepared on potato dextrose agar (Merck, Germany) for 2–3 days by slightly scraping the surface of mature colonies with a sterile cotton swab, soaked in a sterile saline solution containing Tween 40 (0.05 %). The supernatants were adjusted spectrophotometrically to an OD range of 0.09–0.13 (0.5×10^4 to 3.1×10^4 c.f.u. ml^{-1}) at a wavelength of 530 nm, as determined by the quantitative colony count for specifying the viable c.f.u. per millilitre. Conidial suspensions, which mostly consisted of conidia, were diluted 1:50 in RPMI 1640 medium. The microdilution plates were inoculated with 100 ml of the diluted conidial inoculums suspension and incubated at 35 ºC for 48 h; the plates were read visually after agitation. Due to the self-turbidity of VRC-loaded SLNs, results were evaluated using an inverted microscope. Positive control (Only conidial suspension) as well as negative control (Only RPMI medium) and a formulation of SLN without VRC were also run along with the experiments. Moreover, *Paecilomyces variotii* (ATCC 22319) and *Candida parapsilosis* (ATCC 22019) were used as the quality controls. The MIC endpoints were determined as the lowest concentrations of drugs inhibiting recognizable fungal growth (100% inhibition).

2.5 Statistical analysis

In order to find out the differences between various groups, an ANOVA test (SPSS version 18) was performed followed by Dunnett's test. In addition, the Mann-Whitney U test was also applied to test the significant reduction in the MIC caused by VRC-SLNs. The differences were considered statistically significant at $P < 0.05$. All of the results reported here were the mean and standard deviation of at least 3 determinations.

3. Results

3.1. Screening of lipids

The very first steps in selecting the lipids for the formulation of the lipid nanoparticles, was the evaluation of the solubility of the drug in the lipid material. Four lipids were chosen and listed in Table 1. The results showed that no drug crystals can be seen in the sample containing either stearic acid or Compritol.

3.2. Characterization of SLNs

Table 2 presents the particle sizes, zeta potential of the developed SLNs. The results showed that the particle sizes of all SLNs prepared in the current study are in nano-sized range. As VRC-SLNs 4 produced the smallest particle size (286.6 nm) and relatively good zeta potential (-15), therefore it was selected as the best formulation compared to other SLN manufactured in this research. Figure 1 represents the morphology of the best SLN.

3.4. Antifungal susceptibility testing

According to CLSI document M38-A2 susceptibility testing methodology, MIC ranges were obtained for both of VRC and VRC-SLNs against 62 A. *fumigates* strains. The results were interpreted after 48 h incubation at 35°C. Five strains of *A. fumigates* strains were resistant and 57 species were susceptible to VRC. Table 3 shows the detailed results for antifungal susceptibility profile of VRC and VRC-SLNs against 62 *A. fumigates* strains. The formulation of SLNs without VRC had no inhibitory antifungal effect against *A. fumigatus* isolates since all the isolates did grow equally to positive control. The isolates that were resistant to VRC showed a significant decrease in MIC values after using VRC-SLN as their MIC value placed in sensitive range ($P > 0.05$). For VRC- susceptible strains, the MIC_{50}, MIC_{90} and GM value were 0.25, 0.25 and 0.16275 μg/ml, respectively. For VRC- susceptible strains, they were found as 0.015, 0.031 and 0.009636, respectively (Table 3 and Fig 2).

Discussion

VRC is classified as a class II drug according to the Biopharmaceutical Classification System (BCS). The drug's bioavailability is relatively low, and its aqueous solubility is poor at physiological pH, reducing its dissolution rate. Therefore, for this drug to have a maximum therapeutic effect, higher bioavailability and dissolution rates are required [(17)]. To enhance the solubility of VRC and overcome the limitations mentioned above, SLNs can be utilized. SLNs are safe carriers and have low toxicity levels, which is due to their matrix being composed of biodegradable physiological lipids. By using SLNs, lyophilization becomes possible and the bioavailability of poorly soluble molecules such as VRC is improved [(5, 18)].

The existing reports demonstrated that SLNs systems can be used to upload azole agents [(19-21)]; however, to the best of our knowledge, there is not much research and data published on the delivery of VRC using SLNs [(5, 22, 23)]. There are studies focused on the effective ocular delivery of VRC-SLNs (24, 25) but they did not investigated their direct influence on the drug delivery to the fungal cells. It was important to extend the study to see whether VRC can be loaded on SLN and also if it is possible to obtain the desirable properties for VAC-SLNs. Having very small particle size for lipids in SLN formulations can guarantee the close contact between particles and the target tissue, which might increase drug penetration [(13)]. The size of lipid matrices may also enable sustained drug release. The assessment of the most suitable lipid carriers to prepare stable nanoparticles was optimised as the first step. As shown in Table 1, various lipids with different physical and chemical properties were selected. Unlike other lipids, stearic acid and Compritol® 888 ATO performed better in terms of producing a homogenous suspension showing no drug crystals upon heating with the drug. Therefore, stearic acid and Compritol® 888 ATO were selected as the most suitable solid lipid to prepare SLNs. All other lipids as shown in Table 1 failed to dissolve VRC completely. It could be clearly observed from Table 2 that the average particle size and zeta potential decreased in VRC-SLN4 using stearic acid as solid lipid and Span 80 as lipophilic surfactant ($P > 0.05$). It has been shown that the presence of Span 80 can cause a greater drop in the interfacial tension in VRC-SLNs (formulation 4), leading to a more homogenized dispersion with smaller particles and lower zeta potential. These results were in agreement with previously reported studies. It has been reported that lipid type, emulsifier type and their concentrations are parameters that were recognized as major factors influencing the size of solid lipid nanoparticles (26, 27). In the present study, an effective formulation of VRC-SLNs was obtained. As the surfactant can influence the size of

nanoparticles and this is the main reason for a significant reduction in the size of the nanoparticles in the VRC-SLN 4 compared to VRC-SLN 1. [(28)]. The zeta potential is a key indicator of the stability of particle dispersions. So, particles with high zeta potential (negative or positive) are electrically stabilized while particles with low zeta potentials tend to coagulate or flocculate as outlined in table 2 (29). It has been reported that the lipid type, emulsifier type, and their concentrations are the major factors influencing the size of solid lipid nanoparticles [(30)]. Figure 1 exhibits the FESEM photomicrographs of VRC-SLNs 4 as an optimal formulation. These photomicrographs demonstrated that the particles had smooth surfaces and were almost spherical in shape.

It was interesting to note that when VRC-SLNs were applied the values of the MICs decreased significantly compared to VRC on its own, suggesting that the therapeutic dose could be reduced hence the risk of adverse drug effects may be decreased. One possible explanation for the antifungal susceptibility results is the mechanism responsible for the drug resistance recognized in pathogenic fungi [(31, 32)]. The mechanism of drug resistance can happen in three distinct ways, namely reduction of drug concentration that can happen to drug efflux, change in the drug target, and mutation in genome sequence [(33, 34)]. The most important mechanism, which leads to a decrease in drug concentration, is efflux. Several efflux transport systems exist including ABC transporters and MFS transporters that are found in *A. fumigates* [(35)].

The overexpression of pleiotropic drug resistance (PDR) efflux pumps is a major mechanism of drug resistance for azole antifungals [26]. The efflux pumps are members of ATP-binding cassette (ABC) transporters superfamily. Strategies to overcome the problem of PDR includes the design of specific inhibitors of these efflux pumps, or design of antifungal molecules with suitable shields to prevent binding to the efflux pumps. In this study, we benefited from

nanoscaled safeguard of VRC-SLNs that protect the drug from being pumped out by transporter proteins. Moreover, penetration of drug into fungal cells may be facilitated due to the hydrophobic surface of VRC-SLNs.

Conclusion

Voriconazole solid lipid nanoparticles were successfully prepared. These novel delivery systems could face *A. fumigatus* strains that an exhibit different susceptibility to the conventional formulation of VRC was investigated. The results proved that these novel drug formulations may increase the bioavailability and dissolution rate of VRC to enhance the performance of the formulations.

Conflicts of interest

None declared.

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Table 1. Screening of lipids based on solubility of **VRC**.

Lipid	**VRC** (mg) / Solid lipid (100 mg)			
	10	**15**	**20**	**25**
Compritol® 888 ATO	+	+	+	+
Lipocire	+	-	-	-
Precirol® ATO 5	+	+	-	-
Stearic acid	+	+	+	+

Table 2. Physicochemical properties of investigated VRC -SLNs

Formulation	Lipid phase	Emulsifiers	Size (nm)	ZP(mV)
VRC-SLNs 1	CO	Tn+Sn60	313.1±11.3	-11±2.2
VRC -SLNs 2	CO	Tn+Sn80	297.0 ± 20.6	-11±3.0
VRC -SLNs 3	SA	Tn+Sn60	292.2± 14.5	-12±2.5
VRC -SLNs 4	SA	Tn+Sn80	286.6± 4.7	-15±4.1

CO:Compritol Auto 888; **VRC**:Voriconazole; **SA**; stearic acid; **Sn**:Span; **Tn**:Tween; **ZP**:Zeta potential.
Each value represents the mean ±S.D. (n = 3).

Table 3. Effect of new delivery system for VRC on strains of *A. fumigatus.*

Isolates	Number (n)	Antifungal agent	MIC (µg/ml)											MIC range	MIC50	MIC90	GM*	Mechanism Of resistance
			16	8	4	2	1	0.5	0.25	0.125	0.062	0.031	≤0.015					
A. fumigatus	57 (S)	VCZ**	-	-	-	-	-	3	36	-	18	-	-	0.062-0.5	0.25	0.25	0.16275	--------
		VCZ-SLN**	-	-	-	-	-	-	-	-	-	11	46	0.031-≤0.015	0.015	0.031	0.009636	
	5 (R)	VCZ	1	2	1	1	-	-	-	-	-	-	-	2-16	-	-	-	TR34/L98H Amino acid substitution
		VCZ-SLN	-	-	-	-	-	-	-	-	-	2	3	0.031-≤0.015	0.015	0.031	0.013231	

*__GM__: geometric mean MIC.

** **VRC**: voriconazole, **VRC-SLN**: Voriconazole Solid Lipid Nanoparticles

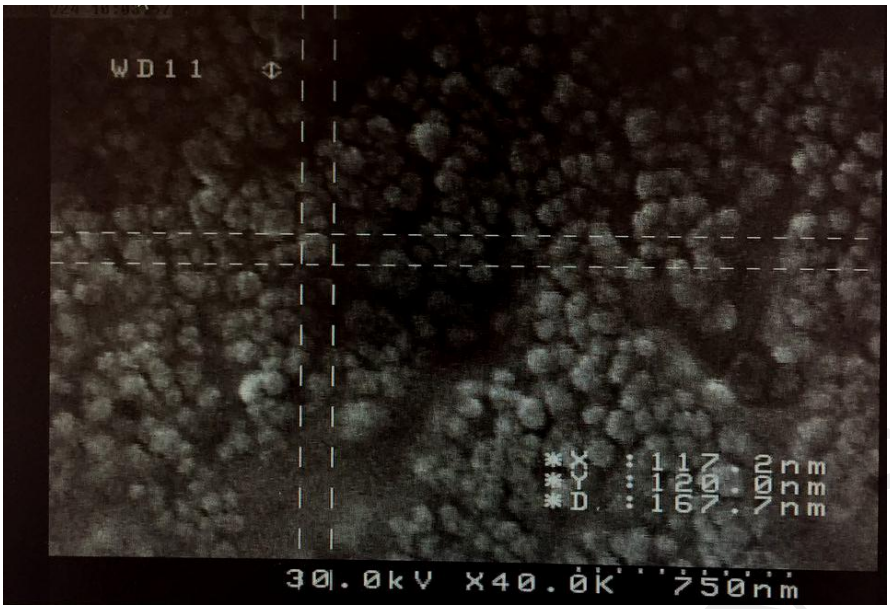

Fig. 1. FESEM micrograph of VRC-SLNs 4. The particles were observed to be almost spherical in shape with a narrow size distribution (Magnification of ×30,000).

Fig. 2. Comparison between the MIC50 of the susceptible strains while treating with VRC and VRC-VRC-SLNs. Using FLZ-NLCs, susceptible isolates showed a significant decrease in MIC values (P value < 0.05).