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DESIGN AND CHARACTERIZATION OF ASCORBIC ACID STABILIZED RIFAMPICIN NANOPARTICLES FOR ORAL DELIVERY

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ABSTRACT

Rifampicin degrades in the acidic environment of stomach and its bioavailability remains problematic in controlling tuberculosis. Previous studies reveal that ascorbic acid is used to stabilize rifampicin in dissolution medium and in plasma sample against its degradation. The present study was aimed to develop nanoparticles of rifampicin using chitosan as polymer and ascorbic acid as stabilizing agent. The nanoparticles of rifampicin were prepared by ionic gelation of chitosan solution with sodium tri-poly phosphate (0.25%) using tween 80 as suspending agent and ascorbic acid as stabilizing agent and evaluated for physico-chemical characteristics, *in vitro* dissolution stability The nanoparticles were 202-250nm in size with polydispersity index 0.2-0.5 and zeta potential + 38 - +42 mV. The encapsulation efficiency and loading capacity of the nanoparticles were 87% and 49% respectively. Ascorbic acid significantly reduced degradation of rifampicin as nanoaparticles when compared to control formulations. The study concludes that nanoparticulate delivery of rifampicin co administered with ascorbic acid as stabilizing agent is beneficial in improving bioavailability of rifampicin.

Key Words: Ascorbic acid, Degradation, Nanoparticles, Chitosan, Tuberculosis, Rifampicin.

INTRODUCTION

Tuberculosis (TB) is an infection caused by M*ycobacterium tuberculosis*. It is the world's second commonest cause of death from infectious disease after HIV/AIDS. WHO declares TB as a public health emergency. The prevalence of TB is higher in south East Asia and sub Saharan Africa as compared to western countries. The prevalence of all forms of TB in India is estimated 5.05/1000 and of smear positive cases 2.27/1000 (Chakraborty *et al*., 2004).

Treatment of TB was initially complicated and challenging as it requires administration of drugs over a long period that resulted in poor patient compliance. WHO recommended a multi drug regimen containing rifampicin (RIF), isoniazid (INH), ethambutol (ETH) and pyrazinamide (PYZ) to shorten the duration of treatment of TB. However rifampicin degrades in the stomach and the degradation has varied from 8.5-50%, during the gastric emptying time for most dosage forms in humans (Shishoo *et al*., 2001) and decomposition of rifampicin is further influenced by the presence of isoniazid in stomach after ingestion (Saranjit *et al*., 2001). To improve this disadvantage isoniazid was administered in enteric coated form to prevent its release in the stomach. Despite these disadvantages, the fixed dose combination (FDC) of rifampicin, isoniazid, ethambutol, pyrazinamide remains in use in the treatment of TB to attract patient compliance; however the bioavailability of rifampicin has become unacceptable in a number of FDC anti- TB formulations.

The past several years have seen the development of a number of rifampicin controlled release formulations

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for the improvement of clinical efficacy of the drug and patient compliance (Lisa C *et al*., 2006). Controlled drug delivery system, nanoparticles, liposomes and microspheres were developed for the sustained drug delivery of anti-TB drugs that have demonstrated better chemotherapeutic efficacy when investigated in animal models (Falk *et al*., 1997). Recently nanoparticulate delivery of anti- TB drugs has assumed significance among the researchers for its known advantages. Nanoparticles are stable solid colloidal particles consisting of biodegradable polymers or lipid materials and range in size from 10 to 1000 nm. Drugs can be absorbed onto the particle surface, entrapped inside the polymer/lipid or dissolved within the particle matrix (Kreuter *et al*., 1993). Oral nanoparticle based anti- TB drug therapy can allow for reduction in dosing frequency for better management of TB. Despite several approaches attempted by researchers in the past, the degradation of rifampicin in the stomach resulting its poor bioavailability from currently available dosage forms remains a concern for effective control of tuberculosis.

Previous study reveals that rifampicin collected in the plasma sample can be stabilized by using ascorbic acid as anti-oxidant. Rifampicin degrades in plasma at ambient temperature, and a 54% loss was observed within 8 hrs and this degradation can be effectively prevented by adding ascorbic acid, thus prolonging stability for up to 12 hrs (Lee G *et al*., 1997). Furthermore, administration of ascorbic acid (1000 mg/day) is recommended in tuberculous patients (Levine *et al*., 1996) as ascorbate concentration of 1mg/day, which is easily reached in blood, prevents the growth of cultures of *M.tuberculosis*. Ascorbic acid is also added to the medium to prevent oxidative degradation of rifampicin during the *in-vitro* diffusion (Gaurav *et al*., 2010) and dissolution (Shishoo *et al*., 1999) studies.

Different polymers have been recommended for preparation of nanoparticles. Biodegradable polymers are preferred as they are eliminated from the body and so polymer induced toxicity is unlikely. Natural biodegradable polymers are more desirable as they are highly biocompatible and are recommended for chronic infections (Lifeng *et al*., 2004). Chitosan is a naturally occurring biocompatible cationic polysaccharide obtained from the deacetylation of chitin. Compared to other natural polymers chitosan has a positive charge and is mucoadhesive and it is used extensively in drug delivery applications (Yongmei *et al*., 2003). Chitosan has the capacity to protect sensitive bioactive macromolecules from enzymatic and chemical degradation during storage (Mao *et al*., 2001). Chitosan has many advantages particularly for developing micro/nanoparticles; it can control the release of active agents; it avoids the use of hazardous organic solvents while fabricating particle since it is soluble in aqueous acidic solution (Tejraj *et al*., 2004). Based on the above factors, the present study attempted to prepare nanoparticles of rifampicin using chitosan as polymer and ascorbic acid as stabilizing agent and investigate the nanoparticles for its physicochemical characteristics, *in vitro* release.

MATERIALS AND METHODS

Materials

Rifampicin was obtained as gift sample from Astha Laboratories Pvt ltd, Hyderabad, India. Biodegradable Chitosan (deacetylation degree 85%) low MW (150KDa), medium MW (300 KDa) and, high MW (600KDa) were gifted by Central Institute of Fisheries Technology, Cochin, India. All other chemicals used were of reagent grade.

Preparation of nanoparticles

The formulation of rifampicin nanoparticles are given in Table 1. The nanoparticles were prepared by ionic gelation of chitosan solution with sodium tri poly phosphate (0.25%) (Calvo *et al*., 1997b) in the presence of tween 80 at ambient temperature with stirring. Rifampicin alone or in combination with ascorbic acid and chitosan was dissolved in 10 ml acetic acid in aqueous solution under magnetic stirring at room temperature for 45 min in the presence of tween 80. 10 ml STPP aqueous solution was added in to chitosan solution . The nanosuspensions were cold centrifuged at 12000g, in a glucose bed for 30 min using Remi centrifuge (R4C-DX, USA).

Characterization of nanoparticles

The supernatant liquid obtained following centrifugation during the preparation of nanoparticle was analyzed by spectrophotometer at 475 nm (Shrutidevi *et al*., 2004) to calculate the % drug entrapment and drug loading. The rifampicin encapsulation efficiency (EE) and loading capacity (LC) of the nanoparticles were calculated as follows (De Campos *et al*., 2001).

$$\text{Encapsulation efficiency} = \frac{\text{Total amount of rifampicin} - \text{Free rifampicin}}{\text{Total amount of Rifampicin}} \text{ x } 100$$

$$\text{Loading capacity} = \frac{\text{Total amount of rifampicin} - \text{Free Rifampicin}}{\text{Weight of Nanoparticle}} \text{X} 100$$

The final suspensions were then frozen and lyophilized at 0.4 mbar and -40 ºC for 5 hrs. The lyophilized nanoparticles were stored in desiccators at 4ºC for further studies.

The morphology of nanoparticles was analyzed by scanning electron microscope (JEOL MODEL JSM 6400). The nanoparticles were mounted directly on the SEM stub, using double –sided, sticking tape and coated with platinum and scanned in a high vacuum chamber with a focused electron beam. Secondary electrons, emitted from the samples were detected and the image formed.

The Zeta potential and size of nanoparticles was measured on a zeta potential analyzer (Zetasizer 3000 HS Malvern instrument U.K). The samples were diluted with

pH 7.4 and placed in eletrophoretic cell and zeta potential measured in the automatic mode. The particle size distribution is reported as polydispersity index. The samples were placed in the analyzer chamber and readings were performed at 25°c with a detected angle of 90 degrees

In-vitro dissolution stability study

In-vitro dissolution stability was performed on rifampicin, rifampicin nanoparticles and rifampicin – ascorbic acid nanoparticles in 0.1 N HCL. The study was carried out by the method described by Shisoo *et al*., (1999). A solution of 0.1N HCL (200 ml) was placed in the vessel of the USP dissolution apparatus No.2 (USP XXIII, 1995) and the medium was equilibrated at 37±0.1°C with stirring at 50 rpm. Rifampicin or nanoparticles were dissolved in and diluted to 100 ml with 0.1N HCL. The resulting solution was transferred immediately to the dissolution vessel at once and 1 ml of sample was withdrawn immediately from a zone midway between the surface of the dissolution medium and bottom of the vessel (0-min sample). Samples were withdrawn at 15,30,45,60 min intervals and filtered through 0.1µm membrane filter immediately and 1 ml fresh 0.1N HCL solution was added in to the system. An aliquot, 1 ml was diluted to 10 ml of 0.1N HCL using cyclomixer (3min). Samples were measured at 475nm in UV-Visible spectrophotometer (Calvo *et al*., 1997). The experiment was run in triplicate and the mean values were recorded as percent drug degradation. The percentage degradation of drug was calculated from the difference between the values of sample drawn at 0 and appropriate time intervals (Mariappan *et al*., 2003).

In-vitro diffusion study

The studies were performed on rifampicin, rifampicin nanoparticles and rifampicin – ascorbic acid nanoparticles in 0.1 N HCL. Sample equivalent to 100mg of rifampicin was redispersed in 10ml 0.1N HCL solution and placed in a dialysis membrane bag with a molecular cut-off of (MWCO 12,000-15,000Da, Himedia, India) which acts as a donor compartment, tied and placed into 10 ml 0.1N HCL solution in a beaker which acts as a receptor compartment .The entire system was kept at 37ºC±0.1°C with continuous magnetic stirring at a rotation speed of 50 rpm. At appropriate time intervals (15, 30, 45, 60min) 1 ml of the release medium was removed through 0.1µm membrane filter immediately and 1 ml fresh 0.1N HCL solution was added in to the system. The amount of rifampicin in the release medium was determined by UV-Visible Spectrophotometer at 475 nm and the percentage release of rifampicin recorded. The experiment was run in triplicate and the mean values were recorded as percent release of rifampicin (Gaurav *et al*., 2010).

Statistical analysis

The data were analyzed by one way ANOVA followed by Tukey's multiple comparison tests with the help of Graph Pad Instat software, version 3.01. All the data were presented as a mean value with its standard deviation (mean±S.D). $P<0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Preparation of nanoparticles

The present study followed ionic gelation method for preparation of nanoparticles using chitosan of different grades. Ionic gelation method is simple to follow and nanoparticles of desirable size are easily obtained. Chitosan was chosen as polymer for preparation of nanoparticles as the polymer is natural, biodegradable, free from toxicity and ideal for chronic infections like tuberculosis (Lifeng *et al*., 2004). Different grades of chitosan were used in order to examine their influence on the physicochemical and release characteristics of drug. Based on the least percent degradation of rifampicin, the concentration of ascorbic acid was limited to the ratio, 100:500mg (rifampicin: ascorbic acid) for the preparation of nanoparticles (Table 2).

Characterization of nanoparticles

SEM analysis showed the nanoparticles as spherical with smooth surfaces and solid dense with no aggregation (Fig.1a, 1b), possibly, a high zeta potential on the surface of the nanoparticles has prevented the agglomeration process. Commonly, zeta potential is an index of the stability of the nanoparticles. Under most conditions, the higher the absolute value of the zeta potential of the nanoparticles, the larger the charge on their surface, leading to stronger repulsive interaction between the dispersed nanoparticles and higher stability and more uniform size (Feng *et al*., 2004). A high potential value of above ±25mV ensures a high energy barrier that stabilizes the nanosuspension (Muller *et al*., 1991). The zeta potential of all formulations ranged from ±32mV to +42mV (Table 3) and these values predict good colloidal stability due to high energy barrier between particles (Mora *et al*., 2010). All nanoparticles showed a positive surface charge due to the influence of amino groups in the polymer. The morphology of all the formulations was uniform and not influenced by molecular weight of chitosan; however, the zeta potential of nanoparticles was affected by the molecular weight of polymer. As the molecular weight of polymer increased the zeta potential of nanoparticles increased significantly ($P<0.05$), due to more reaction sites with electropositive charge available with longer chain length of the chitosan. Poly dispersity index represents the dispersion homogeneity; the range for the PDI is from 0-1.Values close to 0 indicates the homogenous dispersion and those greater than 0.5 indicate high heterogeneity (Mohammed et al., 2010). The PDI for all formulations was between 0.2 and 0.5 (Table 3) which

indicates relative homogenous dispersion. The homogeneity of the dispersion was influenced by the molecular weight of polymer; however maintained with PDI at or less than 0.5.

Particle size plays a critical role in influencing the physicochemical and biological characteristics of nanoparticles. Smaller particles less than 400nm are most preferred in pharmaceutical product development. The particle size of all formulations ranged between 202nm-250nm (Table 3) and increased significantly as the molecular weight of polymer increased. It has been reported that low molecular weight chitosan is more soluble that may aid in the colloidal solubility of nanoparticles in the solution (Fernandez *et al*., 1999) and therefore decreased particle size observed with the lower molecular weight polymer.

The drug encapsulation efficiency and loading capacity of the nanoparticles depend upon the molecular weight of the polymer and the interaction between the drug and the polymer added in the development of nanoparticles. The increase in the molecular weight of chitosan resulted increase in the encapsulation efficiency and loading capacity of nanoparticles significantly (Table 3). The carboxyl group of rifampicin promotes electrostatic interaction with the amino group of chitosan and influence the encapsulation efficiency and loading capacity of nanoparticles. Longer chain of high molecular weight chitosan has more reaction sites and can entrap greater amount of drug when gelated with tripolyphosphate (Yongmei *et al*., 2003). While the physicochemical properties of nanoparticles were significantly influenced by the polymer, such effects were seemingly not affected by ascorbic acid which was used as a stabilizing agent in the study.

In-vitro dissolution stability

The results of *in-vitro* percentage degradation of rifampicin with different concentration of ascorbic acid in pH 1.2 medium are given in Table 2. Rifampicin degraded 33.1% at 15 min, and the degradation increased overtime and reached 42.7% at 60min and our finding supports the view that rifampicin undergoes degradation in the acidic environment of the stomach and the degradation has varied from 8.5%-50% during the gastric emptying time for most dosage form in humans (Saranjit *et al*., 2001). Rifampicin degrades to 3-formyl rifampicin SV (3FRSV) and 1-amino 4 methyl piperazine under acidic pH (Mariappan *et al*., 2003) and at the same time it is more soluble at low pH and therefore well absorbed from the stomach because of its high solubility between pH1 and 2 (Kowalski *et al*., 2004). Ascorbic acid addition reduced the rifampicin degradation at all time point intervals and the percent drug degradation decreased significantly as the concentration of ascorbic acid increased and drug degradation reached minimum (20.40%) with 500mg ascorbic acid and there was no further significant decrease in drug degradation beyond this concentration of ascorbic acid. At 60min, the degradation of drug decreased from 42.7% to 20.40% significantly ($P<0.01$) in the presence of ascorbic acid (500mg). Rifampicin nanoparticles degraded significantly less than rifampicin at all time point intervals and at 60mn it degraded 32.15% as compared to rifampicin (42.7%) ($P<0.05$) (Table 4). Ascorbic acid significantly reduced degradation of rifampicin nanoparticles at all time intervals and, at 60min from 32.15% to 25.92%. Rifampicin – ascorbic acid nanoparticles showed enhanced degradation of drug (25.92%) as compared to rifampicin - ascorbic acid (20.4%), possibly, nanoparticles give rise to larger surface area becoming available for interaction with acidic environment that may facilitate degradation of rifampicin. However nanoparticulate delivery of rifampicin along with ascorbic acid has reduced degradation from 42.7% to 25.92% and therefore nanoparticles approach coupled with ascorbic acid seems beneficial in improving bioavailability of rifampicin. The mechanism underlying improved stability of rifampicin in the acidic environment either by ascorbic acid or by nanoparticles and ascorbic acid together cannot be clearly ascertained.

In-vitro diffusion study

The results of *in-vitro* diffusion study are shown in Table 5. The release of rifampicin was 32.4% at 15 min and increased over time and reached 48.6% at 60 min. Ascorbic acid significantly improved the release of rifampicin at all time point intervals, and at 60 min, from 48.6% to 67.21%. This finding is consistent with our observation in the dissolution stability study and suggests that ascorbic acid may enhance the rate of absorption of rifampicin by further lowering the acidic pH as compared to rate of degradation in the same environment, which needs to be addressed. Rifampicin nanoparticles increased release of rifampicin significantly over time as compared to rifampicin and released 67.23% at 60 min as compared to rifampicin (48.6%), possibly, rifampicin when presented in nanosize may improve the transmucosal transport of the drug through the membrane at a rate faster than rate of degradation of drug in the acidic environment. Ascorbic acid enhanced the release of rifampicin nanoparticles significantly at all time intervals and the release of rifampicin nanoparticles was significantly increased from 66.23% to 74.01% at 60 min. These findings propose the hypothesis that nanoparticles coupled with ascorbic acid may promote absorption of rifampicin faster than its degradation. Besides, chitosan being mucoadhesive may also play a role in promoting absorption of rifampicin.

Table 1. Formulation of rifampicin nanoparticles

S. No	Formula code	Combinations
		Drug: Polymer: Ascorbic acid
1	F1	Rifampicin: Chitosan (150KDa): -
2	F2	Rifampicin: Chitosan (300KDa): -
3	F3	Rifampicin: Chitosan (600KDa): -
4	F4	Rifampicin: Chitosan (150KDa): 500mg
5	F5	Rifampicin: Chitosan (300KDa): 500mg
6	F6	Rifampicin: Chitosan (600KDa): 500mg

Table 2. *In-vitro* percentage degradation of rifampicin with different concentration of ascorbic acid at pH 1.2 medium (mean±SD, n=3)

Time (min)	Rifampicin alone	Rifampicin +Ascorbic acid 125mg	Rifampicin +Ascorbic acid 250mg	Rifampicin +Ascorbic acid 500mg	Rifampicin +Ascorbic acid 1000mg
0	0	0	0	0	0
15	33.1±2.55	23.8±0.31** [a]	15.2±0.37*[b]	14.9±0.20**[c]	15.03±0.31***[d]
30	37.8±1.21	28.8±0.15* [a]	19.5±0.62*** [b]	15.8±0.37***[c]	16.4±0.20** [d]
45	38.5±1.57	32.1±0.47** [a]	29.2±0.72* [b]	18.6±0.52***[c]	18.9±0.34** [d]
60	42.7±1.85	35.0±0.47*** [a]	32.5±0.51** [b]	20.40±1.33***[c]	21.8±0.12*** [d]

***P<0.001, **P<0.01, *P<0.05 ; [a]Significant difference compared to rifampicin alone; [b]Significant difference compared to rifampicin+Ascorbic acid 125mg; [c]Significant difference compared to rifampicin +Ascorbic acid 250mg; [d]Significant difference compared to rifampicin +Ascorbic acid 500mg

Table 3. Physicochemical characteristics of rifampicin loaded chitosan nanoparticle formulations each containing 500mg ascorbic acid (F1- F6) (n=3, mean±SD)

S. No	Formula code	Surface morphology	Particle size(nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)	Loading capacity (%)
1	F1	Smooth & Spherical	202±0.106	0.225±0.19	+42±0.14	80.7±0.014	45.65±0.014
2	F2	Smooth & Spherical	224±0.094	0.230±0.24	+35±0.51	83.57±0.41	47.57±0.014
3	F3	Smooth & Spherical	252±0.440	0.310±0.61	+34±0.71	87.14±0.61	49.39±0.014
4	F4	Smooth & Spherical	205±0.015	0.202±0.09	+42±0.34	82.56±0.014	46.38±0.189
5	F5	Smooth & Spherical	220±0.082	0.232±0.12	+36±0.36	84.03±0.53	47.77±0.038
6	F6	Smooth & Spherical	256±0.230	0.298±0.45	+36±0.34	87.67±0.84	48.09±0.014

Table 4. *In-vitro* dissolution stability data of rifampicin, rifampicin-ascorbic acid, rifampicin nanoparticles and rifampicin-ascorbic acid nanoparticles (F4) at pH 1.2 medium (n=3, mean±SD)

	Percent degradation of			
Time (min)	Rifampicin alone	Rifampicin -Ascorbic acid 500mg	Rifampicin nanoparticles	Rifampicin -Ascorbic acid nanoparticles (F4)
0	0	0	0	0
15	33.1±2.55	14.9±0.20**[a]	15.2±0.37*	15.2±0.20**[b]
30	37.8±1.21	15.8±0.37***[a]	19.5±0.62***	17.6±0.37**[b]
45	38.5±1.57	18.6±0.52***[a]	29.2±0.72*	21.8±0.52**[b]
60	42.7±1.85	20.40±1.33**[a]	32.15±0.51**	25.92±1.33***[b]

***P<0.001, **P<0.01, *P<0.05

[a]Significant difference compared to rifampicin alone; [b]Significant difference compared to rifampicin nanoparticles

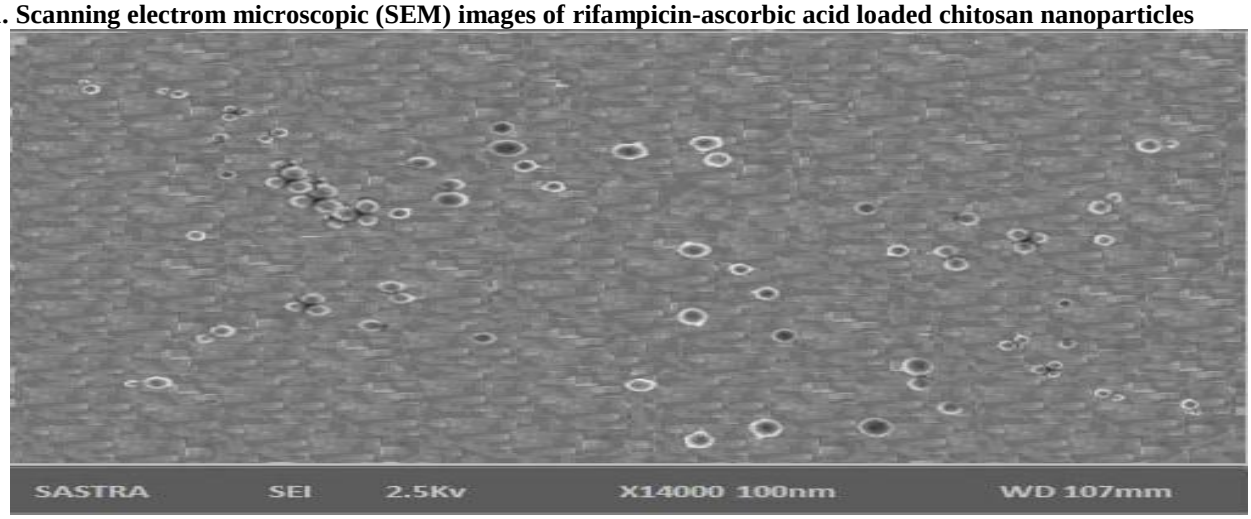
Table 5. *In-vitro* diffusion data of rifampicin, rifampicin-ascorbic acid, rifampicin nanoparticles and rifampicin-ascorbic acid nanoparticles at pH 1.2 medium (n=3, mean±SD)

	Percent release of			
Time (min)	**Rifampicin alone**	**Rifampicin -Ascorbic acid 500mg**	**Rifampicin nanoparticles**	**Rifampicin -Ascorbic acid Nanoparticles (F4)**
0	0	0	0	0
15	32.4±1.05	45±0.30**[a]	48±0.30***	55±0.20**[b]
30	41.2±0.91	56.4±0.32***[a]	60.5±0.62**	64.1±0.37**[b]
45	46.9±1.32	61.1±0.47**[a]	64.8±0.72***	68.4±0.52***[b]
60	48.6±1.15	67.21±0.49***[a]	66.23±0.51**	74.01±1.33*[b]

***P<0.001, **P<0.01, *P<0.05

[a]Significant difference compared to rifampicin alone; [b]Significant difference compared to rifampicin nanoparticles

Fig 1. Scanning electrom microscopic (SEM) images of rifampicin-ascorbic acid loaded chitosan nanoparticles



CONCLUSION

The findings of the study demonstrate that nanoparticulate delivery of rifampicin along with ascorbic acid as stabilizing agent can minimize rifampicin degradation and improves its bioavailability. Besides, ascorbic acid can improve the immune system and thus can effectively control TB infection.

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